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STUDIES ON CLINICAL, HORMONAL AND PATHOLOGICAL CORRELATIONS IN BREAST FIBROADENOMAS

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SUMMARY

Plasma progesterone and estradiol were measured throughout the menstrual cycle in 84 patients with breast fibroadenoma. These *in vivo* data were compared to the levels of estradiol receptor protein (ER)* as assayed *in vitro* on cytosol of tumor tissue obtained at surgery. Plasma progesterone (P) levels for all the patients (11.7 ± 2.8 ng/ml) were significantly lower ($P < 0.001$) than those of normal women (21.0 ± 8.0 ng/ml) studied at the same days of the luteal phase. In addition, plasma estradiol (E_2) levels during the luteal phase were elevated: 289.6 ± 51.5 compared to 180.3 ± 82.0 pg/ml in normal women ($P < 0.01$). The patients were divided in three groups according to the ER levels. The group with high ER concentration (H-ER > 20 fmol/mg protein, 11 cases) was also found to have the highest cell proliferation, whereas in the 49 patients with the lowest ER concentration (L-ER < 7 fmol/mg protein) there was an important stromal reaction. In 28 cases where ER binding site levels varied from 7 to 20 fmol/mg protein (M-ER), the cellular density was intermediate between both groups. The observation of positive estradiol receptor protein in fibroadenomas where the epithelial cellularity is most important emphasizes the role of estradiol in cellular multiplication and hyperplasia. In addition, the observation *in vivo* of an estradiol/progresterone imbalance in all the patients studied indicates that this could be the primary cause favouring the development of benign dystrophy in the mammary gland.

INTRODUCTION

The presence of estradiol receptors in breast cancer is well documented [1–4]. However, few data relate the presence of receptors in benign breast diseases [5–7]. There are two possible explanations for the frequent absence or the small concentration of estradiol receptors in non-cancerous breast tissue: first, benign breast diseases are not hormone-dependent; second, breast is a very heterogenous tissue, in which the receptive structures, i.e. ducts and acini, spread into connective tissue. The answer to these questions might be found by looking for the receptors only in the receptive cells of breast tumor obtained surgically and carefully isolated from connective tissue.

The aim of the present investigation was to determine if the observation made *in vivo* in women with benign breast diseases of an abnormal hormonal status characterized by an unopposed ovarian estrogenic secretion [8] was correlated *in vitro* with an abnormal concentration of cytosolic estradiol receptors (ER) in breast tissue.

MATERIAL AND METHODS

Eighty four women from 18- to 55-years-old with a breast fibroadenoma were studied both *in vivo* and *in vitro*.

In vivo, each patient was studied over a menstrual cycle by determining plasma estradiol and progesterone as previously described [8]. Blood samples were collected during the luteal phase: at days 5, 6 and 7 of the basal temperature plateau. *In vitro*, the fibroadenomas obtained surgically at mid-follicular phase (6th to 10th day of the cycle) were analyzed as follows: after separation from non-tumor tissue, the tumor was cut in two parts—one part was used for pathological studies, the other was kept frozen until processed for biochemical investigations not more than 1 month later.

Pathological investigations. The cellular density of the tumor was assessed by determining the relative proportion of epithelial and stromal cells. Three degrees of cellular density were distinguished: in type I, the proliferation of the acinar epithelial cells was predominant and fibrosis was practically absent; in type III, the fibrosis was so important that the original lobular proliferation could scarcely be recognized as fibroadenoma; in type II, microscopic features were intermediate between type I and type III.

Biochemical study

Chemicals. [2,4,6,7-^{3}H]-estradiol (90 Ci/mmole, N.E.N. Corp.) and the synthetic estrogenic compound [6,7-^{3}H]-R 2858 (50 Ci/mmole, Roussel–Uclaf) were used after purification. Unlabeled steroids estradiol (E_2) and R 2858 were obtained from Roussel–Uclaf. Buffer routinely used was Tris-HCl–EDTA (0.01 M Tris–HCl–1 mM EDTA–12 mM monothioglycerol-

* Abbreviations used in this paper: E_2, estradiol; ER, cytosolic estradiol receptor; P, progesterone; R 2858, moexestrol = 11β-methoxy-17 ethinyl-1,3,5 (10)-estatriene 3,17β-diol.

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glycerol 10% v/v, pH 8.0 at 0°C = G.T.E.M. Buffer). Acid-washed activated charcoal was obtained from Sigma and Dextran T 70 was supplied by Pharmacia. The dextran-coated charcoal (DCC) suspension (0.5% activated charcoal with 0.05% Dextran 70) was stored at 0°C with constant shaking.

