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CONCENTRATIONS OF ESTRONE, ESTRADIOL AND THEIR SULFATES, AND EVALUATION OF SULFATASE AND AROMATASE ACTIVITIES IN PATIENTS WITH BREAST FIBROADENOMA

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In the present studies, the concentrations (mammary tissue and plasma) of estrone (E_1), estradiol (E_2) and their sulfates (E_1S and E_2S), as well as the sulfatase and aromatase activities, were evaluated in patients with breast fibroadenomas. Comparative studies of the evaluation of these parameters were carried out in: (A) tumor tissue, (B) areas surrounding the tumor and (C) areas distant from the tumor (glandular tissue) considered as normal tissue. The concentrations in the tumor tissue (in pmol/g tissue) of E_1, E_2 and E_1S were significantly higher (2–3 times) than in the area of the breast considered as normal. Sulfatase and aromatase activities were found in the breast fibroadenoma tissue. Sulfatase activity was much higher than aromatase (30–150 times) and sulfatase levels were significantly higher in the fibroadenoma tissue than in the area considered as normal. Plasma evaluation of E_1, E_2, E_1S and E_2S concentrations showed no significant differences in relation to those of healthy control women. In conclusion, the high levels of estrogens and their sulfates, as well as the enzymes involved in estrogen formation—sulfatase and aromatase in breast fibroadenoma—contribute to the hypothesis that this disease may be hormone-dependent. *Int. J. Cancer* 70:639–643, 1997.

Fibroadenoma is one of the most frequent manifestations of benign breast disease (Cole *et al.*, 1978; Fleming *et al.*, 1982). The origin of this pathology is not very well established and there is some controversy as to whether it is the consequence of hormonal effects or not. Some workers have reported a significant increase in plasma estradiol, whereas others have observed normal levels (De Boever and Vandekerckhove, 1982; Wang and Fentiman, 1985).

In addition to the biosynthesis of estrogen by the ovaries, at present it is well established that some pathological tissues, for instance breast carcinoma, possess enzymatic systems necessary for the local formation of estrogens (for a recent review see Pasqualini and Chetrite, 1996). Two main pathways involved in estrogen biosynthesis in breast cancer tissues are well documented, these include the ''aromatase pathway'' which transforms androgens into estrogens (Lipton *et al.*, 1987; Perel *et al.*, 1988), and the ''sulfatase pathway'' which converts estrogen sulfates to estrogens (MacIndoe, 1988; Pasqualini *et al.*, 1986, 1992; Santner *et al.*, 1984; Vignon *et al.*, 1980). Information relative to the evaluation of various estrogens (unconjugated or sulfated) in the tumoral tissues of benign breast disease as well as the enzymes implicated in its biosynthesis—aromatase and sulfatase—is very limited.

In the present studies we evaluated the concentrations (mammary tissue and plasma) of estrone, estradiol and their sulfates, as well as the sulfatase and aromatase enzyme activities in patients with breast fibroadenoma. Comparative studies to evaluate these parameters were carried out in: (A) tumor tissue, (B) areas surrounding the tumor, and (C) areas distant from the tumor (glandular tissue), considered as normal.

MATERIAL AND METHODS

Chemicals

[6,7-^{3}H]-Estrone sulfate (specific activity: 49.0 Ci/mmol), [7-^{3}H]-testosterone (SA: 27.7 Ci/mmol, [6,7-^{3}H]-estrone (SA: 41.9 Ci/mmol), [4-^{14}C]-estrone (SA: 57.6 mCi/mmol), [4-^{14}C]-estradiol (SA: 57.0 mCi/mmol), were obtained from NEN (Du Pont de Nemours, Les Ulis, France). Estrone sulfate ammonium salt and β-NADPH were purchased from Sigma-Aldrich (St Quentin-Fallavier, France). Estrone and estradiol were obtained from Steraloids (Touzart et Matignon, Vitry-sur-Seine, France).

