0013-7227/96/$03.00/0
Endocrinology

Vol. 137, No. 7
Printed in U.S.A.

Expression of Aromatase Protein and Messenger Ribonucleic Acid in Tumor Epithelial Cells and Evidence of Functional Significance of Locally Produced Estrogen in Human Breast Cancers*

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ABSTRACT

The expression of aromatase by breast cancer cells and the role of locally produced estrogen in the stimulation of tumor growth has been controversial. The present study was performed to determine the site of aromatization in human breast cancers, using both immunocytochemistry and *in situ* hybridization. The functional significance of locally produced estrogens on growth of the tumor was addressed by measuring aromatase activity and a marker of proliferation (PCNA score). In addition, histocultures of some tumors were carried out to investigate whether testosterone aromatization could stimulate tumor proliferation. Of the 19 tumors investigated, 10 (52.6%) showed significant immunoreactivity to antiaromatase antibody in the cytoplasm of tumor epithelial cells and in surrounding stromal cells. The presence of aromatase mRNA detected by ISH was also located in tumor epithelial cells and stromal cell, and the pattern of expression was the same as with immunocytochemistry. In the ten tumors that showed immunoreaction to aromatase, the average aromatase activity measured in cryosections was 286.5 ± 18.6 (SE) fmol estrogen/mg protein·h, whereas in nine tumors with weak aromatase immunoreaction, the enzyme activity was 154.7 ± 19.3 (SE) fmol estrogen/mg protein·h ($P < 0.05$). The mean PCNA score was 33.8 ± 5.1 (SE)% in strongly stained tumors and 20.8 ± 2.0 (SE)% in weakly stained tumors ($P < 0.05$). Aromatase activity level and PCNA score were significantly correlated. In histoculture of four tumors, estradiol increased the incorporation of [^{3}H]-thymidine into DNA. In two of these tumors, aromatase activity was high and [^{3}H]-thymidine incorporation into DNA was also stimulated by testosterone. In the other two tumors that had low aromatase activity, no such stimulation occurred with testosterone. The results indicate that aromatase is expressed mainly in tumor epithelial cells and that sufficient amounts of estrogen are synthesized by the tumor to produce a proliferative response. It is concluded that estrogen synthesis by cancer cells could play a important role in promoting growth in a significant proportion of breast tumors. (*Endocrinology* **137:** 3061–3068, 1996)

MULTIPLE factors, environmental and endocrine, play a role in the etiology of breast cancer. Among endogenous factors, estrogens are considered to be important in the process of tumor promotion (1). However, the highest incidence of breast cancer occurs in postmenopausal women whose ovarian production of estrogens has ceased. In older women, estrogen synthesis in peripheral tissues such as skin, muscle, and adipose is increased and is the main source of circulating estrogens (2, 3). Estrogens are synthesized by aromatization of androgen substrates via a series of reactions catalyzed by cytochrome P450 aromatase (P-450arom). Although there is no consistent evidence of abnormality in plasma estrogen concentrations in breast cancer patients, concentrations of estradiol in breast tissues from postmenopausal women have been found to be 10 times higher than those in plasma (4, 5). However, the contribution of estrogen produced locally by the normal breast and breast cancers is controversial (6–8). Aromatase activity measured in human breast tumor homogenates has been reported to be relatively low and was thought to be insufficient to catalyze the formation of biologically meaningful amounts of estradiol (9). Studies to determine the location of expression of the enzyme in breast tumors by immunocytochemistry (ICC) using polyclonal antibodies to aromatase have led to conflicting results. Some have reported aromatization only in specialized stromal cells (10, 11). Only one study identified aromatase in epithelial cells (12). However, clinical observations that there may be a correlation between intratumoral aromatization and tumor response to inhibition of estrogen synthesis in patients treated with aminoglutethimide suggest that local production of estrogens might have an important role in tumor proliferation (13, 14).

Because of the above discrepancies, we have used a combination of approaches in the study reported here to resolve these issues. To verify the pattern of immunocytochemical reaction to aromatase, *in situ* hybridization (ISH) was used employing sequence specific probes because even highly specific antibodies have been known to react with some proteins other than those against which they are raised. This can occur because of antigenic similarities. In addition, we measured tumor aromatase activity using cryosections. This was done initially to relate activity to expression detected in

Received January 2, 1996.
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* Part of this work was presented at the 77th Annual Meeting of The Endocrine Society, Washington, D.C., June 14–17, 1995. This work was supported by NIH Grant CA-62483 (to A.B.) and CA-58739 (to J.W.).

frozen sections by immunocytochemitry (15). However, we have found that enzyme activity is severalfold higher in cryosections than homogenates. To determine the functional significance of aromatase in the tumor, cell proliferation was assessed by image analysis and scoring of the immunoreaction to PCNA, a marker of cellular proliferative activity. We also measured the proliferative response of some tumors to steroids by determining the incorporation of [^{3}H]-thymidine into DNA during histoculture.

