Intratumoral Aromatase as a Prognostic Factor in Human Breast Carcinoma[1]

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ABSTRACT

Intratumoral aromatase activity (AA) was measured in 145 samples of human primary breast carcinoma using the tritiated water release assay which quantifies the tritium lost to water during the aromatization of 1β-[^{3}H]androstenedione to estrone. Significant AA was detected in 91/145 (63%) tumors. The possibility of a relationship between AA and a variety of clinical prognostic factors such as estrogen receptors, menopausal status, site, size, and histological grade of tumor was investigated. Possible relationship with time to relapse, overall survival, and survival of patients after relapse were also studied to determine whether intratumoral AA itself was of any prognostic value. There was no relationship between AA and tumor size, site, nodal status, menopausal status or estrogen receptors. However there was a significant correlation between AA and histological grade with an excess of AA-positive tumors having high grade ($P = 0.03$). There was no significant relationship between AA and overall survival ($P > 0.1$), but there was a marginal inverse correlation between AA and time to relapse ($P < 0.1$). A statistically significant correlation was found between AA and survival of patients after relapse ($P < 0.05$).

INTRODUCTION

Estrogens are considered to play a major role in promoting the growth of hormone-dependent breast carcinomas (1, 2). The major sites of estrogen biosynthesis are the ovarian granulosa cells in premenopausal women and adipose tissue in postmenopausal women (3). The conversion of androgens to estrogens is catalyzed by the enzyme complex aromatase and is termed aromatization.

It has been demonstrated that certain breast carcinomas have the ability to produce estrogen through intratumoral aromatase (4–8), indicating that these tumors may possess a possible autocrine or paracrine function in which locally produced estrogen may interact with estrogen receptors in the same or adjacent cells. The levels of detected estrogen synthesis are low but since this occurs within the tumor, the proximity of this source of estrogen production may be significant in stimulating the growth of those hormone-dependent carcinomas. This may be particularly important in postmenopausal women in whom the ovarian contribution of estrogen is markedly diminished or abolished (9, 10). At present very little is known about the clinical or biological significance of intratumoral estrogen synthesis. Miller and coworkers (11) found a significant correlation between the potential of breast carcinoma aromatization and the clinical response to endocrine therapy with the aromatase inhibitor aminoglutethimide, and this study has been supported by the work of Bezwoda and colleagues (12). Other investigators (4, 13–15) found no association of aromatase with estrogen or progesterone receptors. The present study was carried out to quantify the intratumoral AA[3] and to determine whether the

activity was related to a variety of clinical prognostic factors such as estrogen receptors, menopausal status, size, site, and histological grade of tumor. We also compared the aromatase content of the tumor with time to relapse and overall survival of patients to determine whether it was of any value in prognosis.

MATERIALS AND METHODS

Patients and Tissue Samples. One hundred and forty-five primary breast carcinoma samples were obtained from patients who underwent excisional biopsy or mastectomy between 1981 and 1987 in The Royal Marsden Hospital and St. George's Hospital Medical School. The specimens were immediately frozen (at $-196°C$) after removal and stored in a liquid nitrogen bank until analysis. Tumor samples were also examined before and after freezing to evaluate the stability of the enzyme. Medical records were available from 113 patients and were analyzed retrospectively. 83/113 (73%) patients had some type of adjuvant therapy. The data were recorded as follows: date of birth, date of diagnosis, menopausal status, primary and adjuvant treatment received, site and size of tumor (T1: tumor measuring less than 2 cm; T2: between 2 and 5 cm; T3: greater than 5 cm; and T4: any size with direct extension to chest wall or skin), nodal status (axillary lymph nodes involved or not involved histologically), histological grade [assessed by one investigator, B. G., using the criteria established by Bloom and Richards (16)], date of relapse, and death. The duration of follow-up ranged from 1 to 5 years, with a median of 4 years. In all cases diagnosis of carcinoma was confirmed histologically. Estrogen receptors were determined in 126 cases (method described below).

