

Aromatase Localization in Human Breast Cancer Tissues: Possible Interactions between Intratumoral Stromal and Parenchymal Cells

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Abstract

Aromatase is a key enzyme in intratumoral estrogen production required for the production of estrogens through the conversion of serum androgens in postmenopausal breast cancer patients. There have been, however, controversies regarding the intratumoral localization of aromatase in human breast carcinoma tissues. Therefore, we have first examined the intratumoral localization of aromatase mRNA/protein in 19 breast carcinomas using laser capture microdissection/quantitative reverse transcription-PCR (RT-PCR) and immunohistochemistry. Aromatase mRNA and protein were detected in both intratumoral stromal and parenchymal cells in breast carcinoma tissues. Subsequent microarray expression profiling and clustering analyses, in addition to quantitative RT-PCR studies, showed a significant positive correlation between aromatase and estrogen-related receptor α mRNA expression in isolated carcinoma cells. We further examined an interaction between stromal cells isolated from human breast carcinoma tissues and breast carcinoma cell lines using a coculture system to study the biological characteristic of aromatase expression in carcinoma cells. Aromatase mRNA and enzyme activity and 17β -hydroxysteroid dehydrogenase type 1 mRNA in breast carcinoma cell lines, including MCF-7 and SK-BR-3 cells, were up-regulated in the presence of patient-derived 32N or 74T intratumoral stromal cells. The results from steroid conversion assays were also consistent with the findings above. The results of our study also showed that aromatase inhibitors were more effective in inhibiting aromatization induced by coculture in MCF-7 than that in stromal 32N. The examination of the localization of aromatase and its regulation, including the interactions existing between different cell types in human breast carcinoma tissues, may provide important information as to achieving better clinical response to aromatase inhibitors in breast cancer patients. [Cancer Res 2007;67(8):3945–54]

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Introduction

Estrogens play important roles in the growth and invasion of estrogen-dependent human breast carcinomas. While postmenopausal women have low levels of circulating plasma estrogens, the local synthesis or intratumoral production of estrogens that takes place in breast carcinoma tissue itself can lead to higher estrogen levels in the tumor (1, 2). Intratumoral production of estrogens occurs as a result of aromatization of C19 steroids such as androstenedione and testosterone into estrogens, and this is catalyzed by the cytochrome P450 aromatase enzyme (3–5). Previously, the localization of aromatase has been mostly examined using immunohistochemistry with the reported results demonstrating the presence of aromatase protein predominantly in tumoral stromal cells and adipocytes of breast carcinoma tissues (6, 7). However, there have been controversies regarding the cellular localization of intratumoral aromatase with other studies demonstrating aromatase immunoreactivity in carcinoma or parenchymal as well as stromal cells of human breast carcinoma tissues (8, 9).

Aromatase expression is well known to be regulated by various transcriptional factors, including nuclear receptors and their putative ligands in several types of human cells and tissues (10, 11). Both interleukins, such as interleukin (IL)-1, IL-6 and IL-11 released from carcinoma, and/or inflammatory cells have been shown to potentially induce aromatase expression in adipose fibroblast cells (12). However, the correlation between nuclear receptors and aromatase in parenchymal or carcinoma cells of breast carcinoma tissues has remained largely unknown. In addition, there have been no studies reported examining whether the factors released from human intratumoral stromal cells affect aromatase expression of breast carcinoma or parenchymal cells.

Intratumoral aromatase has been considered a viable clinical target for the treatment of estrogen receptor-positive postmenopausal breast cancer patients (13). However, routine evaluation methods for the detection of intratumoral aromatase expression in clinical specimens have not been established. Therefore, in this study, we have first examined the localization of aromatase mRNA in 19 breast carcinoma tissues using laser capture microdissection (LCM), together with quantitative reverse transcription-PCR (RT-PCR), and then examined their correlation with clinicopathologic parameters of the patients. The expression and localization of the aromatase protein were also confirmed by immunohistochemistry using the aromatase monoclonal antibody termed 677 (14, 15). Microarray expression profiling and clustering analyses were also done on both isolated carcinoma and stromal cells obtained from the 19 breast carcinoma cases to identify possible

aromatase-regulating nuclear receptors in human breast carcinoma cells. We then examined the possible effects of isolated stromal cells from breast carcinoma tissues on both aromatase enzymatic activity and mRNA transcripts in breast carcinoma cell lines. Cocultured intratumoral stromal 32N or 74T cells, established from breast carcinoma tissues by primary culture, were used to evaluate the potential effects of carcinoma/stromal cell interactions on aromatase expression and enzyme activity in the carcinoma cells. Effects of coculture of MCF-7 cells with stromal 32N cells on MCF-7 cell proliferation and the inhibitory effects of the aromatase inhibitors on cell proliferation were subsequently investigated to further characterize the biological features of aromatase function in carcinoma or parenchymal cells.

Materials and Methods

Patients and tissue preparation. A total of 42 specimens of invasive ductal carcinoma of the breast were obtained from Japanese female patients from 2002 to 2005 at the Department of Surgery, Tohoku University Hospital and Tohoku Kosai Hospital (Sendai, Japan). The number of subjects examined in each experiment were as follows: 19 cases [54.2 years (range, 37–86; SD, 12.9)] for LCM/quantitative RT-PCR (qPCR) to investigate aromatase localization, 23 cases [55.0 years (range, 36–74; SD, 10.7)] for LCM/microarray studies, and 11 cases [53.1 years (range, 36–77; SD, 10.1)] for LCM/qPCR to validate results of microarray analysis. Nonpathologic breast and adipose tissues adjacent to the carcinoma were also available for examination in 12 out of 23 cases used in LCM/microarray analysis. Relevant clinical data were retrieved from the review of the patient's files. The histologic grade of each specimen was independently evaluated by three of the authors (T. Suzuki, T. Moriya, and H. Sasano), based on the modified methods of Bloom and Richardson (16), according to Elston and Ellis (17). The ethics committees at Tohoku University School of Medicine and Tohoku Kosai Hospital approved the research protocols (2004-144, 2005-068, and 2006-042, respectively), with informed consent being obtained from these patients before surgery in each institution.

Immunohistochemistry. For immunohistochemistry of aromatase, sequential frozen tissues, also used in the LCM analyses, were taken to examine the correlation between mRNA and protein in individual cellular compartments of the breast cancer tissues.

The aromatase monoclonal antibody 677 was raised against native recombinantly expressed human aromatase protein, with details of its characterization and utilization for immunohistochemistry being previously reported by the authors (14, 15). Tissue sections were immunostained by a biotin-streptavidin method with Histofine kit (Nichirei Co. Ltd., Tokyo, Japan). Breast carcinomas tissues were rapidly embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen materials sectioned at a thickness of 3 μ m. The antigen-antibody complex was then visualized with 3,3'-diaminobenzidine solution and counterstained with hematoxylin. Evaluation of aromatase immunohistochemistry was done as previously reported using 10% formalin-fixed and paraffin-embedded tissue specimens (14). The approximate percentage of cells staining (proportion score) were classified into the following four groups: 0, <1%; 1, –25%; 2, –50%; and 3, >50% immunopositive cells. Relative intensity of aromatase immunopositive cells was classified as follows: 0, no immunoreactivity; 1, weak; 2, moderate; and 3, intense immunoreactivity. Aromatase immunoreactivity was evaluated as a total score composed of the proportion score + relative immunointensity score.