Materials and Methods

Cases

Nineteen cases of breast cancer were provided by the Human Tissue Resource Service (Department of Pathology, University of Maryland School of Medicine, Baltimore, MD), within 3–4 h of surgery. Fifteen tumors were infiltrating ductal carcinoma, three were intraductal carcinomas and one was an infiltrating lobular carcinoma. Tissues were immediately rinsed in cold (4 C) 0.1 M PBS, dissected into approximately 1-cm^3 pieces, and blotted dry on filter paper. For biochemical studies, a portion of the tissue was coated with Tissue-Tek O.C.T. cryoprotectant embedding medium (Miles Labs, Naperville, IL), immersed in liquid nitrogen, and stored at −70 C until used. For immunohistochemical studies, the remaining portion of the tissue was fixed in Histochoice MB (Amresco, Solon, OH) for 24 h and embedded in paraffin. Measurement of ER and PR concentrations were carried out as part of the patient's routine diagnosis. The medical records of the patients were reviewed to obtain clinical and histopathological information. Because some of the cases were relatively recent, no significant follow-up was available.

Antibodies

The antiaromatase mouse monoclonal (kindly provided by E. Simpson, University of Texas Southwestern Medical Center, Dallas, TX) was prepared against human placental aromatase (16) and purified using preabsorption on AVID-AL minicolumn (RAININ, Woburn, MA). The specificity of the column purified antibody identified a single band in extracts of several different types of aromatase containing cells with mol wt of about 54K on Western blots (data not shown), which corresponded to that of pure aromatase. The mouse monoclonal antibody PC-10 used to identify PCNA was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Immunohistochemical study

Five-micrometer thick sections were cut from paraffin-embedded tissues and mounted on slides coated with chrome-alum gel. After deparaffination and rehydration in xylene and ethanol, they were placed in 10 mM citrate buffer (pH 6.0) and processed in a microwave oven for three periods of 5 min each. After cooling, the sections were washed twice in distilled water. After incubation for 20 min in 1% hydrogen peroxide/methanol, the slides were washed twice in distilled water and twice in 0.05 M Tris-HCL buffer (pH 7.4). Nonspecific sites were blocked by incubating with 1% normal goat serum in 0.05 M Tris-HCL buffer. After decanting the reagents, the sections were covered with antiaromatase antibody at 8 μg/ml. The samples were incubated overnight in a humid chamber at 4 C. After three washes in 0.05 M Tris-HCL buffer, the slides were incubated for 30 min with biotinylated secondary IgG (Dako Corp, CA) at room temperature. Sections were washed again and incubated with streptavidin peroxidase (Dako) for 30 min at room temperature. After three washes, the sections were incubated with 0.05% 3,3-diaminobenzidine hydrochloride, 0.016% hydrogen peroxide in buffer for 9 min. Sections were washed again and counterstained with Mayer's hematoxylin and coverslipped. Control sections were incubated with 0.01 M PBS and normal mouse IgG instead of primary antibodies. Additional experiments to demonstrate the specificity of the antiaromatase antibody were carried out. The monoclonal antiaromatase antibody (8 μg/ml) was preincubated with Affigel 10 (Bio-Rad, CA) alone or with Affigel 10 that had been coupled with pure human placental aromatase (200 μg/ml). The Affigel was removed by centrifugation, and the supernatants were incubated with sections of placenta and breast cancers.

The results of ICC were evaluated by estimating the percentage of tumor cells with positive cytoplasmic stain. Breast cancers were classified as aromatase positive if at least 15% of the cells were stained, whereas tumors with weak reactivity or staining in less than 15% of the cells were interpreted as negative.

To identify PCNA, the above procedure was used except the anti-aromatase antibody was replaced by the anti-PCNA in 1:200 dilution. Two methods were used to measure the immunostaining of PCNA. The intensity of the immunostaining was scored as a percentage of PCNA-positive cancer cell nuclei (17–20). Faint, diffuse, nuclear staining seen in some tissue sections was not included in the PCNA score. Two sections from each sample were examined, and the entire area of each was screened to find the region with the maximum number of positively stained nuclei. At least 500 nuclei were counted (magnification ×400) from each slide. The number of cells with nuclear staining was independently determined in each tumor by two observers. Aromatase activity assays were performed without knowledge of the immunocytochemical results. The staining intensity of PCNA was also measured using an Axiovert 10 Zeiss microscope with a CCD 72 video camera and Perceptics image analyzer after the section was screened to find 2–3 regions with the strongest immunostaining.

Preparation of radiolabeled cDNA probe with asymmetric PCR

Plasmid that contains a 2.5-kb segment of human aromatase cDNA was kindly provided by Dr. E Simpson (University of Texas Southwestern Medical Center, Dallas, TX) (21). The primers for the aromatase gene bracketed bases 1215–1507 (293-bp PCR product) of the human sequence (22) and were the same as previously used (23). The primer sequences were 5′-^{1215}GAATATTGGAAGGATGCACAGACT^{1238}-3′ and 5′-^{1507}GGGTAAAGATCATTTCCAGCATGT^{1484}-3′. PCR was carried out according to GeneAmp DNA Amplification Reagent Kit instructions (Boehringer Mannheim, IN) using 1 μl of plasmid DNA (1 μg) as template. One microliter of PCR product was added to 5 μl of [^{33}P]dCTP (2000 Ci/mmol; 10 mM dCTP), 1 μl of downstream or upstream primer (25 μM in water), 5 μl 10×PCR buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris/HCl, pH 8.3), 1 μl of dCTP (10 mM), 2 μl dNTP minus dCTP (10 mM each of dATP, dTTP, dGTP), 1 U of *Taq* polymerase and 34 μl water. The mixture was overlaid with 50 μl mineral oil and 50 cycles of PCR were performed (annealing, 45 sec at 60 C; elongation, 90 sec at 72 C; denaturation, 30 sec at 94 C) in an MJ Research (Cambridge, MA) Programmable Thermal Controller. The final 72 C incubation was extended for an additional 10 min to maximize strand completion and the high molecular weight products were isolated by chromatography through G-50 Sephadex.