Aromatase Assay. We have employed the tritiated water assay to measure AA [method of Thompson and Siiteri (17) modified by Ackerman *et al.* (18)]. This is based on the quantification of tritiated water released from 1β-[^{3}H]androstenedione after aromatization to estrone. The radiolabeled androstenedione (specific activity, 28 Ci/mmol) was obtained from New England Nuclear, tested previously for radiochemical purity by thin-layer chromatography in a dichloromethane:diethyl ether system, 9:1, then scanned using a Berthold LB-283 radiochromatogram scanner. Radioactive areas corresponding to standard androstenedione were scraped off and eluted. NADPH (tetrasodium salt), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from BCL, Boehringer Mannheim House, Lewes, UK. Activated charcoal and unlabeled androstenedione were obtained from Sigma Chemical Co. Ltd., Poole, Dorset. Precoated silica gel 60 F254 thin-layer chromatography plates ("Merck" 5715), solvents and other chemicals were purchased from BDH Chemicals Ltd., Poole.

Breast carcinoma tissue (0.5 g) was trimmed from adherent fat, finely minced with scissors, and homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA by a Potter Elvehjem homogenizer at 2500 rpm using three passes with a cooling interval in between. The homogenates were then centrifuged at $1500 \times g$ for 15 min at 4°C. One ml of supernatant was transferred to a reaction vial and incubated with 50 μl of 1β-[^{3}H]androstenedione (1 μCi, 36 pmol). This concentration provided saturating conditions for the study of AA. The reaction was started by adding 0.5 ml of NADPH-generating system [NADPH, 4 mg, glucose-6-phosphate, 8.5 mg, and glucose-6-phosphate dehydrogenase, 5 μl (2 units/ml)] per sample. The incubation was carried out in a water bath at 37°C in air for 30 and 60 min and terminated by transferring 0.75 ml of the mixture at the end of each incubation time to 5 ml of ethyl acetate containing 0.25 ml of 1 mM mercuric chloride. The tubes were allowed to stand for 30 min at room temperature then centrifuged at $1500 \times g$ for 15 min. The supernatant

(ethyl acetate extracts) was discarded and 0.5 ml of trichloracetic acid (30%) was added to the aqueous phase to precipitate the protein. The tubes were left for 30 min then centrifuged as before, and 1 ml of the supernatant was added to 1 ml of a 5% charcoal-0.5% dextran solution. The mixture was incubated for 30 min at 4°C then centrifuged at 1500 × g for 20 min at 4°C. After centrifugation 1 ml of the clear supernatant was added to 10 ml of scintillant fluid and counted in a Packard 300-c scintillation counter. Negative controls consisted of boiled homogenate of the same breast carcinoma and blank tube (substrate and buffer without tissue homogenate). We also incubated breast carcinoma homogenates with and without 4×10^{-6} M 4-OHA in three different assay batches to validate the AA measurement through the inhibition of activity by this specific aromatase inhibitor. The results were expressed as percentage conversion of androstenedione to estrone and as picomoles of estrone formed per gram of protein per hour. The mean values obtained from boiled homogenate and blank control tubes were subtracted from all other samples.

The protein concentration was determined by a modification of Lowry's method (19).

Estrogen Receptors. ER were determined by the dextran-coated charcoal steroid binding assay described previously (20). Values of 15 fmol/mg/protein or greater were classified as positive (ER+) and were estimated by Scatchard plot analysis.

Statistical Analysis. Relapse and survival curves were estimated according to Kaplan and Meier (21), and the significance of any differences was calculated by the Log-rank method (22). The independence of prognostic factors was tested by constructing two-dimensional contingency tables and the statistical significance was determined by χ^2 test.