Patients and biological material

This study was carried out in 15 patients with breast fibroadenoma (aged 17–35 years). None of the patients had a history of endocrine, metabolic or hepatic diseases or had received treatment in the previous 2 months. Each patient received local anesthesia (lidocaine 1%) and 3 regions of the mammary tissue were selected for the various analyses: (A) tumoral tissue; (B) the area surrounding the tumor; and (C) a distant zone (glandular tissue) which was considered as normal. The different tissue specimens removed at the time of surgery were promptly trimmed of fat and connecting tissues. A sample of each tissue was used for histology studies and classified according to its epithelial density. Samples were placed in liquid nitrogen and stored at −80°C until hormonal or enzyme activity analysis.

Venous blood samples from the same patients (8 in the follicular phase and 7 in the luteal phase) were obtained in heparinized tubes and serum was separated by centrifugation and frozen at −20°C prior to hormonal determinations. To analyze variations in estrogens (unconjugated and sulfated) during the ovarian cycle and in healthy pre-menopausal women, blood was obtained from cubital veins of 9 women in the follicular phase and 8 in the luteal phase.

Hormonal analysis

The various estrogens—estrone, estradiol, estrone sulfate and estradiol sulfate—were evaluated in the tissues and plasma by radioimmunoassay (RIA) as previously described (Gelly *et al.*, 1981; Pasqualini *et al.*, 1990). The sensitivity (smallest quantity which is significantly different from zero at $p < 0.01$) of both E_2 and E_1 standard curves was determined to be 5 pg.

In the absence of added estrogen, the dilution of antiserum bound 52% [^{3}H]-E_2 and 48% [^{3}H]-E_1. The antiserum of E_2 was found to have no detectable cross-reaction with E_1, estriol (E_3), dehydroepiandrosterone, pregnenolone or cortisol and similarly the

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antiserum of E_1 did not cross-react with E_2, E_3, dehdyroepiandrosterone or cortisol.

In the tissue. Some 150–250 mg of the different tissues were homogenized in 70% ethanol using an Ultraturrax apparatus (Ika-Werk, Janke and Kunkel, Staufen, Germany) and allowed to precipitate for at least 24 hr at −20°C. [^{14}C]-E_2 (2,000 dpm) and [^{3}H]-E_1S (10,000 dpm) were added to 5 ml of tissue homogenate in order to determine the losses of unconjugated or sulfated estrogen. After centrifugation at 900 *g* for 10 min, the supernatant was decanted and evaporated to dryness, then dissolved in 0.3 ml of distilled water plus 6 ml of ethanol, and precipitated again for 24 hr at −20°C prior to centrifugation at 900 *g* for 10 min. The supernatant was evaporated to dryness and dissolved in 0.5 ml water, then the unconjugated estrogens were extracted with 10 vol of a mixture of ethyl acetate:hexane (3:2, vol/vol). The organic phase was evaporated and dissolved in 0.5 ml of the buffer solution (0.1 mol/l KH_2PO_4, 0.1 mol/l Na_2HPO_4, 0.15 mol/l NaCl, 0.1% gelatine, 0.1% NaN_3, pH, 7.4), then processed for quantitative RIA determination of E_1 and E_2. Antisera for E_1 evaluation were purchased from Immunocorp (Montreal, Canada) and antisera for the evaluation of E_2 were a gift from the Foundation for Hormone Research (Fresnes, France). The aqueous phase was submitted to solvolysis for the determination of E_1S or E_2S. Briefly, 2 ml of ethanol were added to this aqueous phase and deproteinized at −20°C for 24 hr, the protein pellet was removed by centrifugation and the ethanol fraction evaporated. The dry residue was dissolved in 0.9 ml of 0.9% NaCl + 0.1 ml H_2SO_4 2N solution and extracted twice with 3 vol ethyl acetate and incubated overnight at 37°C. After neutralization with a concentrated Na_2CO_3 solution, the dry residue was dissolved in 0.5 ml of water and extracted with 10 vol ethyl acetate:hexane (3:2, vol/vol) and the freed estrone or estradiol were then analyzed and quantified as indicated above. These conditions of solvolysis resulted in 95–98% cleavage of authentic [^{3}H]-E_1S. The percentage recovery of E_1 was 70 ± 7 and that of E_2 67 ± 8. The blank values for E_1 and E_2 from charcoal-treated tissue extracts were negligible.