ISH to mRNA of P450arom

Frozen sections were fixed for 5 min in 4% paraformaldehyde. The sections were then incubated in hybridization solution (50% formamide, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM heparin, 10 mM DTT, 10 mg/ml calf thymus DNA, and 0.5 mg/ml tRNA) containing 2.5×10^5 to 5×10^5 cpm of labeled antisense or sense probes overnight at 42 C. The slides were washed in 1 × SSC at room temperature for 30 min and washed in 0.1 × SSC overnight at room temperature. Slides were then subjected to a 60-min wash at 60 C in 0.1 × SSC and transferred to 0.1 × SSC at room temperature. Slides were dried, dipped in emulsion, and kept in a black slide box for 7–14 days. After development, slides were counterstained with standard hematoxylin.

Aromatase activity

Aromatase activity was measured in cryosections of all breast cancers using the method described for the testis (15). Cryosections (40–50 10 μm frozen sections) of each breast cancer were cut and pooled into a chilled vial and were either assayed immediately or stored at −70 C for not more than 2 days before assay. Pooled cryosections were suspended in 0.6 ml 0.1 M phosphate buffer. To compare the activity in the cryosections with

that of homogenates, portions of four breast carcinoma tissues (0.2 g) were finely minced with scissors and homogenized in 0.6 ml 0.1 M phosphate buffer. A 0.5 ml aliquot of suspension or homogenate was mixed with 1 μCi of [1β-^{3}H]-androstenedione (24.56/mmol) (New England Nuclear-DuPont, Boston, MA) and 0.1 ml of an NADPH generating system (NADP 5 mg, glucose-6-phosphate 20 mg, glucose-6-phosphate dehydrogenase 25 IU/in 0.9 ml phosphate buffer) vortexed vigorously and incubated at 37 C for 2 h. Sections or homogenates incubated without the cofactor were used as a negative controls for each sample. Incubations were terminated by placing the tubes in an ice water bath and adding 2 ml chloroform. Estrogen production was measured from the resulting ^3H_2O produced by release of tritium from the C-1β position during aromatization of the radiolabeled substrate (24, 25). In representative samples, cryosections were also incubated as above with [1,2,6,7(α, β)-^{3}H]-androstenedione (87.5 Ci/mmol) (10 μCi each reaction) in the presence/absence of aromatase inhibitor 4-hydroxyandrostenedione (4-OHA) prepared in our laboratory (24). The products were isolated by methods described previously (15, 24, 25). After incubation, unlabeled (50 μg) and ^{14}C-labeled (6000 dpm) estrone (E_1), estradiol (E_2) were added to act as recovery markers. The steroids were extracted with 4 × 2 ml of ether and separated on 250 μm silica gel plates (Sigma Chemical Co., St. Louis, MO) using the solvent system, ether:hexane (3:1). Estrone and estradiol were eluted from the plate with ether and 40% of each sample was counted in a liquid scintillation counter. The remaining 60% of the E_1 and E_2 spot were acetylated with 1 ml pyridine and 1 ml acetic anhydride incubated at 65 C for 2 h. The reagents were evaporated and the samples chromatographed on TLC again, and areas eluted corresponding to the acetates were eluted and counted in the liquid scintillation counter. Protein concentrations of all cryosection suspensions or homogenates were determined by the method of Lowry (26).

Histoculture of breast tissue and [^{3}H]-thymidine incorporation assay

Fresh tumor tissue was washed with HBSS and divided into 1–2 mm cubes. Four to five pieces were placed on the top of each hydrated gelatin sponge (Upjohn, MI) and incubated in a 24-well microplate in 1 ml Eagle's MEM per well without phenol red with 5% charcoal-dextran treated calf serum alone, containing 1) vehicle; 2) estradiol 10^{-8} M; 3) testosterone 10^{-6} M; 4) dihydrotestosterone (DHT) 10^{-6} M (all from Sigma); 5) an aromatase inhibitor, 4-OHA 10^{-6} M; 6) testosterone plus 4-OHA. Cultures were maintained at 37 C in an incubator with 5% CO_2. After 7 days, the tissue blocks were transferred to new sponges and incubated with [^{3}H]-thymidine (NEN-DuPont) labeling medium (2 μCi/ml/well) for 3 days. The tissue blocks were transferred to 1.5 ml microtube, and 0.5 ml collagenase solution (0.1 mg/ml in 10 mM TE buffer) was added and incubated at 37 C overnight. The collagenase solution was discarded, 0.1 ml proteinase K solution (0.05 mg/ml of 10 mM TE, 0.5% SDS) was added to the tube and incubated at 37 C for 2–3 h. Then, DNA was extracted and dissolved in 0.05 ml TE (pH 7.8). The amount of DNA was quantitated by spectrophotometry (OD_{260}), and the radioactivity of [^{3}H]-thymidine incorporated into newly synthesized DNA was measured in a liquid scintillation counter.