RESULTS

The limit of detection of the assay was 0.02% of conversion of androstenedione to estrone and 1.5 pmol of estrone formed per gram protein/hour. These values were twofold higher than the background values, and any value equal to or greater than these were considered to be positive for AA. The negative controls (boiled tissue homogenate or blank tube: labeled substrate and buffer without tissue homogenate) were always around 0.01%. The addition of 4-OHA to the homogenates of breast carcinoma reduced AA to below 0.02% in all cases. Table 1 compares the results of aromatization by 10 samples of breast carcinomas assayed fresh and at different intervals after freezing. There was no overall significant difference in AA measured in the tumor homogenates before and after freezing. In some samples the activity was similar before and after freezing for different lengths of time. In others the AA was slightly reduced in the frozen samples. In one sample the AA was considerably higher in the frozen sample assayed after being frozen for 2 weeks. The interassay variation was 20.5%.

Significant AA activity was detected in 91/145 (63%) of tumors when the results were expressed in percentage conversion of androstenedione to estrogen and in 73/145 (52%) when the results were expressed as pmol estrone/g protein/h. The AA ranged from 0.02 to 0.7% of conversion and from 2 to 81 pmol estrone/g protein/h. To relate AA with clinical and prognostic factors we have expressed aromatase activity results in pmol of estrone formed/g protein/h.

In Table 2 the relationship between AA and menopausal status, tumor size, site of primary within the breast, histological grade, and ER status are shown. The only factor demonstrating a significant relationship with AA was histological grade, since we observed an excess of high grade tumors (poorly differentiated carcinomas) which were AA-positive ($P = 0.03$). The relationship of AA with time to relapse, overall survival, and survival of relapsed patients was analyzed by life-table (Figs. 1–3). Patients (83/113) received some type of adjuvant therapy

Table 1 *Comparison between AA in human breast carcinomas before and after freezing. Interassay variation = 20.5%; P = N/S*

Sample	Status of tissue	Time (weeks) since freezing	Percentage converted to estrone
1	Fresh		0.03
	Frozen	4 weeks	0.03
	Frozen	14	0.03
2	Fresh		0.03
	Frozen	4	0.02
	Frozen	14	0.02
3	Fresh		0.18
	Frozen	4	0.12
	Frozen	14	0.16
4	Fresh		0.1
	Frozen	2	0.08
5	Fresh		0.05
	Frozen	4	0.03
6	Fresh		0.14
	Frozen	8	0.16
	Frozen	16	0.4
7	Fresh		0.1
	Frozen	5	0.1
8	Fresh		0.02
	Frozen	2	0.4
9	Fresh		<0.02
	Frozen	27	0.02
10	Fresh		0.2
	Frozen	1	0.06

Table 2 *Correlation between aromatase activity and menopausal status, site, size, grade or tumor, and ER content*

Subject	Factor	AA Status Active	AA Status Inactive	Total	χ^2	P=
Menopausal status	Pre	22 (61%)	14	36	1.15	N/S
	Post	59 (71%)	24	83		
Site	UOQ	34 (63%)	20	54	1.14	N/S
	Others	42 (72%)	16	58		
Size	T1	7 (50%)	7	14	4.41	N/S
	T2	51 (74%)	18	69		
	T3	11 (58%)	8	19		
	T4	12 (71%)	5	17		
Grade	1	0 (0%)	3	3		
	2	35 (63%)	21	56	7.116	0.03
	3	34 (85%)	6	40		
ER	+	40 (58%)	29	69	0.4	N/S[b]
	−	36 (63%)	21	57		

[a] UOQ, upper outer quadrant.
[b] N/S, P=0.50.

and 30 (36%) relapsed. Eleven out of 30 (36%) that were not given adjuvant therapy also relapsed. No significant difference was found in the overall survival of AA-positive and AA-negative group. However time to relapse was marginally shorter in the AA-negative group ($0.05 < P < 0.10$) but the survival of patients after relapse appeared longer ($P < 0.05$). Fig. 4 shows the comparison of time to relapse between AA/ER(+) and AA/ER(−). The rate of relapse was similar in both of these groups ($P > 0.1$).