In plasma. Venous peripheral blood samples from patients were obtained in heparinized tubes before surgical intervention, then the serum was separated by centrifugation and frozen at −80°C until hormonal analysis. From each sample, 1 ml was equilibrated for 30 min with [^{3}H]-E_1S (10,000 dpm) and [^{14}C]-E_2 (2,000 dpm) to monitor analytical losses. Unconjugated estrogens were extracted with 10 vol of a mixture of ethyl acetate:hexane (3:2 vol/vol). The organic phase was evaporated, then the extract was dissolved in 0.5 ml buffer solution and processed for quantificative determination of E_1 and E_2 as indicated above. The aqueous phase was deproteinized with 4 ml absolute ethanol, the protein pellet removed by centrifugation and the ethanol solution evaporated, then E_1S and E_2S were extracted and evaluated as indicated above. The smallest amount of E_1 that could be measured in 1 ml plasma was 7 pg and the corresponding value for E_2 was 9 pg. The percentage recovery of E_1 was 65 ± 5 and that of E_2, 61 ± 4.

Enzyme assays

Estrone sulfate-sulfatase. Samples of 100–150 mg from the various areas of the breast were homogenized in 20 mmol/l Tris-HCl buffer solution (pH 7.4) for 15 sec with an ultraturrax apparatus. Estrone sulfate-sulfatase activity was evaluated according to MacIndoe (1988). Briefly, 100 µl of homogenate preparation [0.10–0.12 mg protein, evaluated according to Bearden (1978)] and 200 µl of 20 mmol/l Tris-HCl buffer containing [^{3}H]-E $_1$S at 10^{-8} mol/l were incubated for 30 min at 37°C. The reaction was stopped by addition of 0.3 ml cold 0.1 mol/l Na_2CO_3 containing 5,000 dpm [^{14}C]-E_1 to determine the recovery of the extraction process. The unconjugated steroids were extracted by adding twice 2 ml of toluene. Following freezing of the aqueous phase, the organic phase was transferred to a liquid scintillation vial. After evaporation of the solvent, 3 ml of Opti-fluor (Packard, Rungis, France) were added and the vials were analyzed for [^{3}H] and [^{14}C] content.