Estrogen and progesterone receptor assay

Routine estrogen and progesterone receptor binding assays were performed by the Department of Pathology, University of Maryland Medical System. Aliquots of cytosol were obtained after tumor homogenization and ultracentrifugation and the ER and PR content determined from the radiolabeled steroid bound after removal of the unbound steroid with dextran-coated charcoal. Values were analyzed by the method of Scatchard and ≥9 fmol/mg ER were considered to be positive.

Statistical analysis

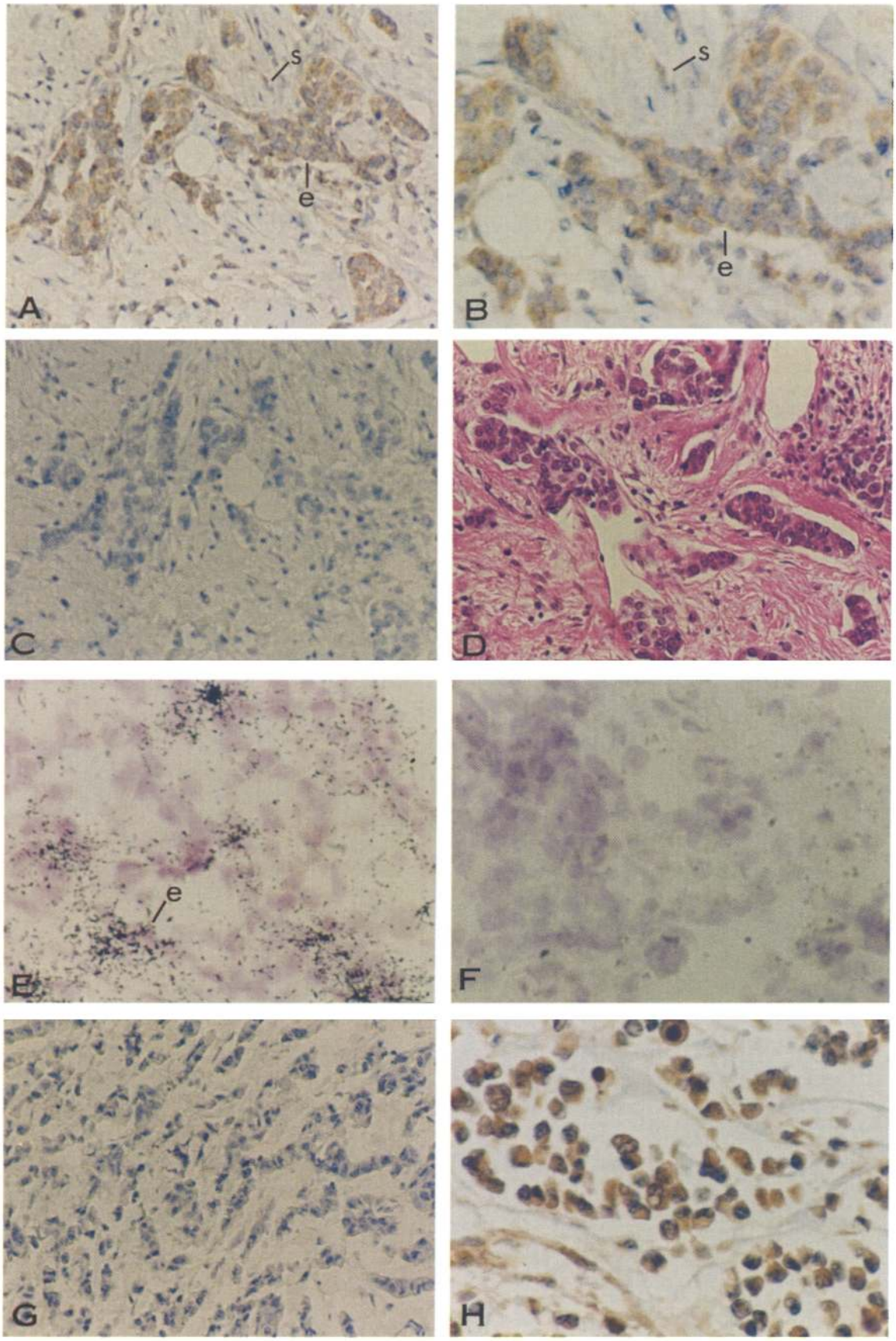
The relationship between the aromatase activity, ER, PR status, and PCNA score of samples classified as having strong or weak aromatase immunoreaction was analyzed using the unpaired Student's *t* test. The relationship between aromatase activity and PCNA score in all tumors was determined using a linear correlation test. The relationship between aromatase immunoreactivity and menopausal status, hormonal receptor values, and histological carcinoma subtypes were performed using the Pearson chi-square test.