Preparation of cytosol fractions. All procedures were performed at 0–4°C. 500 mg of the frozen tissue were crushed with a Thermovac tissue pulverizer, and then homogenized in 2.5 ml of cold buffer with a Polytron PT 10. The homogenate was centrifuged in a 50 Ti rotor of a Beckman $L_2$65 B centrifuge at 105,000 *g* for 1 h. The supernatant (cytosol) had a protein concentration range of 3–8 mg per ml as determined by the method of Lowry *et al.*[9].

Cytosol estradiol binding assay. High affinity binding of estradiol was determined by three techniques: sucrose gradient analysis, a dextran-coated charcoal assay [10] which measures unfilled sites only, and a method allowing the exchange of endogenously bound hormone with [^{3}H]-hormone, in order to determine total estradiol receptor binding sites [11].

Sucrose gradient centrifugation. Linear 5–25% saccharose gradients (3.6 ml) were prepared and kept at 0–4°C for 2–5 h before use, and 200 μl of cytosol were incubated with a 50 μl aliquot of buffer containing 10 nM [2,4,6,7-^{3}H]-E_2 or [6,7-^{3}H]-R 2858 (final concentration 2 nM), agitated and incubated for 4 h at 0°C. Parallel samples were preincubated for 15 min with a 100-fold excess of unlabeled E_2 or R 2858. Suspensions of dextran-coated charcoal (DCC, 500 μl) were centrifuged for 10 min at 2000 *g* to sediment the DCC pellet. The incubated cytosols were then transferred on to the pellets, shaken for 10 min at 0°C and centrifuged for 10 min at 2000 *g*. Aliquots of 200 μl of the resulting supernatant were layered on the sucrose gradients. Centrifugation at 50,000 rev/min for 16 h was performed in a SW-65 rotor of a Beckman L_2 ultracentrifuge. After the centrifugal run, 32 fractions of the gradient were collected in scintillation vials and radioactivity counted in 5 ml of modified Bray's solution [12].

DCC and exchange assays. Cytosols prepared as described above were diluted in G.T.E.M. buffer to approximately 2 mg/ml protein. Aliquots of 100 μl were incubated with 0.1–1.0 × 10^{-4} (final concentration) [^{3}H]-E_2 or [^{3}H]-R 2858 added in a volume of 100 μl buffer and 2% ethanol. Parallel cytosol samples were preincubated for 10 min with 100-fold excess unlabeled R 2858 before incubation with 0.1, 0.5 and 1 nM [^{3}H]-R 2858.

Incubations were carried out in two different ways: 0°C overnight for unfilled ER assay, and 25°C for 5 h and then 0°C overnight for total ER assay. At the end of the incubation period, 500 μl of the DCC suspension were added and adsorption of free steroids carried out at 0°C for 30 min with constant shaking. After centrifugation at 2000 *g* the radioactivity was counted in 500 μl aliquots of the supernatants. The data were analyzed according to the method of Scatchard after subtraction of non-specific binding calculated from the preparations incubated with 100-fold excess of non-radioactive hormone.

Under optimal conditions, i.e. with sufficient adenoma tissue, gradient determination and Scatchard assay with and without exchange were performed, but when surgical samples were too small to yield more than 1 ml of cytosol, assay was limited to sucrose gradient and exchange assay with only four different incubations (0.1–0.5 and 1.0 nM [^{3}H]-R 2858, and 1 nM [^{3}H]-R 2858 with 100 nM unlabeled R 2858.

RESULTS

Estradiol receptors in fibroadenomas

The presence of estradiol binding sites (ER) was observed in 35 of the 84 fibroadenomas studied in the following conditions:

(1) The tissue was obtained surgically at mid-follicular phase when ER is high.

(2) [^{3}H]-R 2858 [13] was routinely used rather than [^{3}H]-E_2 because of its higher affinity for ER and the slow dissociation rate of the complex.

Table 1. Biochemical and clinical characteristics of the three groups of fibroadenomas distinguished according to their cytosol total estradiol binding capacity (H-ER = high ER-capacity, M-ER = mid ER-capacity, L-ER = low ER-capacity)

		In vitro data	Clinical data (luteal phase)		
Groups	Nb	ER (fmol/mg protein)	length (days) cycle (days)	E_2 $\bar{m}$ (pg/ml) ± S.D.	P $\bar{m}$ (ng/ml) ± S.D.
H-ER	11	>20	9/37	278 ± 51	9.8 ± 3.3
M-ER	24	7 < ER < 20	10/36	319 ± 45	13.3 ± 3.2
L-ER	49	<7	9/28	308 ± 100	15.0 ± 8.9
Total	84			289	11.7
$\bar{m}$		—	9.3/33.5	±51	±2.8
SD					
Normal women	20	—	14/29	180 ±80	21 ±6

The ER values are calculated from Scatchard plots after DCC-exchange assays.

