ONCOLOGY

Activity of Aromatase in Breast Cancer Tissue: Role of the Cell Substrate

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The activity of aromatase (estrogen synthetase) in tumor tissue of breast cancer patients was investigated by biochemical and immunohistochemical methods. The detection of medium or high enzyme activity in tumor tissue was virtually the same for both methods. According to immunohistochemical analysis, aromatase activity was localized mainly in malignant epithelial cells, being reliably higher in the tumors of patients in menopause than in those whose cycle was intact. Doubts about the biological role of aromatase in breast cancer tissue may be dispelled if the tissue topography of the enzyme and the individual ratio of epithelial to stromal cellular elements in individual neoplasms of the breast are taken into account.

Key Words: *aromatase; breast cancer*

The first studies of aromatase (estrogen synthetase) activity (AA) in breast cancer tissue, published in mid-Seventies [11], mainly demonstrated the presence of the enzyme in tumor tissue and confirmed the ability of this tissue to synthesize estrogens independently. Prominent studies during the last decade have presented findings on more than 1000 patients and have emphasized the clinical and biological significance of determining AA in breast tumor tissue [4,8,10]. Although some authorities consider this parameter as an important test, characterizing, among other things, the estrogen receptivity of tumors and their sensitivity to hormone therapy [2,4,10], opinions on this matter differ. One possible cause of disagreement (and sometimes even quite contrary opinions) [7,8,10] may be that in all the studies cited AA in tumor tissue homogenates was determined biochemically, making it impossible to identify the role of individual cell components in the local (intratumor) biosynthesis of estrogens. Meanwhile, two studies using the immunohistochemical method yielded remarkably different results. In one of these reports interaction of antibodies with aromatase (and accordingly specific staining) was detected mainly in the epithelial tumor cells [6], whereas in the other it was more often observed in the stromal (spindle) cell elements [13].

We set out to form our own ideas about AA in breast cancer tissue by using both biochemical and immunohistochemical methods.

MATERIALS AND METHODS

Samples of tumor tissue obtained during surgery in 41 patients with breast cancer aged 25 to 77 years were examined. Clinical stages I and II were diagnosed in the majority of patients. In 27 patients AA was measured by the biochemical method [11,

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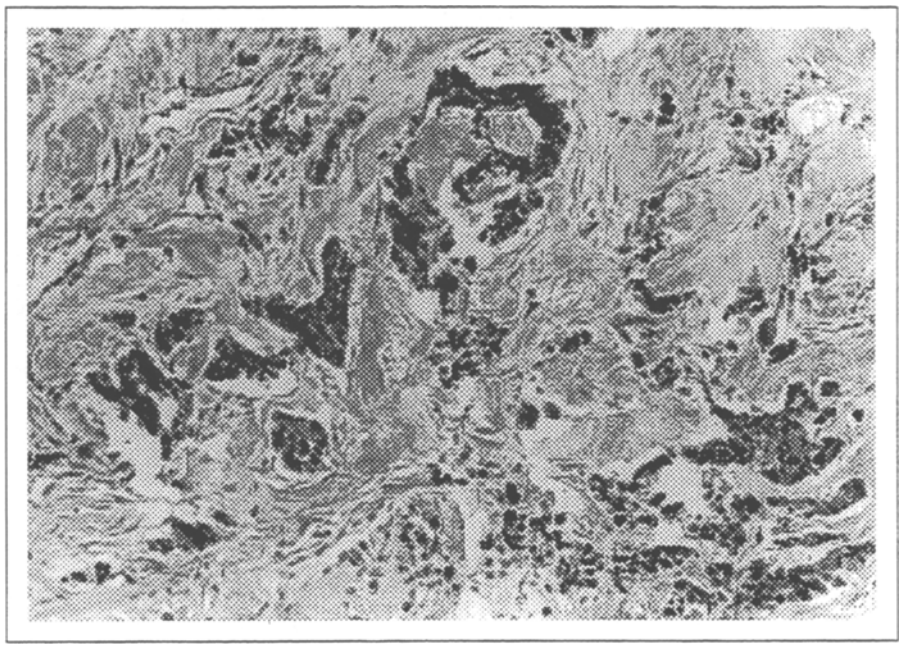

Fig. 1. Immunohistochemical detection of aromatase in breast cancer tissue (predominant staining of epithelial cells). Staining intensity ++++, ×80, hematoxylin staining of nuclei.