Other antibodies used in this study for characterizing clinicopathologic parameters of the cases are as follows: monoclonal antibodies: ER α (ER1D5; Immunotech S.A., Marseilles, France), progesterone receptor (MAB429; Chemicon International Inc., Temecula, CA), and Ki-67 (MIB1; DakoCytomation Co. Ltd., Kyoto, Japan); and rabbit polyclonal antibody: HER-2/*neu* (Ao485; DakoCytomation). The rabbit polyclonal antibody for 17 β -HSD1, 17 β -HSD5, and monoclonal antibody for steroid sulfatase (STS) were kindly provided by Dr. Poutanen (University of Oulu, Finland), Dr. Lu (Laval

University Hospital Center, Québec, Canada) and Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), respectively. These antibodies were used for immunohistochemistry in 10% formalin-fixed and paraffin-embedded tissue specimens of the cases. To score estrogen receptor (ER), progesterone receptor (PR), and Ki-67, more than 1,000 carcinoma cells from each case were counted independently by the same three authors as described above (Y. Miki, T. Suzuki, and H. Sasano), and the percentage of immunoreactivity as a labeling index (LI) was subsequently determined. The cases with <10% ER α LI or PR LI were designated ER- or PR-negative breast carcinomas according to the report by Allred et al. (18). For scoring of HER-2/*neu*, 17 β -HSD1, 17 β -HSD5, and STS (19–22), two groups were tentatively identified (0, no immunoreactivity and 1, positive carcinoma cells).

Total RNA extraction from breast tissues and cDNA synthesis. Total RNA was carefully extracted from 12 breast carcinoma specimens from both carcinomas and adipose compartments of the breast cancers in addition to nonpathologic breast tissues adjacent to the carcinoma using the TRIzol method (Invitrogen Corporation, San Diego, CA). A reverse transcription kit (Superscript II Preamplification system; Invitrogen) was used in the synthesis of cDNA.

Real-time RT-PCR. Real-time PCR was carried out using the LightCycler System (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences of aromatase, *ERR α* , and *RPL13A* were shown previously (23). Primer sets of *GCNF*, *HNF-4 α* , *VDR*, *TR β* , *TR α* , *HSD17B1*, *HSD17B2*, *HSD17B3*, *HSD17B4*, and *HSD17B5* were designed using OLIGO Primer Analysis Software (Takara Bio Inc., Shiga, Japan). cDNAs of known concentrations for target genes and the housekeeping gene, ribosomal protein L13a (*RPL13A*) were used to generate standard curves for real-time quantitative PCR to determine the quantity of target cDNA transcripts. The mRNA level in each case was represented as a ratio of *RPL13A* (%; refs. 20, 21, 23).

Laser Capture Microdissection. Nineteen breast carcinoma cases were frozen-sectioned at a thickness of 8 μ m. Approximately 5,000 cells were laser-transferred from the carcinoma cells and the intratumoral stromal cells under light microscopic examination. For LCM/microarray expression profiling, after initial recovery and resuspension of the RNA pellet, a DNase digestion was done for 2 h at 37°C using 10 units of DNase (GenHunter, Nashville, TN) in the presence of 10 units of RNase inhibitor (Invitrogen), followed by extraction and precipitation. The pellet was resuspended in 27 μ L of RNase-free H₂O and used for high-density cDNA array analysis.

Microarray analysis in isolated carcinoma cells. Twenty-three breast carcinomas were available for examination of gene expression patterns using microarray analysis following isolation by laser capture microscopy. Total RNA was extracted from ~5,000 carcinoma cells prepared by LCM procedures as described above. Sample preparation and processing were done essentially as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc., Santa Clara, CA), with the exception that the labeled cRNA samples were hybridized to the complete human U133 GeneChip set (Affymetrix), including 22,215 and 22,577 genes. Relative levels of gene expression were calculated by global normalization. All gene expression data were clustered, and results were visualized using GeneSpring 7.2 (Agilent Technologies, Inc., Santa Clara, CA).

In this study, we focused on nuclear receptors that may modulate aromatase expression in carcinoma cells. Out of 44,792 genes, 88 genes were selected from the gene expression profiling for further analysis by reference to the web database of Nuclear Receptor.¹¹ Each case of breast carcinoma was ordered according to the level of aromatase gene expression determined by microarray and clustering analysis between each gene. Data from these categories and aromatase were subjected to hierarchical clustering analysis and visualization using the Cluster and TreeView programs (Stanford University, Palo Alto, CA; ref. 24) to generate tree structures based on the degree of similarity, as well as matrices comparing the levels of expression of individual genes in each sample examined. In addition, we further examined the correlations between the levels of aromatase mRNA expression and seven genes that were most closely

¹¹ NucleaRDB (April 2005, release 5.0); <http://www.receptors.org/NR/index.html>.

associated with aromatase in carcinoma cells isolated by LCM from 11 cases of human breast carcinoma.

Breast cancer cell lines and culture conditions. Human breast carcinoma cell lines MCF-7, T-47D, ZR-75-1, and SK-BR-3 and the human choriocarcinoma cell line BeWo were provided from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Human breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 and mouse preadipocyte 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA). The MCF-7, T-47D, ZR-75-1, and 3T3-L1 cell lines were maintained in RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO) or Leivobitz's L-15 medium (Invitrogen) for the MDA-MB-231 and MDA-MB-468 cells and supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS). BeWo placental cells were maintained in Ham's F12 medium (Invitrogen) supplemented with 15% FBS. Primary stromal cells employed in this study were designated 74T and 32N and were isolated using collagenase treatment from human breast carcinoma tissues (25) and maintained in RPMI 1640 with 10% FBS.

Coculture system. For physical separation of stromal and carcinoma cell lines, transwell cultures were established in six-well plates or 100-mm dishes using Transwell Permeable Supports (0.4 μ m pore; Corning, Incorporated, New York, NY). MCF-7 and SK-BR-3 cells were cultured in transwell chambers in the absence or presence of 32N, 74T, and 3T3-L1 cells and were cultivated on the bottom of the plates or dishes. After 24 h of cultivation using this coculture system, carcinoma and stromal cells were separated, and each component was examined in the aromatization assay, estrogen production assays, or by real-time RT-PCR. The cells after coculture with other cells were designated with subscript CO (i.e., MCF-7_{CO}, 32N_{CO}). After these assays, viable cells were counted by the trypan blue exclusion (TBE) assay, and the total RNA was extracted using the TRIzol method described above.