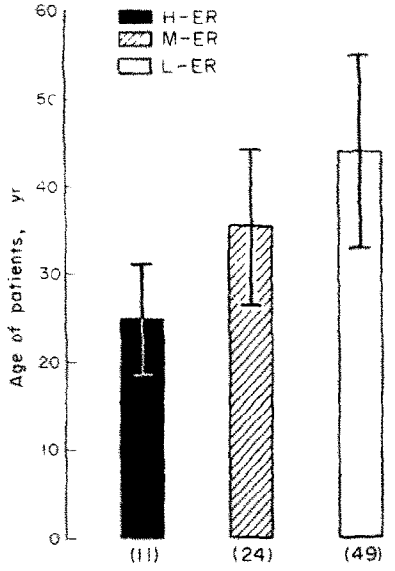

Fig. 1. Age distribution of patients in the three groups of fibroadenomas: H-ER (ER > 20 fmol/mg protein), M-ER (7 < ER < 20 fmol/mg protein) and L-ER (ER < 7 fmol/mg protein).

(3) The results provided are the ER values obtained from Scatchard plots after DCC-exchange assays which measure the total estradiol binding sites.

As regards the concentration of binding sites (Table 1) 3 groups of tumors could be delineated: one group yielded high ER content (H-ER: 11 cases) with a maximum of 105 fmol/mg protein and a mean value of 35 fmol/mg protein. A second group yielded a medium ER content (M-ER: from 7 to 20 fmol/mg protein, 24 cases). In the third group (49 cases) the ER content was low (L-ER < 7 fmol/mg protein).

The results of DCC-exchange assay were confirmed by the sucrose density gradients: fibroadenoma ER levels greater than 6–7 fmol/mg cytosol protein by

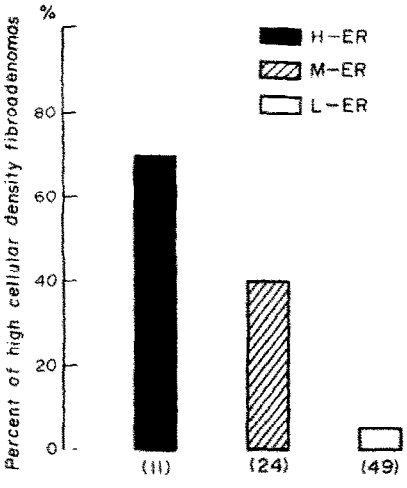

Fig. 2. Percentage of breast fibroadenomas with high cellular density (cellular density of type I) in the three group of patients: H-ER (ER > 20 fmol/mg protein), M-ER (7 < ER < 20 fmol/mg protein) and L-ER (ER < 7 fmol/mg protein).

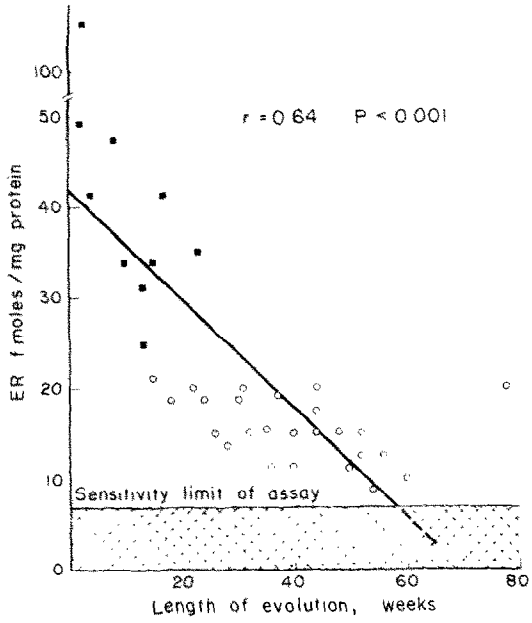

Fig. 3. Linear correlation ($P < 0.001$) between the estradiol cytosol binding sites calculated from Scatchard plots after DCC-exchange assays (ER = Y) and the length of the fibroadenoma evolution between discovery and surgery (χ) in the 11 patients of the H-ER group (■) and the 24 patients of the M-ER group (○). ER = 7 fmol/mg cytosolic protein is the limit of sensitivity of the assay.

DCC-exchange assays have a saturable 8S peak on sucrose gradient.

Age of patients (Fig. 1)

In group H-ER, the age of patients ranged from 18 to 32 years with a mean of 25 years. In group M-ER, the patients were 26 to 44 years old with a mean of 36 years, whereas in group L-ER the patients were older (32 to 55 years, mean: 44 years).

