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ENDOGENOUS STEROID HORMONES AND LOCAL AROMATASE ACTIVITY IN THE BREAST

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Summary—To test the hypothesis of an increased activity of the enzyme aromatase in adipose tissue from affected when compared with non-affected quadrants of patients with breast cancer, the aromatase activity has been measured in tumour and fatty tissues dissected at specific sites from the breasts of 16 patients. Activity was measured after extensive purification of the product formed. Results, expressed in fmol/g of tissue, did not show a higher activity in the affected vs the non-affected quadrants. In the tumours, higher activities were found when expressed per g of tissue. Per mg of DNA, an indicator of the number of cells, tumour enzymatic activity was lower than in fatty tissues. The relations between the products of aromatase, oestrone and oestradiol in the various tissues point to the importance of additional enzymatic processes, especially of the reductive 17β-oestradiol dehydrogenase, in the accumulation of high quantities of oestradiol in the malignant tissue.

INTRODUCTION

In the aetiology of human breast cancer nutritional [1] and reproductive factors [2] play a major role. The age-specific incidence of this malignancy shows very large variations in populations in different countries [3].

Recently De Waard and Trichopoulos [4] put forward a unifying hypothesis in which the known risk factors for breast cancer were considered jointly in one pathogenetic framework, related to the formation of pre-cancerous lesions. Early menarche and tall body height are thought to be related to the formation of breast lesions with an increased carcinogenic potential. In their concept, these anatomically undefined "pre-cancerous" lesions are produced as a response to energy-rich nutrition in childhood.

The relation between an important reproductive factor, such as age at first pregnancy, and breast cancer is based, in the hypothesis, on a slowing down of the formation of "pre-cancerous" lesions by the terminal differentiation of mammary gland epithelium during pregnancy. The pre-cancerous lesions which were generated during the perimenarcheal years and adolescence may be promoted to clinical breast cancer in subsequent years under the influence of a number of factors, among which oestrogens appear to play a major role [4]. In postmenopausal women the oestrogenic effects of obesity possibly act as growth-enhancers of oestrogen-responsive tumours. In agreement with this suggestion is the finding that the incidence of oestrogen receptor (ER) positive tumours is higher in Western than in non-Western countries [5], and higher in tumours from obese women than in those from lean women [6] within Western populations.

Biochemically the supposed role of oestrogens has attracted much attention [7, 8]. Differences in the blood levels of oestrogens in women with and without tumours have not been found. However, because oestrogens have to be taken up by cells, interest has focused more recently on factors responsible for the accumulation of oestrogens in mammary tissues. Since the first demonstration [9] of very high intratissular concentration of the active oestrogen, oestradiol in the breast, it has become established that these intratissular steroids do not simply reflect plasma levels. The stability of oestradiol levels in malignant tissues, independent of the menopausal status of women [10, 11], and the absence of relations between plasma and intratissular oestradiol concentrations in postmenopausal women [10, 12] demonstrate that indeed local factors must be involved in the accumulation of relatively high concentrations

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of oestrogens in mammary tissues, particularly in postmenopausal women.

Normal as well as malignant breast tissues contain the enzymes necessary for the biosynthesis of oestrogens, in addition these tissues also contain enzymes related to the catabolism of oestrogens [13]. The formation of the active oestradiol is dependent mainly on two enzymes, the aromatase and the reductive oestradiol 17β-oxidoreductase, both present in mammary tissues [14–17]. Recently, an important role for local aromatase activity in adipose tissue in relation to the site of a tumour was postulated for human breast cancers [18], based on higher aromatase activity in adipose tissue in the breast quadrant carrying a tumour, compared with the other quadrants.

In this paper a similar study will be described, carried out in adipose and tumour tissues obtained from breast cancer patients immediately after operation. These samples were obtained from women living in Poland, a country with a 50% lower incidence of breast cancer in comparison with Western European countries [3].

MATERIALS AND METHODS

Patients

Malignant tissue and adipose tissue samples were obtained from 16 patients after total mastectomy in the Medical Academy in Wroclaw, Poland; 6 of these patients were premenopausal and 10 postmenopausal. Patients had not received any hormonal or chemotherapy before operation. After removal of the breast, the tissue was placed on ice.