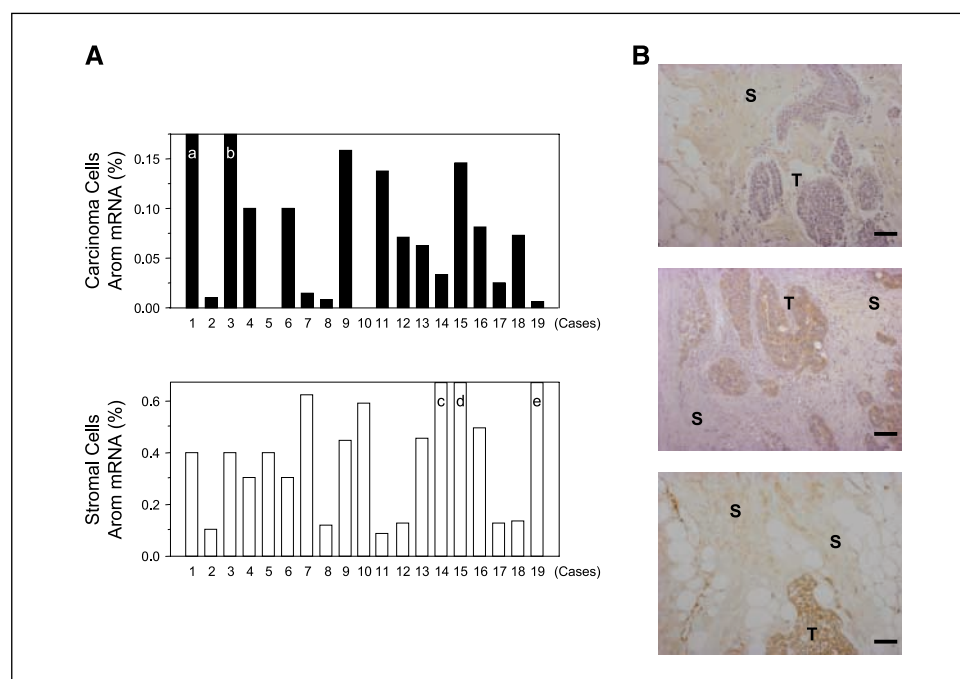
Aromatization assay. Several previous studies have shown that the aromatase enzyme activity and/or mRNA levels were low or not detectable in the breast carcinoma cells such as MCF-7 (26–29). Therefore, in this study, we employed 6 α -methylandrosterone-4-ene-3,17-dione (MeAD) assay as a quantitative evaluation of aromatization activity (30). The conversion of MeAD, an androgen analogue, into estrogen analogue (6 α -methyl estradiol) by aromatase was shown to be highly specific, and an evaluation of accurate aromatase activity could become possible with the measurement of its estrogen analogue produced (30). From the results of comparative studies using rat ovary tissue, the quantitative limit of detection of the MeAD assay

was approximately 2-fold higher than that of the ³H-water release assay (data not shown).

The aromatization assay using MeAD was done as described previously (30). MeAD and 6 α -methyl estradiol (MeE2) were generously provided by Dr. Numazawa (Tohoku Pharmaceutical College, Sendai, Japan). Estradiol-3-pentafluorobenzyl-17 β -methylpyridinium ether (E2-PFBY) was synthesized at Teizo Medical Co., Ltd. (Kanagawa, Japan). We used LC-MS-grade water, methanol, and acetonitrile for liquid chromatography–tandem mass spectrometry (LC-MS/MS). The LC-MS/MS devices and measurement conditions were as follows: an API 4000 electrospray ionization mass spectrometer (MDS SCIEX; Applied Biosystems (Foster City, CA)) was used. For high-performance liquid chromatography (HPLC), an Agilent 1100 chromatograph (Agilent Technologies) was employed. HTC PAL autosampler (CTC Analytics AG Industriestrasse, Zwingen, Switzerland) and an X Terra MS C18 column (3.5 μ m, 2.1 \times 150 mm) were employed. Measurement was done in the positive ion mode. For the quantification of MeE2-PFBY, we used a product ion (*m/z* 353.2) produced from a precursor ion (*m/z* 558.3). On the other hand, product ion of ¹³C-E2-PFBY was *m/z* 109.9 produced from precursor ion at *m/z* 548.2. For the quantification of MeE2, after estrone, ¹³C₄ (Hayashi Pure Chemical Industries, Inc., Osaka, Japan) was added to the medium, and the mixture was mixed. The extract was dissolved in methanol and reduced with 1% NaBH₄. The sample was dried and treated with pentafluorobenzyl bromide under 0.8% KOH ethanol. Then, the reactive solution was diluted with purified water, and a derivative was extracted with ether. The sample was dried under reduced pressure and mixed with 2% 2-fluoro-1-methylpyridinium-*p*-toluenesulfonate/dichloromethane and 10% triethylamine. E2-PFBY derivative was eluted with 10% formic acid solution/acetonitrile (2, 8). The elution vehicle was removed under reduced pressure, dissolved in 0.1 mL of the mobile phase, and used as a sample for LC-MS/MS.

Estrogen production assays. MCF-7 and MCF-7_{CO} cells were incubated at 37°C in FBS-free RPMI 1640 containing 10 nmol/L of androstenedione or testosterone as substrates for 24 h. Concentrations of estrone and estradiol were evaluated by LC-MS/MS analysis (31). All the cells treated with substrates were counted by TBE assay. After addition of 100 pg of androstenedione-²H₇ (C/D/N Isotope Inc., Quebec, Canada), estrone-¹³C₄, and estradiol-¹³C₄ (Hayashi Pure Chemical Industries) as internal standards, steroids were extracted with diethyl ether from the media. The separated organic layer was evaporated and then dissolved in picolinic anhydride in

Figure 1. A, aromatase mRNA level in each case (1 to 19) of breast carcinoma (top) and stromal (bottom) cells. There were no detectable levels of aromatase in the breast carcinoma cells from two cases (5 and 10). For the following samples, the values of aromatase mRNA were *a* = 0.20%, *b* = 0.30%, *c* = 0.91%, *d* = 6.23%, and *e* = 1.02%. Aromatase immunoreactivity scores for each case (1 to 19) of breast carcinoma (black) and stromal (white) cells. There were no aromatase immunoreactivities observed in breast carcinoma cells from four cases (7, 12, 16, and 18). B, immunohistochemical localization of aromatase in human breast carcinoma tissues. Aromatase immunoreactivity was detected predominantly in carcinoma cells (top), only in stromal cells (middle), or in both carcinoma and stromal cells (bottom). S, stromal cells; T, carcinoma cells. Bar, 50 μ m.



tetrahydrofran solution with triethylamine. After application to a Bond Elut C₁₈ column, steroid derivatives were eluted with 80% acetonitrile solution. In this study, we used liquid chromatography (Agilent 1100; Agilent Technologies) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems) operated with electron spray ionization in the positive-ion mode, and the chromatographic separation was done on Cadenza CD-C₁₈ columns (3 × 150 mm, 3.5 μm; Imtakt Corporation, Kyoto, Japan). Ion spray voltage was 4.5 kV, and turbo gas temperature was 450 °C in ionization conditions. The estrogen levels in each case are presented as picograms per milliliter per 10⁶ cells.