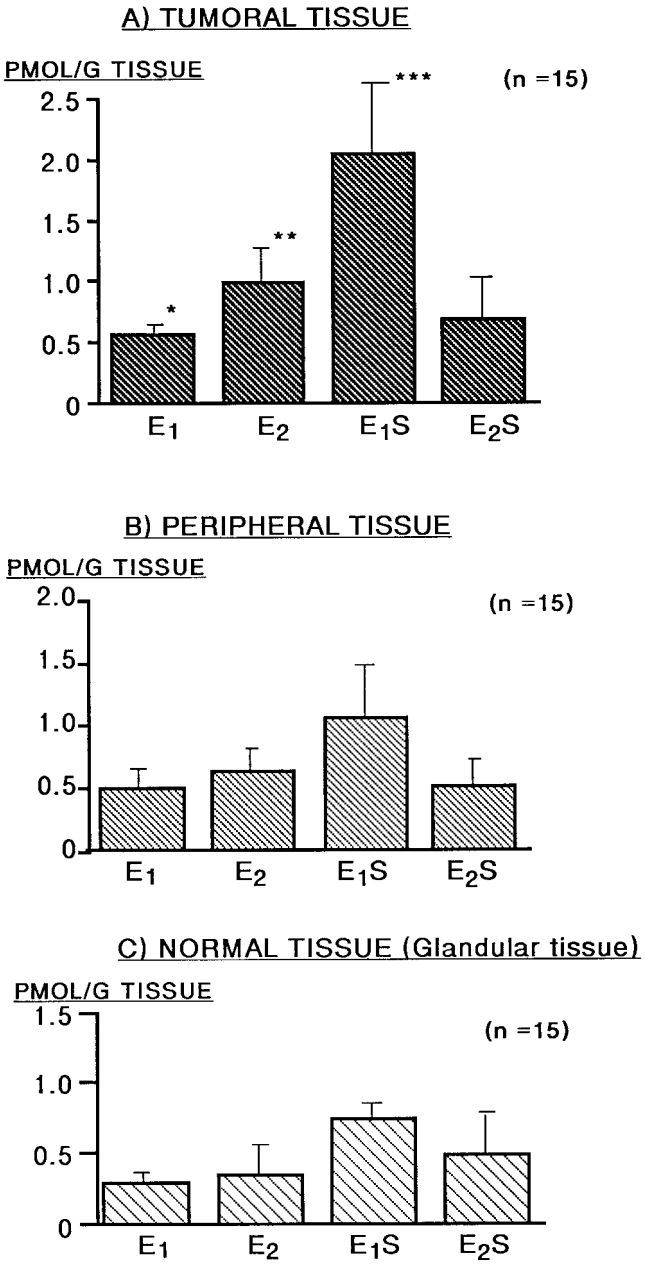

FIGURE 1 – Concentrations of estrone (E_1), estradiol (E_2), estrone-sulfate (E_1S) and estradiol-sulfate (E_2S) in different tissular areas (tumoral, peripheral and normal) of patients with breast fibroadenoma. The different estrogens were evaluated as indicated in the text. Values (in pmol/g tissue) are expressed as the mean ± SEM (n = 15). *$p \leq 0.01$ *vs.* E_1 in the area of the breast considered as normal (C). **$p \leq 0.005$ *vs.* E_2 in the area of the breast considered as normal (C). ***$p \leq 0.001$ *vs.* E_1S in the area of the breast considered as normal (C).

All determinations were performed in duplicate. E_1S-sulfatase activity was expressed in pmol of E_1 formed per mg of protein/hr. The reaction rates were linear within the incubation times and the protein range employed. The apparent Michaelis-Menten constant (Km), determined by the method of Lineweaver and Burk (1934), using 5 measurements of initial velocity of E_1 production at 37°C and pH 7.4 over a range of substrate concentrations between 2 and 20 µmol/l of E_1S, at a single point (30 min), is Km: 10.63 ± 1.10 µmol/l and the Vmax: 13.93 ± 0.87 nmol/mg protein/hr.

TABLE I – PLASMA EVALUATION OF ESTRONE (E_1), ESTRADIOL (E_2) AND THEIR SULFATES (E_1S AND E_2S) IN PATIENTS WITH BREAST FIBROADENOMAS AND IN NORMAL WOMEN

	n	E_1 (pmol/ml)	E_1 (pmol/ml)	E_1S (pmol/ml)	E_2S (pmol/ml)
Normal					
Follicular phase	9	0.183 ± 0.048	0.274 ± 0.100	1.86 ± 0.65	0.288 ± 0.110
Luteal phase	8	0.233 ± 0.033	0.495 ± 0.117	3.84 ± 0.45	0.420 ± 0.068
With breast fibroadenoma					
Follicular phase	8	0.173 ± 0.730	0.335 ± 0.120	1.64 ± 0.76	0.278 ± 0.019
Luteal phase	7	0.336 ± 0.018	0.520 ± 0.170	3.61 ± 0.68	0.400 ± 0.110

Values are expressed as the mean ± SEM.

TABLE II – RATIO CONCENTRATION OF TUMORAL AND NORMAL TISSUES TO PLASMA OF ESTRONE (E_1), ESTRADIOL (E_2) AND THEIR SULFATES (E_1S, E_2S) IN PATIENTS WITH BREAST FIBROADENOMA

	Tumoral tissue	Normal tissue
E_1	3.32*	1.68
E_2	3.03*	0.96
E_1S	1.20*	0.42
E_2S	2.55	1.71

The ratio (g/ml) corresponds to values obtained for each estrogen in the tumoral tissue (pmol/g) or the area of the breast considered as normal (glandular tissue) divided by the plasma concentration (pmol/ml, follicular phase). Data represent average values obtained with 8 patients.

*$p \leq 0.01$ *vs.* normal tissue.