In addition to a specimen of the tumour, five different samples of breast adipose tissue were dissected by the pathologist from each patient, one specimen of each of the three non-affected quadrants and two from the tumour-bearing quadrant. Of the last two samples one was dissected at a distance of 1–2 cm (designated fatty tissue close, fc) and the second at 5–7 cm (fatty tissue distant, fd) from the tumour. The specimens of the non-affected quadrants were taken from the periphery of these quadrants. Under Results the non-affected quadrants will be presented as fatty tissue of the upper inner (fui), upper lateral (ful), lower inner (fli) and lower lateral (fll) quadrants. All specimens were trimmed of connective tissue by the pathologist and stored at −80°C until analysis. Just prior to the analysis individual tissue samples were sliced in small parts and minced to avoid the effects of tissue heterogeneity as much as possible.

Aromatase activity

Aromatase activity was measured similarly to the method described by Miller and colleagues [18], with modifications in the purification schedule of the product oestradiol. Except where indicated, all procedures using tissues were performed at temperatures <4°C.

In brief, the aromatase measurement consists of homogenization of aliquots of 0.5 g of the previously minced tissue for 45 s at −196°C using a Mikrodismembrator (Braun, Melsungen, Fed. Rep. Germany); the tissue powder was suspended in 3 ml of 0.01 M phosphate buffer (pH 7.4) containing 500 U aprotinine/ml; transferred to a centrifuge tube and centrifuged for 2 h at 105,000 *g*. The fatty layer at the top of the tube was removed and the pellet and supernatant were gently homogenized by sonication for 60 s. The resulting suspension was used for incubation using 0.37 MBq [1,2,6,7-^{3}H]testosterone (Radiochemical Centre, Amersham, England) diluted with unlabelled testosterone, at a final concentration of 75 nmol/l. The total incubation volume was 7 ml, in addition to the homogenate it contained 2.5 mmol/l NADP, 15 mmol/l glucose-6-phosphate and 25 U glucose-6-phoshate-dehydrogenase, dissolved in 0.01 M phosphate buffer (pH 7.4).

After 2 h at 37°C, 60 Bq [^{14}C]oestradiol (internal recovery standard) plus 100 μg unlabelled oestradiol and 100 μg testosterone were added. Steroids were extracted from the incubation medium by adsorption to previously activated C18 columns and elution with 3 ml methanol. After evaporation the residue was dissolved in 0.2 ml ethanol and diluted with 2.5 ml toluene and 2.5 ml petroleumether. The solution was extracted with 5 ml of 0.4 N NaOH. This phenolic separation is used to separate the oestrogens from the excess of substrate. Oestrogens were extracted from the alkaline solution with diethylether (2 × 5 ml) and after evaporation of the ether, the residue was subjected to chromatography on Sephadex LH20 columns (0.5 g; toluene–methanol, 92:8), capable of separating oestradiol from other oestrogens. The ^{3}H/^{14}C-ratio was measured in a small aliquot of the oestradiol fraction.

After evaporation the remaining oestradiol was acetylated (acetic anhydride 6 drops + pyridine 3 drops, 1 h at 60°C), subjected to TLC

(cyclohexane–ethylacetate, 70:30) and again a 10% aliquot of the eluted oestradiol diacetate was counted to determine the $^3H/^{14}C$-ratio. After deacetylation (1 ml methanol + 0.25 ml 2% K_2CO_3, overnight at 37°C), chromatography was performed on Sephadex LH20 columns and measurement of the ratio, followed by methylation (dimethylsulphate in alkaline solution) and chromatography (TLC in cyclohexane–ethylacetate, 50:50) and measurement of the ratio. Finally, the methylated oestradiol was acetylated, chromatographed (TLC in cyclohexane–ethylacetate, 50:50) and all remaining radioactivity was counted.

This purification schedule proved to be effective in removing all radioactivity not belonging to authentic oestradiol. The $^3H/^{14}C$-ratio did not change after methylation, as shown in Fig. 1. In Fig. 1 the ratio of each derivative has been given relative to that in the 3-methyl-oestradiol, obtained in 12 of the tissue samples from the study described in this paper. The additional, final acetylation and chromatography did not change the ratio. The ratio of the last measurement was used to calculate the conversion of testosterone to oestradiol, expressed in fmol of product during the incubation of 2 h.