Cell proliferation assay. After coculture with stromal cells for 24 h, MCF-7 cells treated with 10⁻⁹ to 10⁻⁷ mol/L testosterone or androstenedione for 24 h were trypsinized and harvested in phenol red- and FBS-free medium in 96-well plates (3 × 10⁴ cells/mL). Androstenedione and testosterone (10⁻⁹ to 10⁻⁷ mol/L) were added for 24 h. Cell proliferation was evaluated using the WST-8 method (Cell Counting Kit-8; Dojindo Inc., Kumamoto, Japan; ref. 32). We also examined the effects of aromatase inhibitors on cell proliferation by methods described above. Both steroidal (exemestane 10⁻⁸ mol/L; Pfizer Inc., New York, NY) and nonsteroidal (letrozole 10⁻⁸ mol/L; Novartis Pharma AG, Basel, Switzerland.) aromatase inhibitors were used in the androgen-treated MCF-7_{CO} and MCF-7 cells for 24 h.

Statistical analysis. Statistical analysis was done using the StatView 5.0 J software (SAS Institute Inc., Cary, NC). Values for patient's age, tumor size, Ki-67 LI, and mRNA levels for aromatase are represented as the mean ± SD. Simple regression analysis was employed to assess the correlations between aromatase and ERRα mRNA expression levels. An association between the degree of mRNA expression of aromatase and these parameters for each individual case was evaluated using one-way ANOVA and the Bonferroni test. Statistical differences between aromatase mRNA expression and ER status, PR status, menopausal status, stage, lymph node status, histologic grade and HER-2/*neu*, 17β-HSD1, and 17β-HSD5 immunoreactivities were all evaluated in a cross-table using the χ² test.

Results

Distributions of aromatase mRNA transcripts in breast tissues. The level of aromatase mRNA expression (mean ± SD; *n* = 12) was significantly higher in both carcinoma (1.327 ± 1.394%) and adipose tissues (2.103 ± 1.790%) than in non-neoplastic breast tissue (0.106 ± 0.095%) adjacent to carcinoma. The aromatase level in placenta tissues as a positive control was 80.770 ± 31.867% (mean ± SD; *n* = 3; data not shown).

Localization of intratumoral aromatase mRNA transcripts in breast carcinoma tissues. From 19 breast carcinoma cases, aromatase mRNA transcripts were detected in intratumoral stromal cells from all 19 cases and in the carcinoma cells from 17 of these cases (Fig. 1A). The mean value of aromatase mRNA transcript level was significantly higher (*P* = 0.009) in intratumoral stromal cells (0.348 ± 0.238) than in the carcinoma cells (0.112 ± 0.090). No significant correlation was detected in the aromatase mRNA level between stromal and carcinoma cells (*r* = 0.132, *P* = 0.592). Aromatase immunoreactivity was detected in the cytoplasmic compartment of both intratumoral stromal and carcinoma cells (Fig. 1B and C). Aromatase immunoreactivity was absent in the carcinoma cells of four cases.

Correlation between aromatase mRNA levels in intratumoral stromal and carcinoma cells and clinicopathologic status in breast carcinoma patients. An association between the intratumoral aromatase mRNA levels in stromal and/or parenchymal/carcinoma cells and clinicopathologic parameters in 19 breast carcinoma cases are summarized in Table 1. Two cases of aromatase-negative carcinoma cells were assigned as 0.000%. Aromatase mRNA levels in stromal cells were positively correlated with histologic grade (*P* = 0.032). Aromatase mRNA levels in

Table 1. Correlation between aromatase expression levels and clinicopathologic parameters in 19 breast carcinomas

| Parameter | <i>n</i> | Carcinoma cells | | Stromal cells | |
|------------------|----------|-----------------|----------|---------------|----------|
| | | Mean ± SD | <i>P</i> | Mean ± SD | <i>P</i> |
| Histologic grade | | | | | |
| I + II | 14 | 0.098 ± 0.085 | | 0.342 ± 0.228 | |
| III | 5 | 0.014 ± 0.014 | 0.072 | 2.002 ± 2.847 | 0.032* |
| ER status | | | | | |
| + | 11 | 0.116 ± 0.087 | | 0.374 ± 0.239 | |
| - | 8 | 0.029 ± 0.031 | 0.015* | 1.146 ± 2.080 | 0.234 |
| PR status | | | | | |
| + | 12 | 0.107 ± 0.089 | | 0.337 ± 0.228 | |
| - | 7 | 0.033 ± 0.032 | 0.051 | 1.320 ± 2.190 | 0.134 |
| 17β-HSD1 status | | | | | |
| + | 10 | 0.134 ± 0.090 | | 0.413 ± 0.230 | |
| - | 9 | 0.028 ± 0.029 | 0.007* | 1.101 ± 2.100 | 0.373 |
| 17β-HSD5 status | | | | | |
| + | 11 | 0.096 ± 0.104 | | 0.361 ± 0.164 | |
| - | 8 | 0.062 ± 0.052 | 0.437 | 1.266 ± 2.225 | 0.24 |
| STS status | | | | | |
| + | 12 | 0.074 ± 0.068 | | 0.435 ± 0.292 | |
| - | 7 | 0.102 ± 0.135 | 0.592 | 1.723 ± 3.012 | 0.138 |
| EST status | | | | | |
| + | 8 | 0.068 ± 0.066 | | 0.462 ± 0.337 | |
| - | 11 | 0.087 ± 0.094 | 0.683 | 0.891 ± 1.790 | 0.609 |

*Significantly different; in percent.