Results

A strong immunoreaction to the aromatase monoclonal antibody was observed in the cytoplasm of tumor epithelial cells and surrounding stromal cell in 10 of 19 breast cancers. However, in all cases, the tumor epithelial cells comprised the major portion of cells reactive to the antiaromatase antibody. The nine other tumors with weak or no immunostaining were interpreted as negative. Microphotograph representatives of aromatase positive and negative breast tumors are displayed in Fig. 1 A, B, and G, respectively. When antiaromatase antibody was replaced by an irrelevant mouse IgG, no immunoreaction was seen in the same tissue (Fig. 1C). When the antiaromatase monoclonal antibody was preincubated with uncoupled Affigel 10 only, there was no effect on immunostain production. Absorption of the antibody to Affigel-aromatase beads abolished staining in both the placental and tumor samples. As identified previously, exclusive and intense staining of the syncytiotrophoblast of the chorionic villi of placenta (25), confined staining of the interstitial Leydig cells of the testis (15), ovarian granulosa cells (27) and intense staining of epithelial cells of ducts in the TDLUs of normal breast (28) was observed with this antibody using the above conditions and also indicated that it was reacting solely with aromatase.

Three out of four breast cancers used for ISH showed strong labeling of P-450arom mRNA in the tumor cells (Fig. 1E). The labeling pattern of ISH paralleled that of the ICC. Labeling was intense and almost all tumor cells were labeled, whereas moderate labeling was observed in the surrounding stromal cells. There was almost no labeling in the tumor epithelial cells using sense probe (Fig. 1F). Aromatase activity in these tumors was 344.1, 248.8, and 238.1 fmol estrogen/mg protein·h and was in the range of the mean aromatase activity in cryosections of the ten tumors that showed strong immunoreaction to the aromatase antibody (286.5 ± 18.6 SE fmol estrogen/mg protein·h). The fourth tumor showed weak immunoreaction. Aromatase activity of this sample was 88.5 fmol estrogen/mg protein·h, which was similar to the mean aromatase activity (154.7 ± 19.3 SE fmol estrogen/mg protein·h) in the nine tumors showing weak aromatase immunoreaction. Weak specific cellular labeling was seen in this tumor sample. The difference between the aromatase activity in tumors with strong immunoreactivity was significantly greater than in those with weak immunoreactivity ($P < 0.05$).

The aromatase activity in cryosections was compared with that of homogenates of four breast tumors by the radiometric assay using [1β-^{3}H]-androstenedione as substrate. The enzyme activity was found to be severalfold greater in cryosections than in the corresponding homogenates (Fig. 2). Aromatase activity in cryosections of representative tumors was also measured using the isolated product assay after incubation with [1,2,6,7-^{3}H]-androstenedione as substrate. As shown in Table 1, there was agreement between values measured by the two methods. Furthermore, when sections of the



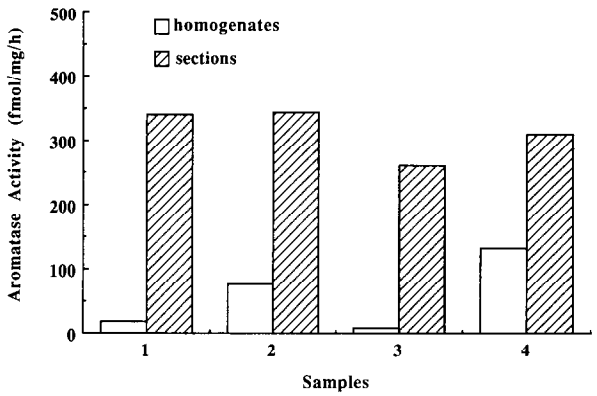


FIG. 2. Comparison of aromatase activity in cryosections *vs.* homogenates of human breast cancers. Aromatase activity was measured by the ^3H_2O release assay described in *Materials and Methods* using 40–50 frozen sections (10 μm) or 200 mg homogenized tumor tissue of each of 4 breast cancers. All measurements were performed in duplicate in the same assay.

TABLE 1. Comparison of aromatase activity in cryosections of breast tumors measured by the radiometric method and the isolated product assay

Tumor no.	Estrogen production (fmol/mg protein/h)		
	^3H_2O	Estrone	Estradiol
1	332.1	29.9	361.4
1 + 4-OHA	0	0.5	5.5
2	133.9	121.9	52.4
2 + 4-OHA	0	9.4	14.7

Cryosections (40–50 10 μm frozen sections) of each breast cancer were incubated with 1 μCi of [1β^3H]-androstenedione and 0.1 ml of an NADPH-generating system at 37 C for 2 h. Incubations with aromatase inhibitor (4-OHA) or without the cofactor were used as negative controls. Estrogen production was measured from the resulting ^3H_2O produced by release of tritium from the C-1β position during aromatization. The value for the sample incubated with 4-OHA was subtracted. Cryosections were also incubated as above with [1,2,6,7(α,β)^{3}H]-androstenedione (10 μCi each reaction). After incubation, unlabeled and ^{14}C-labeled estrone (E_1) and estradiol (E_2) were added. The steroids were extracted and separated on TLC with ether:hexane (3:1). Estrone and estradiol were eluted, acetylated, and chromatographed on TLC again. The corresponding acetates were eluted and radioactivity measured.

tumor were incubated testosterone plus aromatase inhibitor, 4-OHA, estrogen production was inhibited by 90–95%.