Cellular density in tumors (Fig. 2)

The greatest epithelial cell proliferation was observed in group H-ER (70% of type I) whereas in group L-ER 5% of the tumors were of type I, 40% of type II, and 55% type III. In group M-ER, the proportion of tumors of type I, II and III was almost identical (40, 30 and 30%, respectively).

Length of evolution of the tumors at the time of surgery (Fig. 3)

The exact time of evolution is impossible to determine; nevertheless the patients' reported time of tumor discovery to surgery was taken as an approximate duration of evolution.

The greatest concentration of estradiol-binding sites (group H-ER) was observed in patients with a short duration (15 days to 5 months). By contrast, in patients with low concentrations of estradiol binding sites (group L-ER) the length of evolution of the tumor was more than 9 months with a mean of 4.3 years. In group M-ER the length of evolution was intermediate (mean: 7 months).

Hormone assays

The length of the menstrual cycle varied in the 3 groups of patients from 26 to 43 days with a mean of 32.1 ± 5.2 days (H-ER), 33.2 ± 6.0 days (M-ER) and 34.0 ± 7.2 days (L-ER), but these variations were not significant.

There was no difference between the 3 groups of patients as regards the length of the luteal phase (thermal plateau) 9.0 ± 1.3, 9.6 ± 1.9 and 9.0 ± 2.8 days, respectively, but they were significantly shorter than the luteal phase of the normal women (13.2 ± 0.8 days; $P < 0.05$).

During the luteal phase, the mean plasma estradiol levels of the patients were, respectively, 278 ± 51, 319 ± 45 and 308 ± 100 pg/ml. These three values do not differ significantly ($P = 0.5$) but each of them is significantly higher ($P < 0.001$) than the values observed in normal women (180 ± 80 pg/ml). As for plasma progesterone, the mean values observed in patients during the luteal phase, respectively 9.8 ± 3.3, 13.3 ± 3.2 and 15.0 ± 2.2 for the groups H-ER, M-ER and L-ER, were significantly lower than those observed in normal women (21.0 ± 6.0 ng/ml). These results clearly indicate that in the patients studied plasma progesterone levels were uniformly low during the luteal phase whereas over the same period plasma estradiol levels were elevated.

DISCUSSION

Recent work from this laboratory reported that patients with benign breast diseases had an inadequate corpus luteum [8] characterized by normal secretion of estradiol contrasting with an abnormally low secretion of progesterone. In the present series of patients, progestational inadequacy was also observed but in addition there was an increased plasma estradiol concentration, as reported by Backström and Cartensen in premenstrual tension [14]. These results suggest that in patients with benign breast disease, and particularly with recent fibroadenomas, the hormonal status is characterized by a clearly persistent estrogenic stimulation unopposed by adequate progesterone secretion. The presence of estradiol receptors in receptive cell of fibroadenoma with great cellular density gives some support to the hypothesis that benign breast diseases are estrogen-dependent. Such an hypothesis was previously reported by Cortes-Gallegos *et al.*[15] who found a higher concentration of estradiol in breast tissue from patients with benign mastopathy than in breast tissue from normal women.

To explain the fact that the results obtained by other groups are less convincing than ours, we suggest that the relatively high concentration of estrogen binding sites observed in the fibroadenomas studied in this work is largely due to a precise microdissection of the receptive structures of the tumor and also to methodological considerations—particularly the use of an exchange method for the actual determination of specific estradiol binding cytosolic sites present in the tumor.

In this regard it is interesting to note that the greatest concentration of estradiol binding sites was observed in tumors with high epithelial cellularity and very low fibrosis. These tumors were generally recently developed, in the youngest women, and with a short length of evolution between discovery and surgery. Similar observation was made by Rosen *et al.*[7] in a shorter series of 23 fibroadenomas.

The persistent estrogenic stimulation noted both *in vivo* and *in vitro* in patients with breast fibroadenomas, particularly in cases with important cellular proliferation, emphasize the role of estradiol in cellular multiplication and hyperplasia [16–18]. In addition the observation *in vivo* of an inadequate progesterone secretion by the corpus luteum emphasizes the role of progesterone in antagonizing the effect of estradiol on cellular growth and differentiation [19–21]. The importance of estradiol/progesterone imbalance in the development of endometrial hyperplasia and adenocarcinoma is, indeed, well documented [22, 23]. The present observation supports the hypothesis that a similar hormonal component may provide a setting in mammary gland favorable to the development of benign dystrophies. In addition there are now enough clinical data available to suggest that progesterone and progestins are effective in the treatment of many benign breast diseases [24], particularly in cases where cellular hyperplasia is predominant and fibrosis still absent [25].

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