14] based on ^3H_2O release from the androgen precursor ^3H-1β-androstenedione (New England Nuclear, specific activity 25.4 Ci/mmol), in 22 by the immunohistochemical method [6] using rabbit polyclonal antibodies to aromatase, kindly made available by Prof. E. Simpson (USA), and in 3 patients by both methods.

For the biochemical method, tumor tissue delivered to the laboratory was immediately frozen in liquid nitrogen and thus stored prior to use. Before the study 100-200 mg of tissue were minced with scissors and ground in liquid nitrogen, the resulting powder was homogenized in 1-2 ml of 12.5 mM K-phosphate buffer (pH 7.5), and the homogenate was centrifuged for 15-20 min at 2000 rpm; 0.2 ml of supernatant was taken for the test, and 0.05 ml for protein measurement after Lowry. Besides the above-mentioned androgen precursor, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and dithiothreitol (Sigma or Boehringer Mannheim) were used in the test. The reaction mixture was incubated 1-2 h at 37°C, and "heavy" water and steroids were separated by successive extraction with cold chloroform and activated carbon (Serva). Radioactivity was measured in a dioxane scintillator using a Mark III liquid scintillation β-counter (Nuclear Chicago).

For the immunohistochemical method, tumor tissue (operation material) was fixed in liquid containing formaldehyde and embedded in paraffin. Before use, 5-μ slices were prepared from paraffin blocks and fixed on slides with alumgel. Then the following steps were performed: the preparations were freed of paraffin by xylol and alcohol and of endogenous peroxidase by 3% H_2O_2; nonspecific binding was eliminated by treatment with normal porcine serum diluted 1:5; antibodies to aromatase (20 μg/ml, 0.1 ml/2 μg per glass) were added; second (antirabbit) antibodies were added; treatment with PAP complex; development with diaminobenzidine (brown staining of aromatase complex); blue staining of cell nuclei with hematoxylin. Slices of human placental tissue were taken as a positive control of specificity; for negative control the step involving applying antibodies to aromatase was omitted. The immunohistochemical reaction was assessed semiquantitatively in scores (from 0 to ++++) as regards the staining intensity and fraction of stained cells.

The data were statistically processed using nonparametric and parametric statistical methods: Student's t and P tests and the Wilcoxon-Mann-Whitney test.

RESULTS

AA in breast cancer tissue determined by the biochemical method varied from 0 to 1900 fmol estrogens per 100 mg protein/h with a mean value of 263.2±111.7 fmol per 100 mg protein/h. All the data may be divided in 2 groups: with an AA of 0 to 68 fmol per 100 mg protein/h (8 patients, 36.3% of all biochemical measurements of AA) and from 90 to 1900 fmol per 100 mg protein/h (14 patients, 63.6% of cases). The value of 90 fmol per 100 mg protein/h was chosen as an arbitrary cutoff point separating patients with high/medium and low AA in tumor tissue. No clear-cut relationship was found between patient age, menstrual status (including the

TABLE 1. Distribution of AA in Tumor Tissue as Shown by the Histochemical Method

	Staining intensity				
	0	+	++	+++	++++
Number of cases (% of total)	3 (14)	5 (23)	4 (18)	2 (9)	8 (36)
Distribution by groups characterizing enzyme activity, %	36		64		

duration of menopause), body weight, history of smoking, and cancer stage, on the one hand, and AA values measured by the biochemical method in breast cancer tissue, on the other, although there was a tendency for AA to increase in patients aged over 50 and in those with stage I-II cancer (data not presented).