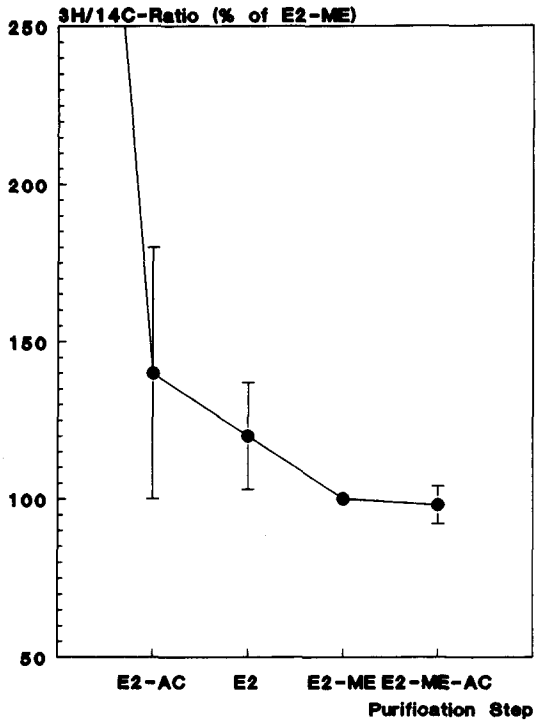

Fig. 1. The $^3H/^{14}C$-ratio measured in aliquots of oestradiol or its derivatives are shown during the process of purification of the product oestradiol formed after incubation of the breast tissues with testosterone. The purification procedure is described under Materials and Methods.

Oestrogen concentrations in tissues

A different aliquot of the minced samples was used to measure the endogenous concentrations of oestrone and oestradiol using procedures described previously [19].

Statistical analysis

Differences between results on aromatase activity were tested using the non-parametric Wilcoxon's rank sum test. Correlations were calculated using Spearman's rank test.

RESULTS

Aromatase activity in tumours and fatty tissues

Significant aromatase activity could be detected in all samples studied, with large differences between the individual specimens. Aromatase activities, in fmol of oestradiol generated during 2 h, expressed per g wet weight of the original tumours or of the adipose tissues, are given in Fig. 2. Compared with the activity in adjacent adipose tissue (fc), a higher aromatase activity was found in the tumours ($P < 0.002$). Also, in the other specimen of adipose tissues, dissected from the affected quadrant (fd), aromatase activity was lower than in the tumours ($P < 0.001$), all activities are expressed per g wet weight of tissue. No significant differences were found in the activities between tumours from pre- and postmenopausal women (not shown).

Comparison of the enzyme activities in the adipose tissues, obtained from different quadrants, shows the lowest activity in the fatty tissue relatively close to the tumour. However, comparison of the activities in the samples obtained from each individual patient did not show a significant difference between the various segments of adipose tissues, due to the fact that some samples of adipose tissue close to the tumour did show relatively high aromatase activity. In 15 patients all 6 tissue specimens per breast were available: in 7 of them the lowest aromatase activity was measured in the fc sample; in another 5, in the fd specimen; whereas in 4 patients the highest activity was measured in either the fc or the fd specimen. The differences between samples from pre- and postmenopausal women were not significant.

The expression of the aromatase in fmol/g wet weight of tissue of course does not take into account the differences in the cellular composition of tumour and adipose tissue. Therefore,