In nineteen cases, the expression of PCNA was observed within the nuclei of tumor epithelial cells (Fig. 1H). However, the score and intensity of immunoreaction to PCNA were higher in the 10 aromatase positive tumors than for the 9 weakly staining samples. In aromatase positive tumors, the mean intensity (7143.0 ± 1321.4 SE) was 1.8-fold higher than tumors with low aromatase expression and activity (3964.8 ± 352.8 SE). The mean ± (SE) of PCNA score of the two groups was 33.8 ± 5.1% and 20.8 ± 2.0%, respectively ($P < 0.05$). Thus, the PCNA score and the mean aromatase activity for strongly immunoreactive tumors were significantly higher than weakly immunoreactive tumors ($P < 0.05$) (Fig. 3). In addition, the aromatase activity for all tumors correlated significantly with PCNA score ($r = 0.61$, $P < 0.005$). However, statistical analysis using the Pearson chi-square test showed no significant correlation between aromatase activity or PCNA and the ER status, the PR status, the menopausal status, or the histological carcinoma subtypes ($P > 0.05$) (Table 2).

Four breast carcinomas were available immediately after surgery and were used in histoculture to determine their response to steroids. Addition of estradiol to the culture medium increased [^{3}H]-thymidine incorporation into DNA, more than 2-fold in all four tumors (Fig. 4). In two of the samples, addition of testosterone also increased incorporation of [^{3}H]-thymidine into DNA more than 2-fold. This stimulation by testosterone was inhibited by aromatase inhibitor 4-OHA (24). The levels of aromatase activity in these two samples were 248.8 and 238.1 fmol estrogen/mg protein·h and similar to the mean aromatase activity of the strongly stained tumors. In the other two samples, no effect of testosterone was observed. In these tumors, aromatase activity was 88.5 and 90.2 fmol estrogen/mg protein·h, and in the same range as the mean activity of tumors with weak immunoreaction. There was no effect of DHT, progesterone, or 4-OHA alone on [^{3}H]-thymidine incorporation into DNA in any of the samples (data not shown).

Discussion

The results described demonstrate the presence of aromatase in breast tumor epithelial and stromal cells. Discrete clusters of cells, mainly tumor cells, were immunoreactive to antiaromatase antibody and hybridized to sequence specific probes for P-450arom mRNA. Furthermore, tumor epithelial cells also reacted with the antibody to PCNA, a maker of proliferation, in contrast to other cells. The functional significance of tumor aromatase was indicated by the correlation between measurements of aromatase activity and PCNA in the tumor samples. Thus, the PCNA score was significantly higher in tumors with high aromatase activity. This was supported by the finding that estrogens were synthesized from testosterone in amounts sufficient to stimulate proliferation ([^{3}H]-thymidine incorporation into DNA) in histoculture.

In previous investigations (10–12), polyclonal antibodies prepared against human placental aromatase were used to locate aromatase expression in breast tumors. Two out of three of these studies reported that only stromal cells, but not

FIG. 1. Aromatase, PCNA expression in human breast tumors. Aromatase expression was detected by immunohistochemistry (ABC) with antihuman, anti-PCNA monoclonal antibodies in fixed paraffin-embedded human breast cancer sections. Aromatase mRNA detected using ISH was performed on frozen sections with ^{32}P-labeled single-strand cDNA probes. All sections were counterstained with hematoxylin. A and B, Strong intense staining is present in tumor epithelial cells (e). Moderate staining can be seen in surrounding stomal cells(s) (×200, ×400). C, Control section of human breast carcinoma stained with irrelevant mouse IgG. Neither tumor cells nor stromal cells are stained (×200). D, H+E staining of the breast carcinoma (×200). E, The tumor epithelial cells are strongly hybridized to aromatase mRNA antisense probe (×400). F, Control section of human breast carcinoma hybridized with sense probe (×400). G, Breast carcinoma not immunoreactive to antiaromatase antibody counterstained with hematoxylin. The background stain was minimal as in most tumors studied (×200). H, Most of cell nuclei of tumor epithelial cells are positive for PCNA (×400).

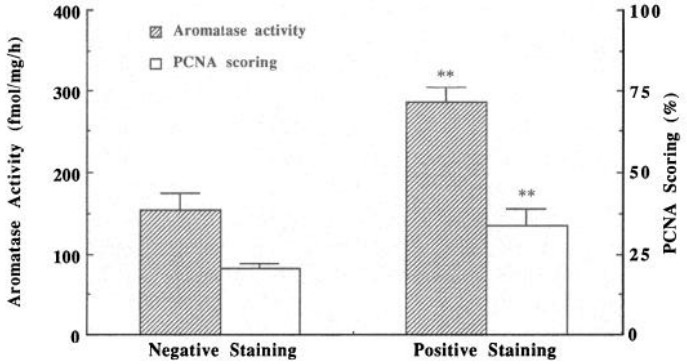


FIG. 3. Comparison of the mean aromatase activity, PCNA score in tumor sections. Comparison of mean aromatase activity, PCNA score in tumor sections that had either a positive (n = 10) or negative (n = 9) reaction to the aromatase monoclonal antibody. Aromatase activity was measured by the ^3H_2O release assay using 40–50 frozen sections (10 μm) of each breast cancer. All measurements were performed in duplicate in the same assay. PCNA score was assessed as a percentage of PCNA-positive immunostaining cancer cell nuclei. The PCNA scores of aromatase positive tumors were significantly higher than in aromatase negative tumors; **, $P < 0.05$.