Using the immunohistochemical method, we discovered that specific staining indicating the presence of aromatase is observed more frequently in epithelial than in stromal cells (Fig. 1). Malignant cells stain more intensively than normal epithelial breast cells. A positive correlation was observed between staining intensity and the fraction of cells staining "for aromatase" in the same tumor tissue preparations (Spearman ranked correlation coefficient +0.87). In 12 out of 22 cases (54.5%) more than half of all cells were specifically stained in the preparations examined. Five variants of the reaction were distinguished by the intensity of staining. The distribution of these variants by their incidence is presented in Table 1. Evidently, the incidence of detection of tumors with a low (0 or +) or, on the contrary, a medium or high (++, +++, and ++++) AA corresponds to that detected by the biochemical method. Nonetheless, direct comparison of the results of AA measurements by the biochemical and immunohistochemical methods in the same 3 breast cancer patients did not show a reliable coincidence of the results.

According to the intensity of staining the immunohistochemical method showed a reliably higher AA in tumor tissue of menopausal patients (2.79± ±0.38, 12 patients) than in those with an intact cycle (1.50±0.40, 10 patients, $p<0.01$). Analysis of the relationship between the immunohistochemical findings and specific features of the patient organisms and the tumor proper did not reveal a clear-cut relationship between AA and menopause duration, excess weight, estrogen level in the blood, and disease stage. However, a significant increase was detected in AA in tumor tissue of patients aged over 50 (data not presented).

The results suggest that an AA, which may be considered medium or high is detected in approximately 60% of all breast tumors by both the biochemical and the immunohistochemical method. Although this conclusion is in line with some published reports [7,8,10], it should be noted that the values detected by the biochemical method and presented in the literature vary widely: from 0-70.5 fmol/mg protein/h [8] to 0.05-2.07 pmol/mg protein/h [2]. The threshold value, below which all AA values should be considered negative (low), varies as well. Some authorities [2] consider this threshold to be 50 fmol/mg protein/h, while for others [8] it is 5 fmol/mg protein/h, and for still others [7] 1.5 fmol/mg protein/h. Evidently, such differences have to do with features of the selected biochemical method, from the type of androgen precursor (androstenedione, testosterone) to the reaction principle ("heavy" water release, isolation of the estrogen fraction), as well as with the particular technique employed, such as thin-layer, column, or other type of chromatography, the method of extracting the reaction product, etc. Using the biochemical method, we selected 0.9 fmol/mg protein/h (90 fmol/100 mg protein/h) as the threshold, because with the two-hour incubation we used, it was for such or higher AA values that the number of counts per sample as a rule surpassed at least two times (in accordance with published recommendations [4,8]) the number of counts in the control (all reagents minus test tissue homogenate).

Classification on the basis of the above-mentioned criterion showed a correlation between the mean values of AA measured by the biochemical and immunohistochemical methods and the frequency of the subdivision into tumors with low and medium/high activity. However, individual comparison of the results in 3 cases failed to demonstrate such a correlation. One possible explanation for this discrepancy is as follows: the biochemical method measures AA in the tumor mass *in toto,* whereas the immunohistochemical method, it turned out, demonstrates a preference for one of the cellular elements contained in the tumor (for epithelial cells, according to our findings and some others [6]), and the individual ratio of different cell types in a tumor can affect the result. In the same way we can account for the fact that, although the immunohistological method showed an increase of AA in tumors of menopausal patients in our studies, no such relationship was, as a rule,

observed with the biochemical method [7,8,10]. Evidently, the elevation of AA in breast cancer tissue in menopausal patients results from its increase solely (or predominantly) in malignant epithelial cells, because, for example, the content of estrogens drops in menopause in fatty tissue of the breast (in which AA is localized mainly in the stromal cells but not the fat cells [5]), but not in breast tumor tissue [3].

The mere fact of a higher AA level in tumor material from patients in the menopausal period is additional proof of differences in the hormonal metabolic status and biological characteristics of breast tumors in patients of pre- and postmenopausal age [12]. No matter what the causes of AA localization mainly in the epithelial ([6] and our findings) or stromal cells [13] (maybe due to the use of different antibodies to aromatase in the studies cited), the interaction of individual elements of a heterogeneous cell population of breast cancer on the basis of paracrine and autocrine effects is indisputable [9] and merits further study; one topic for future research is AA regulation and inhibition in tumor tissue of patients of different menstrual status.

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