Aromatase. Aromatase activity in homogenates of the different areas of breast tissue was determined by isolation of [^{3}H]-E_1 and [^{3}H]-E_2 after incubation with [^{3}H]-testosterone according to Miller *et al.* (1974). Briefly, tissue homogenates (0.10–0.12 mg protein) were incubated for 2 hr at 37°C in 20 mmol/l Tris-HCl buffer solution (pH 7.4), [^{3}H]-testosterone at 10^{-8} mol/l and 1 mmol/l NADPH. The reaction was stopped by the addition of ethanol containing [^{14}C]-E_1 and [^{14}C]-E_2 (5,000 dpm of each) to monitor procedural losses. The amount of estrogens (E_1 and E_2) obtained was determined after separation by thin-layer chromatography in ethyl acetate:cyclohexane (1:1, vol/vol). All determinations were performed in duplicate. Results are expressed in pmol of estrogens formed per mg protein/hr. Formation of estrogens from testosterone was linear for the duration of the experiments (3 hr) and the amount of product formed was a linear function of the amount of enzyme (preparation) incubated. The apparent Michaelis Menten constant, determined by the method of Lineweaver and Burk (1934) using 6 measurements of initial velocity of estrogen production at 37°C and pH 7.4 over a range of substrate concentrations between 2 and 20 µmol/l of testosterone, at a single point (2 hr), is Km: 16.25 ± 3.75 µmol/l and the Vmax: 35.85 ± 4.20 pmol/mg protein/hr.

Statistical analysis

Results were expressed as the mean ± SEM. Statistical comparisons were made using the *t*-test. The value of $p \leq 0.01$ was considered significant. For the different parameters studied, the intra-assay coefficient of variation was less than 8%, and the inter-assay coefficient of variation was less than 12%.

RESULTS

(I) Estrogen concentrations

In the different areas of the breast. Figure 1 gives the levels of estrone (E_1), estradiol (E_2), estrone sulfate (E_1S) and estradiol sulfate (E_2S) in different areas of the breast in patients with fibroadenoma. It should be noted that: (1) the concentrations of estrogen sulfates, particularly of E_1S, were relatively high; (2) the level of E_1S was significantly higher in the tumoral tissue than in the peripheral tissue or the area of the breast considered as normal; (3) the levels of E_1 and E_2 were significantly higher in the tumoral tissue than in the area of the breast considered as normal (glandular tissue).