TABLE 2. Characteristics of 19 breast cancer patients and tumor aromatase expression

	Aromatase positive	Aromatase negative	Total
Menopausal status			
Premenopause	3	3	6
Postmenopause	7	6	13
Estrogen receptor			
Positive	6	3	9
Negative	4	6	10
Progesterone receptor			
Positive	5	3	8
Negative	5	6	11
Histology			
Infiltrating ductal	8	7	15
Intraductal	2	1	3
Infiltrating lobular	0	1	1

There was no significant difference between any groups (Chi-square test, $P > 0.05$).

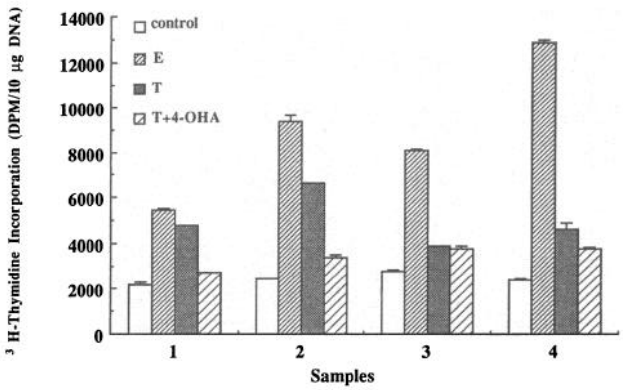


FIG. 4. The effect of estradiol (E_2), testosterone (T), and 4-hydroxyandrostenedione (4-OHA) on the growth of cultured human breast cancer tissue. Four to five tumor pieces (1–2 mm) were incubated on a gelatin sponge for 7 days in medium alone with E_2 10^{-8} M, T 10^{-6} M, 4-OHA 10^{-6} M or T+4-OHA 10^{-6} M. The tissue blocks were then incubated with [^{3}H]-thymidine for 3 days. The amount of DNA radioactivity was measured. The aromatase activity of sample 1 and sample 2, which showed positive immunoreaction to antiaromatase monoclonal antibody was 248.8, 238.1 fmol/mg protein·h, respectively, is shown, as well as the aromatase activity of samples 3 and 4, which showed negative immunoreaction were 88.5, 90.2 fmol estrogen/mg protein·h, respectively.

tumor epithelial cells, contained aromatase in breast cancers (10, 11). In the study by Esteban *et al.* (12), aromatase was identified in both tumor epithelial cells and stromal cells. In the present study, we used several different methods to verify our immunocytochemical findings. First, the column purified, antihuman aromatase, monoclonal antibody identified a single band with mol wt of about 54K on Western blots that corresponded to that of pure aromatase (data not shown) in extracts of several different types of aromatase containing cells. Second, this antibody identified the same structures in frozen sections and in Histochoice MB-fixed, paraffin-embedded tissue sections of the same samples. Thus, the pattern of staining in paraffin-embedded tissue was identical to those of our previous reports of frozen sections from human placenta (25), testis (15), ovary (27), and normal human breast (28). However, the quality of the sections and the intensity of the stain reaction was better in the microwaved, paraffin-embedded samples. Third, when the antibody was neutralized with antigen (pure aromatase), no staining of any structures was observed in either placental or breast tumor sections. Fourth, because aromatase is not secreted from its site of synthesis (29), and as we previously reported identification of P-450arom mRNA in normal breast and breast cancer tissue (23), we used ISH to P-450arom mRNA with sequence specific probes to verify aromatase expression in tumors. The concordance between the ICC and ISH results clearly indicates that aromatase is expressed mainly in epithelial cells. The discrepancy in the results of immunolocalization between our study and earlier reports may be due to differences in specificity of the primary antibodies and/or methodology used for ICC (10, 11).

Previous studies used homogenates to measure aromatase activity in human breast cancers (6, 7, 30–32). Because of the low levels of aromatase activity reported, the contribution of *in situ* estrogen synthesis to the total level of hormone found in breast carcinomas has been the subject of debate (9). However, it appears that the level of enzyme activity may have been previously underestimated in earlier studies. We found that greater activity could be detected when cryosections, rather than homogenates, were incubated in the biochemical aromatase assay (Fig. 2) and that sufficient estrogen was produced to activate the ER as suggested by the correlation between aromatase activity and PCNA, and by [^{3}H]-thymidine incorporation into DNA in histoculture.

Although we have not investigated the reason for the higher aromatase activity in the cryosections than homogenates, the use of thin tissue sections into which substrate and cofactors easily diffuse and which remain frozen until they are incubated, may preserve the enzyme activity. In contrast, the process of homogenization before the incubation may lead to loss of some activity. When aromatase activity was measured by the isolated product assay shown in Table 1, it is interesting that, at least in the tumor with high aromatase activity, the product was mainly estradiol, the more potent estrogen, rather than estrone that is

the circulating estrogen in postmenopausal women. Although further studies are necessary, this supports previous findings by others and that estradiol is locally produced (4, 5).