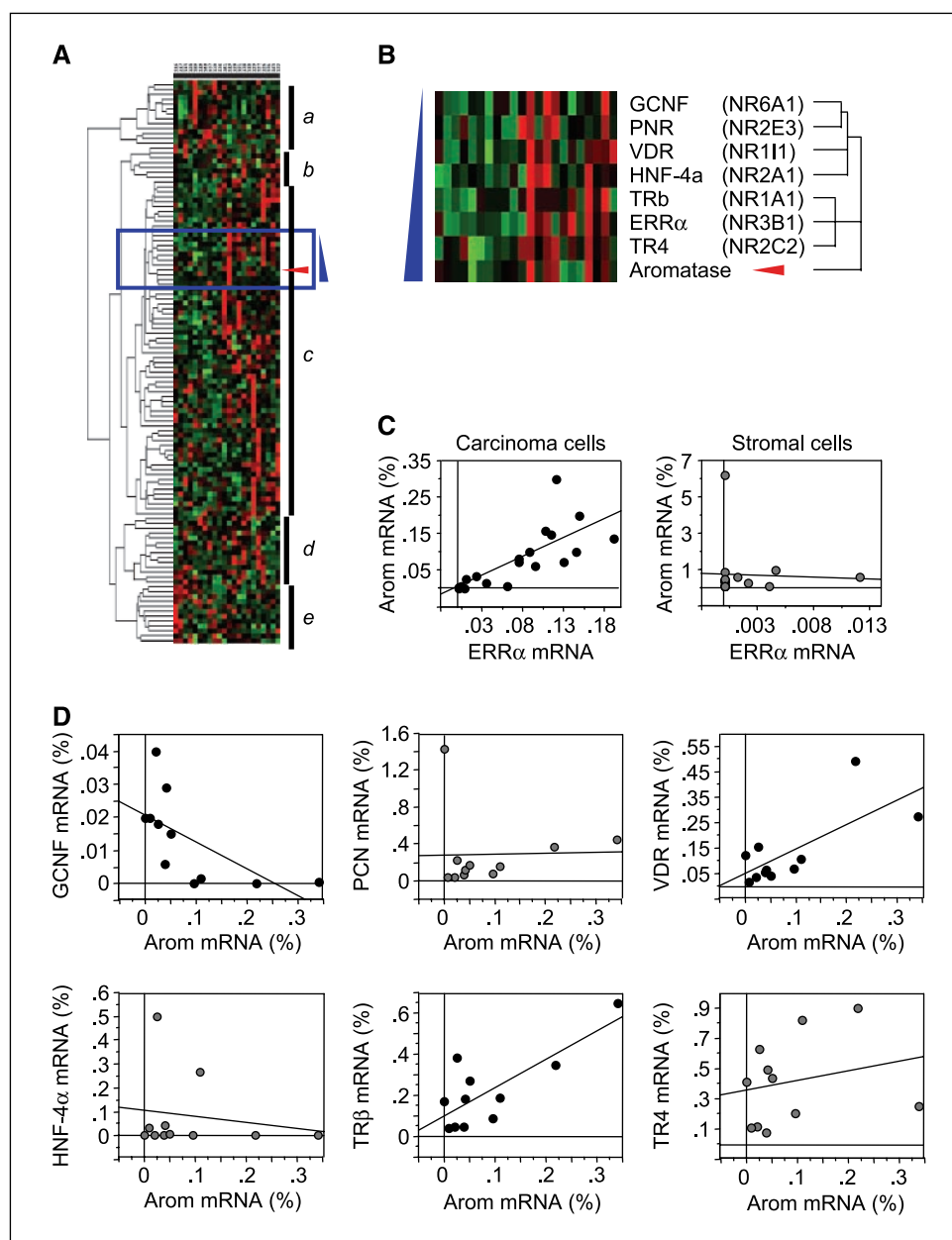


Figure 2. A and B, clustering analysis of microarray expression profiles of nuclear receptor genes. The cluster associated with the aromatase gene was composed of 59 genes (A, group c). More closely associated with the aromatase gene (A, square) were 12 genes (B). C, a statistically significant positive correlation was detected between aromatase and ERR α gene in 19 cases of (left) carcinoma cells ($r = 0.748$, $P = 0.002$), but not in (right) stromal cells ($r = 0.044$, $P = 0.860$). D, statistically significant correlations were detected between aromatase and ERR α ($r = 0.74$, $P = 0.01$; data not shown), VDR ($r = 0.73$, $P = 0.02$), TR β ($r = 0.62$, $P = 0.04$), and GCNF ($r = -0.64$, $P = 0.03$) genes in 11 cases of carcinoma cells. No statistically significant correlations were detected between aromatase and PNR ($r = 0.02$, $P = 0.95$), HNF-4 α ($r = -0.17$, $P = 0.61$), and TR4 ($r = 0.24$, $P = 0.48$) gene in 11 cases of carcinoma cells.

parenchymal/carcinoma cells were positively correlated with ER status ($P = 0.015$) and 17 β -HSD1 status ($P = 0.007$), but not with histologic grade ($P = 0.072$). No significant association was detected between aromatase mRNA level in stromal or carcinoma cells and age, tumor size, lymph node status, PR status, HER-2/*neu* status, Ki-67 LI, 17 β -HSD5 status, and STS status in this study.

Microarray analysis evaluated by hierarchical clustering. The results of focused clustering analysis subclassified 88 genes into five well-defined expression profiles or groups (Fig. 2A, groups a-e). The aromatase gene was included in group c (59 genes). The following seven genes were most closely associated with aromatase in group c (Fig. 2B): GCNF (NR6A1), PNR (NR2E3), VDR (NR1I1), HNF-4 α (NR2A1), TR β (NR1A1), ERR α (NR3B1), and TR4 (NR2C2).

Correlation between aromatase and nuclear receptors in isolated breast carcinoma cells. A statistically significant positive correlation was detected between aromatase and ERR α ($r = 0.74$,

$P = 0.01$), VDR ($r = 0.73$, $P = 0.02$), and TR β ($r = 0.62$, $P = 0.04$) in parenchymal/carcinoma cells of 11 human breast carcinoma cases (Fig. 2D). A statistically significant negative correlation was also detected between aromatase and GCNF ($r = -0.64$, $P = 0.03$) in these isolated parenchymal/carcinoma cells (Fig. 2D).

There were no significant increases or decreases of mRNA levels for the nuclear receptors GCNF, PNR, VDR, HNF-4 α , TR β , ERR α , and TR4 in MCF-7_{CO} compared with MCF-7 cells (data not present).

Correlation between aromatase and ERR α expressions in breast carcinoma cells. A statistically significant positive correlation was detected between aromatase and the ERR α gene (Fig. 2C) in 19 cases of parenchymal/carcinoma cells ($r = 0.748$, $P = 0.002$), but not in stromal cells ($r = 0.044$, $P = 0.860$).

In breast carcinoma cell lines, ERR α mRNA was detected in all cell lines examined in this study (Fig. 3A). Relatively low levels of

ERR α mRNA were detected in both stromal 32N and 74T cells. ERR α mRNA was also detected in placental BeWo cells.

Aromatase mRNA expression levels in human intratumoral stromal and carcinoma cells. Aromatase mRNA was detected in both stromal 32N and 74T cells isolated from human breast cancer tissues examined in this study, but the levels were lower than that of placental BeWo cells (Fig. 3B). Relatively low levels of aromatase mRNA was detected in mouse preadipocyte 3T3-L1 cells. Both 32N and 74T cells also exhibit aromatase enzyme activity, and there were no significant differences found between the aromatization levels in the 32N and 74T cells ($P = 0.140$).