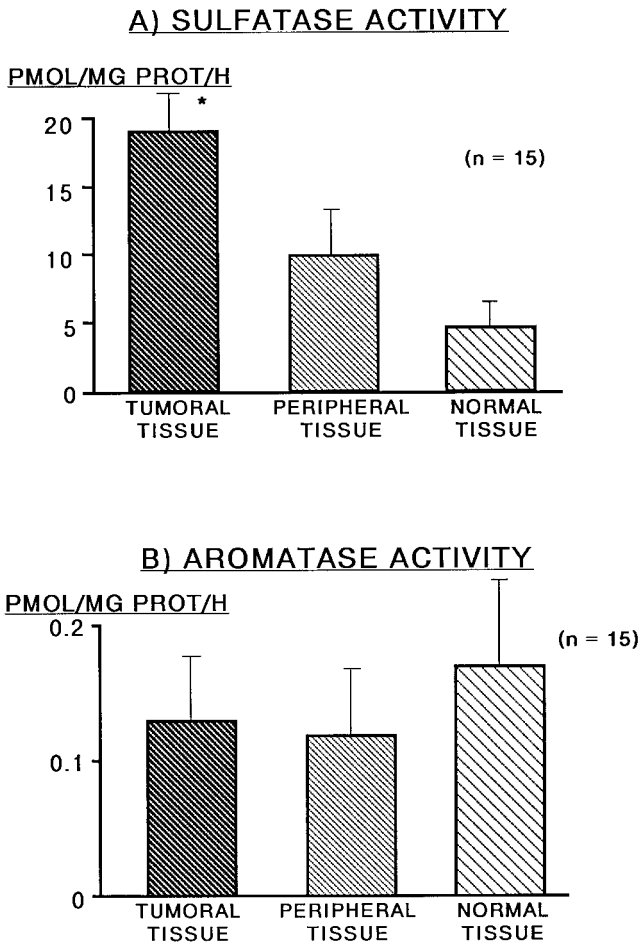

FIGURE 2 – Estrone-sulfate sulfatase and aromatase activities in different areas of breast fibroadenoma. Estrone-sulfate sulfatase and aromatase activities in the tissues of breast fibroadenoma patients were evaluated as indicated in the text. Values (in pmol/mg protein/hour) are expressed as the mean ± SEM. *$p \leq 0.001$ *vs.* estrone-sulfate sulfatase value in tumoral tissue.

In the plasma. Table I gives the levels of E_1, E_2 and their sulfates in the patients' plasma. No significant difference was observed in the concentrations of the different estrogens evaluated in relation to those of healthy control women. A comparison of the estrogen levels in the tumor tissues and plasma shows: (1) high concentrations of E_1 and E_2; (2) similar E_1S concentrations related to the tumoral area; (3) lower concentrations of E_1S in the normal area of the breast than in plasma.

Table II compares the concentration ratios of tumoral and normal tissues to plasma (follicular phase) of E_1, E_2 and their sulfates in

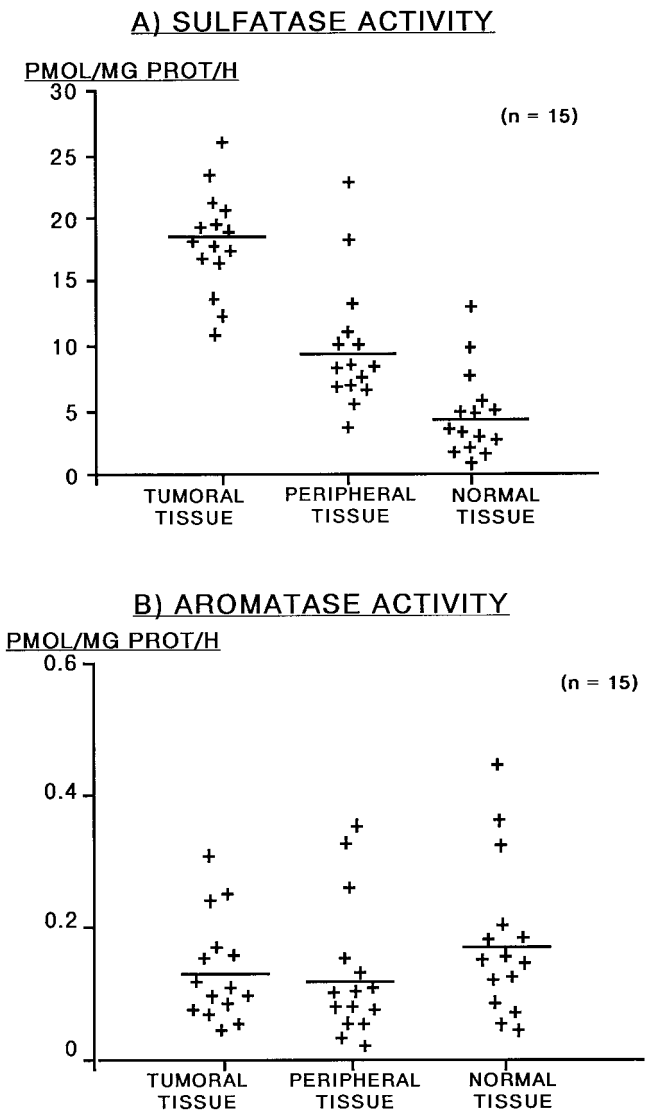

FIGURE 3 – Individual determinations of estrone-sulfate sulfatase and aromatase activities in different tissues of breast fibroadenoma patients. Estrone-sulfate sulfatase and aromatase activities in the tissues of breast fibroadenoma patients were evaluated as indicated in the text.

patients with fibroadenoma. As observed, the gradient of tumoral tissue to plasma increased significantly for E_1, E_2 and E_1S compared to the gradient obtained with the area of the breast considered as normal.