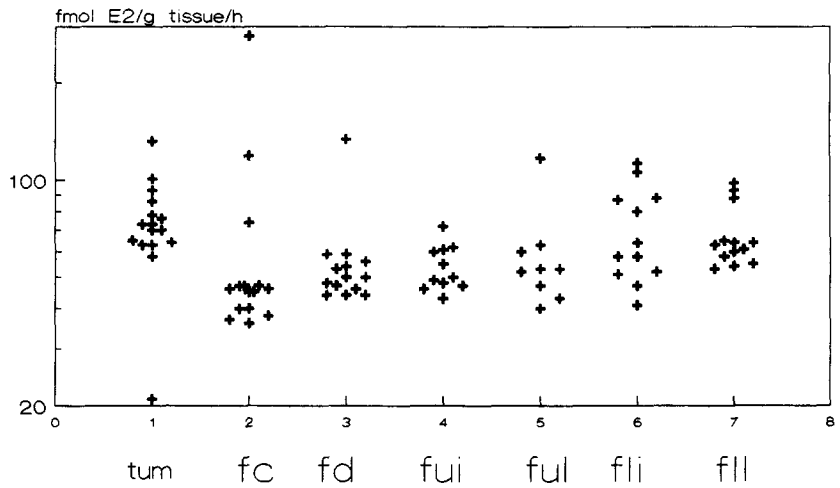

Fig. 2. Individual results on aromatase activity in the various samples of breast tissue as measured in the study described. E2 = oestradiol, other abbreviations are given under *Patients*.

the aromatase activity was calculated also per mg of DNA, as an indicator of the number of cells studied. In particular, adipose tissue contains very low quantities of DNA/g of tissue, as each cell contains large quantities of triglycerides. As a consequence, aromatase activity/mg of DNA in adipose tissues was much higher than in tumours. As illustrated in Fig. 3, aromatase activity in fmol of product/mg of DNA·h, with one exception, in the individual patients was significantly higher in fatty tissue dissected close to the tumour in the affected breast quadrant.

Concentrations of endogenous oestrogens

As described previously [8], the concentrations of endogenous *oestradiol* in tumours of Polish women were very similar in pre- and postmenopausal women; median concentrations were 0.57 and 0.60 pmol/g of tumour. *Oestrone* levels declined from 0.65 pmol/g of tumour in premenopausal women to 0.29 pmol/g in postmenopausal women. The oestradiol concentration in the tumours was higher than in either fatty or non-malignant tissues, obtained from the same women. In contrast, oestrone levels were somewhat lower in the tumours as compared with the fatty or normal epithelial samples. The results on concentration measurements in malignant and in normal adipose tissues of Polish patients are shown in Fig. 4.

A highly significant correlation between the oestradiol and oestrone concentrations in the fatty tissues was calculated: $r = 0.79$ ($P < 0.01$) and $r = 0.73$ ($P < 0.01$) in pre- and postmenopausal women, respectively. In the tumours the two oestrogens were correlated in premenopausal women, $r = 0.56$ ($P < 0.05$), but not in the samples from postmenopausal women, $r = 0.16$, NS.

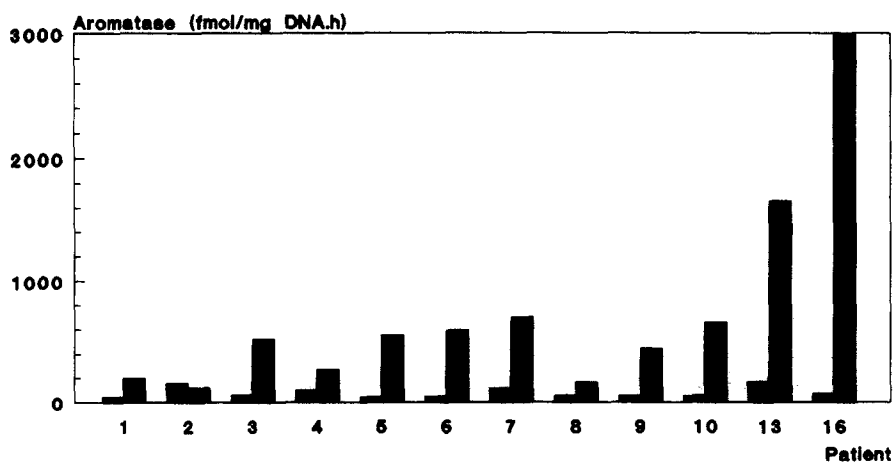

Fig. 3. Aromatase activity (expressed per mg of DNA) in breast cancer tumours (■) and adjacent fat tissue (■).