Aromatase mRNA was detected in all breast carcinoma cell lines examined in this study (Fig. 3B). High levels of aromatase mRNA were detected in SK-BR-3 and MDA-MB-468 breast carcinoma cell lines, but the levels were lower than that of the 74T and 32N stromal cells and placental BeWo cells. We therefore used the higher aromatase mRNA expressing SK-BR-3 cells and the low aromatase mRNA expressing MCF-7 cells for further examinations. The aromatase mRNA level of native MCF-7 cells was significantly lower than that of native SK-BR-3 cells ($P = 0.041$). However, the levels in native SK-BR-3 cells were significantly lower than those of 32N ($P = 0.032$) or 74T cells ($P = 0.044$).

Effects of coculture on aromatase mRNA and activity levels in human intratumoral stromal and carcinoma cells. The results of the effects of coculture of breast cancer cell lines on the 32N and 74T cell aromatase activity are summarized in Fig. 3C to E. Aromatase mRNA level and activity in both 32N and 74T were significantly increased by cocultivation with MCF-7 but not with SK-BR-3 cells.

The results of the effects of coculture of stromal cells on MCF-7 and SK-BR-3 aromatase level are summarized in Fig. 3D and E. Both aromatase enzyme level (Fig. 3D) and mRNA level (Fig. 3E) in MCF-7_{CO} (after coculture with both 32N and 74T) were significantly higher than the levels found in monocultures of MCF-7 cells. There were no significant differences between native SK-BR-3 cells alone

and SK-BR-3_{CO} (Fig. 3D and E). There were also no significant differences found in the aromatization levels between MCF-7_{CO} and SK-BR-3_{CO}. Aromatase activity/mRNA expression in both MCF-7_{CO} and 3T3-L1_{CO}, however, was not increased after coculture with MCF-7 and 3T3-L1 (Fig. 3C-E).

Effects of aromatase inhibitors on MCF-7 or stromal cells. Both 10^{-8} mol/L exemestane and 10^{-8} mol/L letrozole inhibited the increase in aromatization activity of the MCF-7_{CO} (Fig. 4A). Both 10^{-8} mol/L exemestane and 10^{-8} mol/L letrozole also inhibited the increase in aromatase activity of 32N_{CO} compared with the aromatase activity level of the 32N cells alone (Fig. 4A). The aromatization activity levels of MCF-7_{CO} following treatment with the aromatase inhibitors in MCF-7_{CO} were significantly lower than the aromatase activity level found in native MCF-7 cells (Fig. 4A).

The results of the cell proliferation assays are summarized in Fig. 4B to D. In monocultures of MCF-7 cells, there were no changes in the number of cells after 24 h incubation with 10^{-7} to 10^{-9} mol/L androstenedione, whereas 10^{-7} mol/L testosterone significantly increased the number of cells after 24 h. In MCF-7_{CO}, there were significant increments in the number of cells after 24 h treatment with 10^{-7} mol/L androstenedione and 10^{-8} to 10^{-7} mol/L testosterone. The cell numbers of MCF-7_{CO} treated with 10^{-8} mol/L androstenedione and 10^{-8} mol/L testosterone were significantly higher than those found in monocultures of MCF-7 cells. All of these increases of MCF-7_{CO} cell proliferation were inhibited following treatment with 10^{-8} mol/L exemestane (Fig. 4C) or 10^{-8} mol/L letrozole (Fig. 4D).

Estrogen production and expression levels of 17 β -hydroxysteroid dehydrogenases in MCF-7. The results of estrogen production assays are summarized in Table 2A and B. Following the treatment with androstenedione (10^{-9} mol/L; 286.4 mg/mL) as the aromatase substrate, the rate of conversion into both estrone and estradiol in MCF-7_{CO} was higher than that in MCF-7 cells alone. The rate of conversion into estradiol but not into estrone in

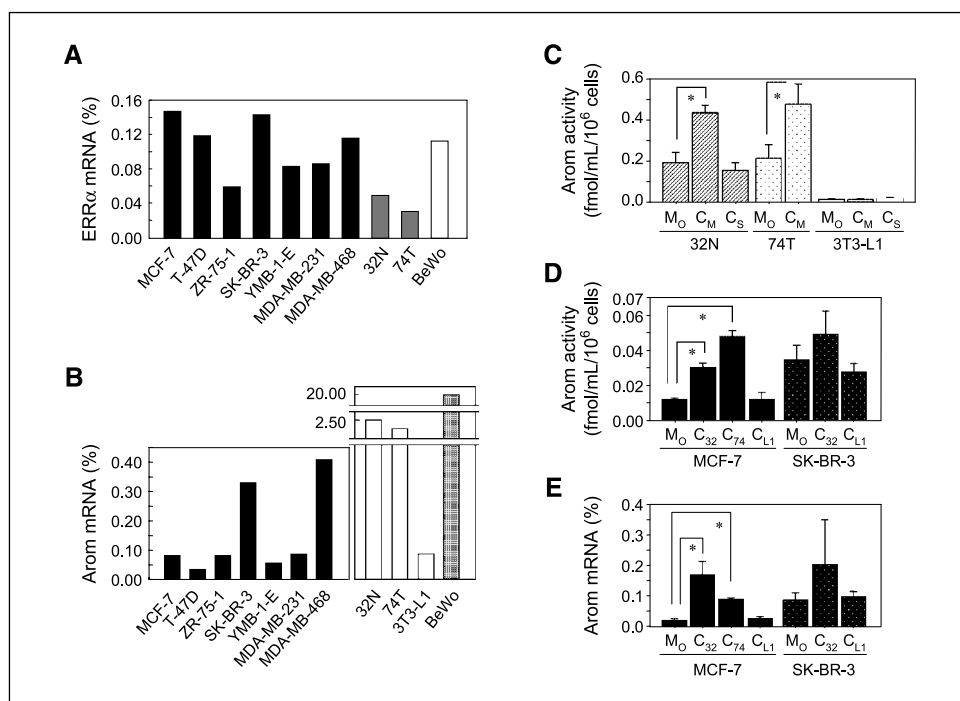
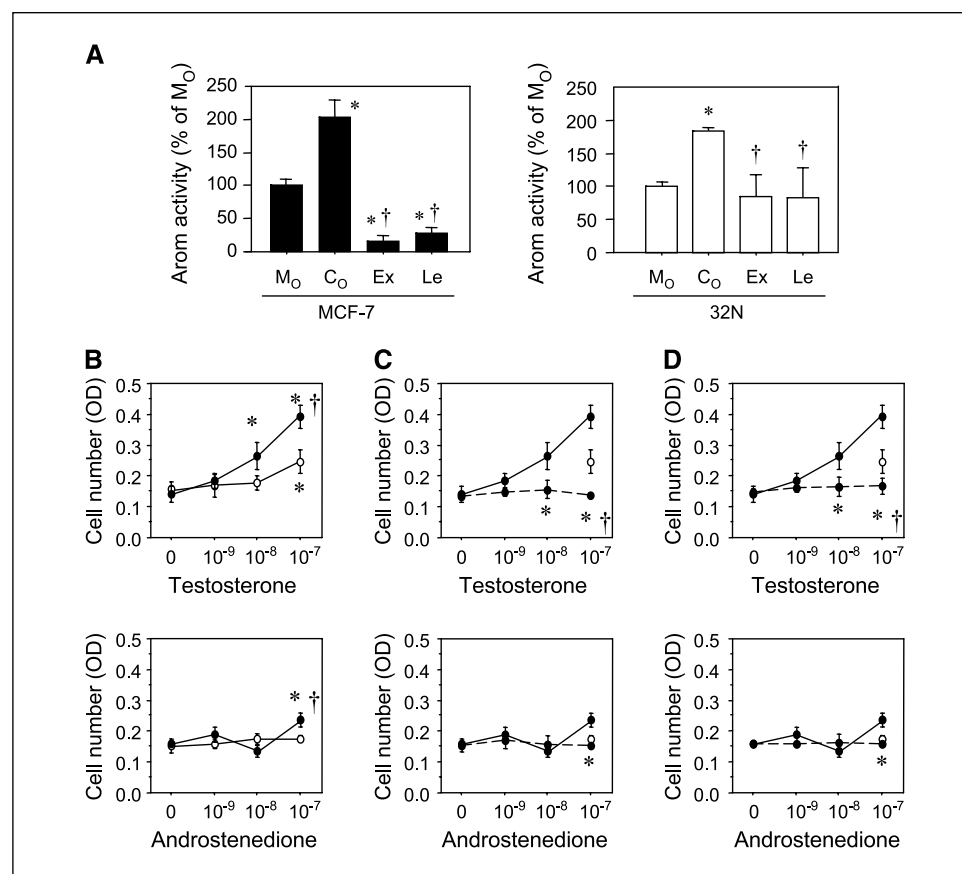