Tumor epithelial cells, rather than other cell types, were found to react with the antibody to PCNA as well as the aromatase antibody. PCNA is a highly conserved 36-kDa, acidic, nuclear polypeptide that plays a critical role in the initiation of cell proliferation (33). Its expression is elevated almost exclusively during S-phase of the cell cycle (34). The validity of using PCNA immunostaining on paraffin embedded human breast cancers as a marker of cell proliferation, and its quantitation by the score method has been reported in numerous studies (17–20, 35). Although PCNA immunostaining was evident in most tumor samples, the finding that the score correlated with aromatase activity provides support for the local production of estrogen by some tumors having functional significance.

Further evidence that aromatase activity in some tumors is sufficient to synthesize estrogen in amounts that produced a proliferative response is provided by an increase in [^{3}H]-thymidine incorporation into DNA in histoculture of tumors incubated with testosterone. This conclusion was supported by 1) inhibition of the testosterone stimulation in incubations with the aromatase inhibitor, and 2) lack of effect of the nonaromatizable androgen DHT.

Aromatase immunolocalization in the normal human breast was also determined by the same antibody as well as by a polyclonal antibody prepared to a sequence of the aromatase protein (27). The immunoreaction to both antibodies produced the same pattern of staining that was in concordance with messenger RNA (mRNA) expression of aromatase examined by ISH in normal human breast. Aromatase mRNA and protein was localized in epithelial cells in TDLUs, also in surrounding stromal cells, epithelial cells lining cysts and scattered stromal cells in parenchyma outside the TDLUs. The average aromatase activity for seven normal human breast samples was 163.0 fmol/mg estrogen protein· h. Thus, it appears that aromatase is expressed in the normal breast epithelium, the cells that are the site of origin of carcinomas. Although the role of locally produced estrogen in the normal human breast is unclear at the present time, it is probably not a feature acquired during transformation to malignancy. In breast cancer, aromatase could have an autocrine function whereby the tumor epithelial cells directly stimulate their growth by producing estrogens. The production of estrogens by tumor cells might also have paracrine actions by increasing the synthesis or release of growth factors and proteins from surrounding cells that can act to stimulate the tumor cells. There is evidence that tumor aromatase activity *in vivo* may be enhanced by several cytokines, growth factors, and proteins (36–39). IGF-1, IL-1, and IL-6 have been reported to stimulate aromatase activity in cultured tumor derived fibroblasts suggesting paracrine systems that may also stimulate tumor aromatase activity and proliferation.

Although there was a significant correlation between high aromatase activity and cell proliferation, not all tumors were estrogen receptor positive. Indeed, no consistent relationship between tumor aromatase activity and estrogen receptor status was found in previous studies (32, 40, 41). We found a tendency for tumors possessing relatively high aromatase activity to be estrogen receptor positive. Thus, 6 of 9 estrogen receptor positive tumors expressed aromatase, whereas 6 of 10 estrogen receptor negative tumors lacked aromatase. Miller *et al.* (41) also observed that there was a significant trend that favored an association between aromatase activity and the presence of ER, although tumors expressing active aromatase included both ER+ and ER- tumors. It is difficult to postulate a role for estrogens produced *in situ* in tumors lacking estrogen receptors. One mechanism that might account for a proliferative response to aromatase products in apparently ER negative tumors could be that estrogen receptors are down regulated in the presence of high intracellular concentrations of estrogen and therefore not measurable by the binding assay. Alternatively, the locally synthesized estrogens may bind to different receptors than those routinely detected. For example, Matsuda *et al.* (42) reported that estrogens mimic the ligand for the erbB2 protein and estrogen played an important role in erbB2-mediating signaling. The c-erbB2 protoncogene encodes a 185-kDa transmembrane glycoprotein that shows significant structural similarity to the EGF receptor (43, 44). The c-erbB2 is frequently amplified and the protein product is overexpressed in a number of mammary carcinomas (45–48). Ligand-dependent activation of the kinase activity is thought to contribute to tumor progression.

In conclusion, the study provides several lines of evidence that aromatase is expressed mainly in epithelial cells of breast cancers as well as surrounding stromal cells. Thus, the distribution of the immunoreaction to two different antiaromatase antibodies was the same in each tissue. In addition, the monoclonal antibody produced the same pattern of staining irrespective of whether frozen sections or microwaved, paraffin embedded tissue were used with little or no nonspecific staining. ISH with sequence specific probes occurred in the same location as the immunoreaction to the antiaromatase antibodies. Significantly higher aromatase activity was measured in sections in which strong immunoreaction was apparent. The finding that aromatase in the tumor is correlated with increase in a marker of proliferation (PCNA) suggests that tumor aromatase has functional significance. In a small number of samples of breast tumors in histoculture, proliferation was stimulated as indicated by increased [^{3}H]-thymidine incorporation into DNA, when testosterone was incubated with tumors containing high aromatase activity compared with those with low aromatase activity. We concluded that estrogen produced by tumors may have been underestimated in previous studies and that its synthesis by cancer cells could play an important role in promoting growth in a significant proportion of breast cancers.

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