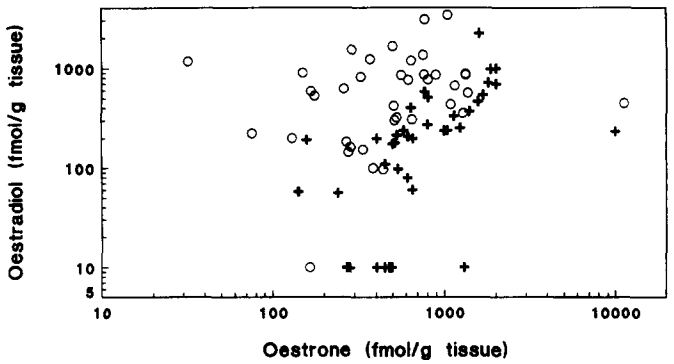

Fig. 4. Relationship between oestradiol and oestrone concentrations in tumour (○) and fatty tissue (+) measured in Polish breast cancer patients.

DISCUSSION

This study confirms the presence of aromatase activity in tumour as well as in adipose tissues in the human breast. The biological significance of this activity and of the differences between various segments of the breast cannot easily be interpreted, because measurements of the enzyme have been undertaken using optimal conditions *in vitro* which cannot be extrapolated simply to the *in vivo* situation. Still, comparative studies of aromatase activity in different segments of the same organ are of value since they are able to indicate local differences in activities relevant to growth stimulation and promotion of breast tumours in the human.

Therefore it is remarkable that the results of the present study are not in line with the other available similar study [18]. In the present study no evidence was obtained for a higher aromatase activity in adipose tissue of the breast obtained from the tumour-bearing quadrant; in only 4 of 15 patients the aromatase was higher in fatty tissue of the affected quadrant, whereas in 12 of them the lowest activity was measured in one of these specimens (fc and fd).

In order to explain the divergent results several factors can be of influence:

(a) The technique used to measure aromatase. In adipose tissues the Scottish study [18] relies on the so-called [^{3}H]water-release assay, in which the release of the marker at the 1β-position is used as a parameter for aromatization, whereas the study in this contribution relies on the amount of the product produced by the aromatization reaction. Since the introduction of the [^{3}H]water assay, discrepancies have been described pointing to the fact that the water-release assay may result in too high values [20].

(b) The tissue fraction used in the experimental studies. O'Neill *et al.* [18] incubated only the particulate fraction of the tissues after homogenization, whereas the whole tissue fraction except for the fat has been used in this study.

(c) The origin of the breast tissue samples. In this study all tissues have been obtained from Polish women, belonging to a population with a lower incidence of breast cancer. Before focusing on this explanation, the first two points mentioned must be excluded because only then a relation between etiology and aromatization can be considered.

In the Scottish study no real, unbiased comparison can be made between the aromatase activity in the tumour and those in the fatty tissues as different techniques have been used for its measurement in different tissues [21]. From our study it appears that the way of expressing the aromatase activity is of utmost importance. Expressed per g wet weight of either tissue, the tumour has the highest activity. The expression of an activity/mg of (soluble?) protein causes additional problems because a clear description is required on the exact composition of the fraction in which the protein has been determined. Particularly in homogenates containing insoluble proteins (particles), calculation per mg of protein can cause problems. A probably more realistic expression per mg of DNA, taking into account the number of cells in the samples, reveals that the activity in the tumours of the breast is lower than in adipose tissues. In *in vivo* situations the formation of products is not only determined by

the enzymatic activity, in addition the substrate concentration and the availability of co-factors will have an influence on product formation in the tissues. Thus, it remains to be established which role adipose breast tissue plays in the biosynthesis of oestrogens.

In vivo oestrone and/or oestradiol are products formed by the action of aromatase. Measurement of these steroids reveals that their concentration/g of tissue (fat contains oestrogens) is higher in the tumours than in the adipose tissue and in the non-malignant epithelial tissue [8] of the individual patients. The good correlation between the two oestrogens in fatty tissues (Fig. 4) is in favour of a common origin of these steroids. The absence of correlation, particularly in the tumours of postmenopausal women, indicates that in these tumours additional factors, responsible for an increase in oestradiol formation, play an important role. The recent finding on secretion of peptides by breast stromal fibroblasts [16], capable of stimulating specifically the synthesis of oestradiol from oestrone, demonstrates that the role of the reductive 17β-oestradiol dehydrogenase could be of major importance.

Thus, if the results of our study on lower aromatase activity in fatty tissues of the affected quadrants of the human breast may be extrapolated to the *in vivo* situation, the role of aromatase may be less important than as yet unidentified factors, controlling the specific formation of oestradiol around the tumour.

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