Figure 3. A, ERR α mRNA was detected in all breast carcinoma cell lines examined in this study, stromal 32N and 74T, and BeWo (in percent of RPL13A). B, aromatase levels in breast carcinoma cell lines and breast cancer-derived stromal 32N and 74T cells, mouse preadipocyte 3T3-L1 cells, and placental BeWo cells. C-E, the effects of coculture on aromatase mRNA level and enzyme activity in breast carcinoma and stromal cells. C, aromatase mRNA level in 32N, 74T, and 3T3-L1 cells; D, aromatase enzyme activity in MCF-7 and SK-BR-3 cells; E, aromatase mRNA level in MCF-7 or SK-BR-3 cells; M₀, monoculture; C, after coculture with 32N (C₃₂), 74T (C₇₄), 3T3-L1 (C_{L1}), MCF-7 (C_M), or SK-BR-3 (C_S) cells.

Figure 4. A, the effects of aromatase inhibitors on aromatase enzyme activities in MCF-7 (left) and 32N cells (right). Ex, treatment with 10^{-8} mol/L exemestane; Le, treatment with 10^{-8} mol/L letrozole; *, $P < 0.05$ versus M_0 ; †, $P < 0.05$ versus C_{32} or C_M ; in percent of RPL13A (aromatase mRNA level) or femtomoles per milliliter per 10^6 cells (aromatase enzyme activity). B, cell proliferation of MCF-7_{CO} and MCF-7 cells treated with testosterone (top) and androstenedione (bottom). ●, MCF-7_{CO}; ○, MCF-7 cells; *, $P < 0.05$ versus vehicle control (0 nmol/L); †, $P < 0.05$ versus MCF-7 treated with 10 or 100 nmol/L androgens. C and D, cell proliferation following treatment with the aromatase inhibitors, (C) exemestane and (D) letrozole. ●, solid line, MCF-7_{CO}; ●, dashed line, MCF-7_{CO} treated with aromatase inhibitor; ○, MCF-7; *, $P < 0.05$ versus MCF-7_{CO}; †, $P < 0.05$ versus MCF-7.



MCF7_{CO} was also higher than that observed with MCF-7 cells alone, following the treatment with testosterone (10^{-9} mol/L; 288.4 mg/mL) as the aromatase substrate.

The results of 17β -hydroxysteroid dehydrogenases mRNA levels in cocultures and monocultures of MCF-7 cells are summarized in Table 2C. The 17β -HSD1 mRNA level in MCF-7_{CO} was significantly higher than that found in MCF-7 cells. The 17β -HSD2 mRNA level in MCF7_{CO} was significantly lower than that found in MCF-7 cells. There were no significant increases or decreases of other types of 17β -HSD types such as types 3, 4, and 5 in MCF-7_{CO}.

Discussion

Lu et al. (33) previously reported localization of aromatase protein and mRNA using immunohistochemistry and mRNA *in situ* hybridization, respectively, in the same breast cancer specimens. They showed that the aromatase protein and mRNA expression was predominantly detected in parenchymal cells (33). Further studies have shown the localization of aromatase protein using immunohistochemistry in human breast tissues (6–9), but the reported results have been markedly different between the different laboratories. These discrepancies in the cellular localization of intratumoral aromatase expression in human breast carcinomas may be due to the different aromatase antibodies and probe sequences employed in these different studies. This is the first study to show aromatase mRNA expression in the different cellular compartments of human breast carcinoma tissues following isolation using laser capture microscopy and subsequent qPCR analysis. The results of the combined LCM/qPCR study in our study

showed that intratumoral aromatase in human breast cancer is expressed in both stromal and carcinoma or parenchymal components of the tissue. This finding confirms results of previous immunohistochemical study using the monoclonal antibody 677 done in 10% formalin-fixed and paraffin-embedded materials. We have also done immunohistochemistry using this monoclonal antibody 677 in frozen tissue sections adjacent to those in which LCM/qPCR analysis was conducted and have evaluated the immunoreactivity using the scoring system developed on 10% formalin-fixed and paraffin-embedded tissue specimens (14). Parenchymal/carcinoma cells are the major cell types of breast cancer tissues, and estrogens produced *in situ* by carcinoma cells could effectively activate the ER in the nuclei of carcinoma cells via an autocrine mechanism. Stromal cells also express aromatase, and thus, this source of intratumoral estrogen biosynthesis and subsequent estrogen-dependent cell proliferation is considered significant. An important aspect is the potential interplay that may exist between the carcinoma and stromal cell compartments. Therefore, we did further characterization of the potential regulation of aromatase in parenchymal or carcinoma cells in human breast cancer tissues.