DISCUSSION

The metabolic conversion of androgens to estrogens by human breast carcinomas has been reported by several groups (4–8). The data presented in this study confirm those earlier published findings. AA in our investigation ranged from 2 to 80 pmol estrone/g protein/h in active tumors and this figure is in agreement with the data reported by Lipton (14) that was 5–
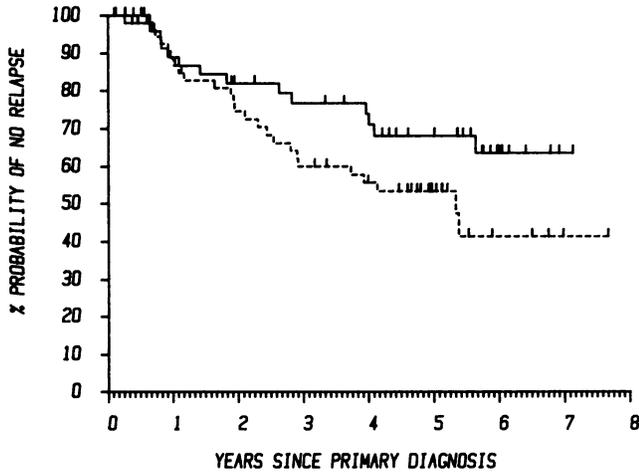
Fig. 1. Relapse rates of 52 patients with breast carcinoma containing AA (———) and 61 without AA (- - -). $\chi^2 = 3.01; P < 0.1$.
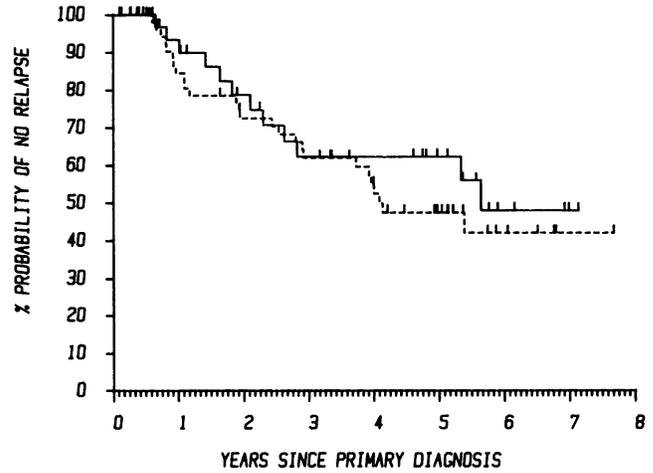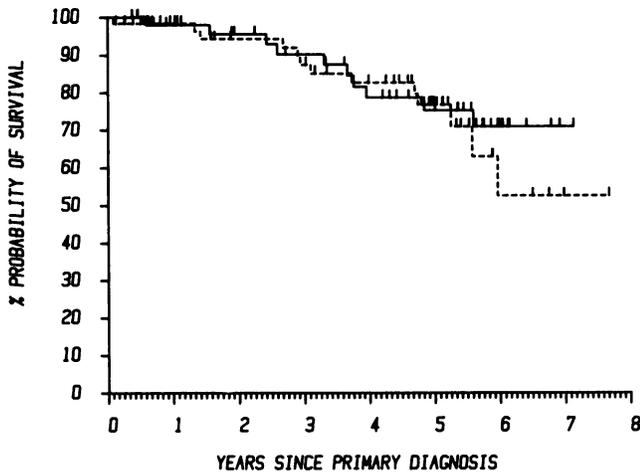

Fig. 2. Overall survival rates of 52 patients with breast carcinoma containing AA (———) and 61 without AA (- - -). $\chi^2 = 0.38; P > 0.1$.
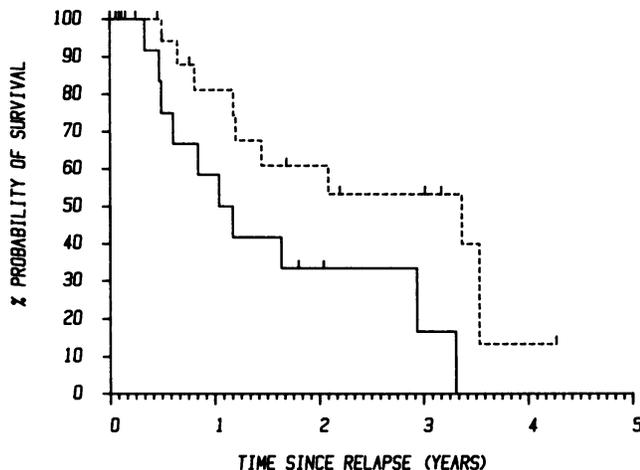

Fig. 3. Survival of relapse patients in 14 cases of breast carcinoma containing AA (———) and 24 without AA (- - -). $\chi^2 = 3.97; P < 0.05$.