(II) Estrone sulfate-sulfatase and aromatase activities

As the formation of estrogens in breast carcinoma is mainly ''via aromatase'' which converts androgens into estrogens, and ''via sulfatase'' which converts estrogen sulfates into estrogens, it is interesting to evaluate these 2 activities in the breast tissue of patients with fibroadenomas. Figure 2 indicates the activities of these 2 enzymes in the different areas of the breast (n = 15), and the individual values are indicated in Figure 3. The data indicate that: (1) the sulfatase activity is 30–150 times greater than that of aromatase; (2) the sulfatase activity is significantly higher in the tumor than in the other areas of the breast; (3) no significant differences were observed for the aromatase activity in the 3 explored regions of the breast.

DISCUSSION

The present data show very clearly that breast fibroadenomatous tissues contain high levels of E_1, E_2 and their sulfates, and that these concentrations are significantly higher than in the tissular area of the breast considered as normal (glandular tissue). In addition, the tumoral tissue and plasma (g/ml) ratios are significantly high for E_1, E_2 and E_1S. Analysis of the concentrations of various estrogens in the plasma shows no significant difference compared to the values for healthy control women. These quantitative findings of estrogen levels in plasma agree with those of others who also observed that, in breast fibroadenoma patients, plasma estrogens do not increase (De Boever and Vandekerckhove, 1982). However, in another study, plasma E_2 values were higher than in normal women (Martin *et al.*, 1978).

Another aspect of these studies concerns the enzyme activities of sulfatase and aromatase; the data show that sulfatase was significantly higher (30–150 times) than aromatase. With regard to sulfatase activity, the values were particularly intense in the area of the tumoral tissue. However, no significant difference for the various regions of the breast was found for aromatase. It should be noted that, in another series of studies (data not shown) we found that these tissues also contain 17β-hydroxysteroid dehydrogenase, the enzyme which converts E_1 into E_2.

The present information extends the ''intracrine organ'' concept, according to which a target tissue can produce a hormone in addition to the classical endocrine organ (*e.g.*, the ovary). A similar concept of ''intracrine tissue'' is well documented for breast cancer (MacIndoe, 1988; Pasqualini *et al.*, 1986, 1992, 1995, 1996; Vermeulen *et al.*, 1986). How can this local production of estrogens be controlled? At present, information on this point is limited, but an interesting observation is that, in benign breast disease, the production of progesterone was subnormal during the luteal phase of the menstrual cycle (Mauvais-Jarvis *et al.*, 1979). In this connection, it is worthy of note that progesterone can modify the action of E_2 in endometrial tissue by increasing the metabolism of E_2 to E_1, an effect mediated by an increase in 17β-hydroxysteroid dehydrogenase activity (Tseng *et al.*, 1977).

Estrogen receptors (ER) which, as is very well known, are involved in the mechanism of E_2 action, are present in a relatively high proportion in fibroadenoma. They were detected in a percentage ranging from 11 to 55% by Allegra *et al.* (1979), with an overall incidence of 38%. Progesterone receptors were also present; Martin *et al.* (1979) observed that of 88 fibroadenomas, 18 possessed receptors to progesterone (PR). Studies of the levels of ER and PR during the menstrual cycle in patients with fibroadenoma show that ER increase throughout the follicular phase, where maximal values are found in the preovulatory phase, and decrease during the luteal phase. PR were high in the follicular phase (Kuttenn *et al.*, 1981). The data give additional information on the hypothesis of hormone dependence of breast fibroadenoma.

In conclusion: (1) High concentrations of E_1, E_2 and E_1S are found in breast fibroadenoma tissue and the levels are significantly higher than in the area of the breast considered as normal tissue; (2) breast fibroadenomatous tumor contains the enzymatic systems necessary for the biosynthesis of estrogens; (3) sulfatase activity is significantly higher in the tumor than in the area considered as normal.

These studies will contribute to the hypothesis that breast fibroadenoma originates from hormones, particularly estrogens, and may open new possibilities in the treatment of this disease using anti-hormones.

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