Aromatase mRNA in adipose stromal cells was shown to be increased by coculture with MCF-7 cells (27). The results of previously reported studies all showed that various aromatase-stimulating factors (ASF), such as IL-1, IL-6, IL-11, IL-6 soluble receptor, tumor necrosis factor- α and prostaglandin E_2 , etc. (10, 34, 35), are released from parenchymal or carcinoma cells, which resulted in the up-regulation of aromatase expression in stromal cells (including adipostromal cells, preadipocytes, or fibroblasts) within

Table 2.**(A) Estrogen production levels in MCF-7 and MCF-7_{CO}**

| Cells | Substrates | Concentrations ^{*,†} | |
|---------------------|------------|-------------------------------|------|
| | | E2 | E1 |
| MCF-7 | AD | 0.02 | 0.18 |
| | TST | 0.07 | 0.16 |
| MCF-7 _{CO} | AD | 0.04 | 0.34 |
| | TST | 0.15 | 0.05 |

(B) Estrogen production ratios in MCF-7 and MCF-7_{CO}

| Cells | Substrates | Conversion (%)* | |
|---------------------|------------|-----------------|--------|
| | | AD→E1 | TST→E2 |
| MCF-7 | AD | 0.05 | 0.28 |
| | TST | 0.63 | 0.02 |
| MCF-7 _{CO} | AD | 0.09 | 0.41 |
| | TST | 0.44 | 0.04 |

(C) mRNA levels of 17 β -HSDs and 5 α -reductases in MCF-7 and MCF-7_{CO} cells

| Cells | mRNA levels (mean \pm SD), % | | | | |
|---------------------|--------------------------------|-------------------|-------------------|-------------------|-------------------|
| | HSD17B1 | HSD17B2 | HSD17B3 | HSD17B4 | HSD17B5 |
| MCF-7 | 0.178 \pm 0.076 | 0.040 \pm 0.005 | 0.066 \pm 0.114 | 0.016 \pm 0.027 | 0.187 \pm 0.091 |
| MCF-7 _{CO} | 0.553 \pm 0.047 | 0.009 \pm 0.016 | 0.170 \pm 0.061 | 0.014 \pm 0.024 | 0.323 \pm 0.172 |
| <i>P</i> | 0.047 ‡ | 0.030* | 0.233 | 0.927 | 0.293 |

Abbreviations: AD, androstenedione; E2, estradiol; E1, estrone; TST, testosterone; MCF-7_{CO}, after coculture with 32N for 24 h.

*Mean of $n = 2$.

† In picograms per milliliter per 10⁶ cells.

‡ Significant difference; $n = 3$; in percent of RPL13A.

human breast carcinoma tissues. Therefore, the possible effects of ASFs secreted from stromal cells on aromatase expression in parenchymal or carcinoma cells remained largely unknown possibly due to the reported low or no detectable aromatase enzyme levels of MCF-7 cells (26–29). Exogenous human epidermal growth factor (26), transforming growth factor (26), and keratinocyte growth factor (36) have all been reported to stimulate aromatase activity in MCF-7 cells. In our study, coculture of MCF-7 cells with stromal cells derived from human breast carcinoma tissues markedly induced the aromatase expression and activity in MCF-7 cells. Although we have not investigated which of the specific ASFs influence the expression of endogenous aromatase in MCF-7 cells, the ASFs described above could all be secreted from fibroblastic stromal cells adjacent to carcinoma, especially at the sites of stromal invasion, and have an influence. The precise ASFs involved would require further investigations to clarify their actual role.

The signals mediated through various nuclear receptors, including orphan nuclear receptors, have been postulated to influence aromatase activity and expression in breast carcinoma or parenchymal cells (10, 11, 37, 38). In our study, we showed that aromatase expression was closely associated with ERR α , VDR, GCNF, and TR β expression in breast carcinoma cells. ERR α has been previously

reported to be a positive regulator for aromatase gene expression in SK-BR-3 breast carcinoma cells (11), but not in the 3T3-L1 preadipocyte cells (39). ERR α is also known to bind to silencer elements located between promoter I.3 and II of the aromatase gene, which results in increased aromatase transcript levels in SK-BR-3 cells (11). The results of our study, including microarray expression profiling analyses, as well as those of a previously reported study (23), all showed that ERR α expression is positively correlated with aromatase expression in human breast carcinoma or parenchymal cells, but not with stromal cells or whole breast tissue containing both carcinoma and stromal cells (23). Therefore, ERR α is considered a key regulator of intratumoral estrogen production in human breast carcinoma or parenchymal cells, but not necessarily in stromal cells. However, there have been no studies reported on the possible correlations between aromatase gene expression and VDR, TR β , or GCNF genes. VDR was well known as one of the estrogen target genes with an estrogen-responsive element in its promoter lesion (40). Therefore, the expression of VDR may be induced by estrogens synthesized by aromatase in human breast carcinoma cells. A statistically significant negative correlation was detected between aromatase and GCNF. GCNF was reported to be able to inhibit ERR α -mediated transactivation in human placental

choriocarcinoma cell lines (41). Therefore, GCNF may inhibit aromatase expression through the down-regulation of ERR α -mediated transactivation. The significance of TR β expression in breast carcinoma cells has remained largely unknown. The mRNA levels of ERR α , TR β , and GCNF in MCF-7 cells were, however, not increased following the coculture with stromal cells. Therefore, ASFs released from stromal cells may increase aromatase mRNA transcript levels through an interaction with these nuclear receptors above, but it awaits further investigations for clarification.

In the estrogen production assays, a relatively high rate of conversion into estradiol was detected in MCF-7_{CO}, but not MCF-7 cells alone following the treatment with testosterone as the aromatase substrate. Aromatase catalyzes testosterone into estradiol but not into estrone, whereas estrone is converted from androstenedione by aromatase (42). In our study, the level of estrone was decreased in MCF-7_{CO} compared with MCF-7 alone following the treatment with testosterone. Estrone is therefore considered to be converted from testosterone via androstenedione by 17 β -HSD2. We also showed that the low level of 17 β -HSD2 mRNA and the high level of 17 β -HSD1 were detected in MCF-7_{CO}, but not MCF-7 alone. These findings all suggest that the rate of conversion into estrone in MCF-7_{CO} was lower than that in MCF-7 cells alone following the treatment with testosterone but not with androstenedione as the aromatase substrate. Interleukins were also shown to regulate 17 β -HSDs mRNA and activity in human breast carcinoma cells (39, 43). Therefore, 17 β -HSD1 expression may also be regulated by the factors released from stromal or carcinoma cells in addition to aromatase.

The coculture system used in our study could provide important information with regard to the evaluation of the intratumoral microenvironment such as cell-cell interactions and these soluble factors (44). It is also considered a useful model for examining the effect of medications on breast carcinoma patients. The adhesive microenvironment, including cell-matrix interactions and cell-cell interactions, plays an important role in the development of both the normal mammary gland and breast carcinoma (44). The results of our study also showed that aromatase inhibitors were more effective on aromatization increased by coculture of MCF-7 cells than in stromal 32N cells alone. These results suggest that the aromatase in parenchymal or carcinoma cells is the more important target for aromatase inhibitors in breast cancer patients because of the more effective decreases in aromatase activity in carcinoma cells. Therefore, it is clinically important to evaluate the localization of aromatase in breast carcinoma tissues to evaluate the possible efficacy of aromatase inhibitor treatment. However, it awaits further examinations to clarify all the interactions among the different cell types in human breast carcinomas.

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