70.5 pmol/g protein/h and Santen (23) 5–80 pmol/g protein/h. However the figures obtained by Bezwoda (13) were significantly higher than the ones referred above (80–730 pmol/g protein/h).

The use of frozen breast carcinoma samples in this study did not show significantly less AA in comparison with fresh tissue

Fig. 4. Relapse rates of 37 patients with breast carcinoma who are AA/ER+ (———) versus 61 patients who are not (- - -). $\chi^2 = 0.56; P > 0.1$.

which validates the use of liquid nitrogen-stored tissue to measure AA in breast carcinomas. The small degree of variability may be due to tumor heterogeneity.

The suppression of aromatization in tumors to below the assay detection limit achieved by the addition of 4-OHA (4 × 10^{-6} M) and the linearity of conversion of androstenedione to estrone over the incubation time also support the validity of this method to measure AA. We also validated this method by measuring AA in 21 samples of breast carcinomas employing a modification of the method outlined by Miller et al. (4) and Newton et al. (24) which measures the radiolabeled E1 and E2 after aromatization. No significant difference in the levels of AA was noted between both methods (results not shown). The lack of correlation between AA and ER in our investigation is in agreement with results obtained by Lipton et al. (14) who analyzed a similar number of cases and by other investigators (13, 16) that have reported smaller series. In contrast Miller et al. (25) found a good correlation between ER and AA in 54 cases. The different methodology for measuring AA used in that study may contribute to this disparity. Our report did not confirm Lipton's (14) suggestion that AA content is lower in large tumors. The good correlation we found between AA and poorly differentiated breast carcinomas has not previously been recorded but the importance of intratumoral oestrogen synthesis to the growth of these tumors is in doubt since in general high grade breast carcinomas have lower ER levels (26). While this manuscript was being prepared Lipton et al. (27) reported a negative correlation between AA and the degree of differentiation of the tumor. However he analyzed a smaller series and employed a different methodology to estimate the degree of differentiation of the tumor. The focus of interest in this investigation was to determine the relationship between AA with survival, and disease free interval (time to relapse). 60% of patients in this series developed local recurrence or distant metastatic disease over a period of 6 years. The Log-rank test was marginally significant showing an inverse correlation between the relapse rate and AA. Patients (78/114) were given adjuvant endocrine therapy (aminoglutethimide or tamoxifen). However such treatment, independent of the type of drug given, had no effect on the overall survival or time to relapse ($\chi^2 = 1.47; P > 0.1$). No association was found between AA and overall survival, but there was a minor statistical difference after relapse, the patients with tumors containing AA surviving shorter than those with aromatase-negative tumors with a rate of survival of 32% at 2 years as against 62% in the AA-negative

group. This result was contrary to expectations since tumors that retain the capacity for oestrogen synthesis might be expected to grow faster and therefore relapse earlier but have a longer survival after relapse by responding better to endocrine therapy. However some of our patients had received adjuvant therapy and in order to answer this question we now need to study only patients who had not received such therapy. It will be difficult to determine the relationships between survival after relapse, effects of endocrine treatment, and AA without a controled randomized trial. We are uncertain of the reason for finding a correlation between the AA and the degree of anaplasia of the tumor. Further, larger series will be needed to confirm this finding. In summary, therefore, our present study fails to record a relationship between AA and survival following diagnosis of breast cancer. This suggests that the ability to locally synthesize oestrogen has little consequence for the prognosis of patients with breast cancer. The marginally significant difference in relapse-free and post-relapse survival will need to be further investigated in a larger series of patients.

ACKNOWLEDGMENTS

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