Uptake and Concentration of Steroid Hormones in Mammary Tissues

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INTRODUCTION

Since it was first demonstrated that human breast tumors can be influenced clinically by changes in the endocrine milieu,[1] many studies have been done to identify a relationship between these tumors and hormones. Epidemiologic studies and experimental research in animals have established a role for steroid hormones (estrogens, androgens, and/or progesterone) in the etiology of the disease[2].

Regarding estrogens, many investigations using urinary excretion patterns, plasma levels and production rate measurements in women with breast cancer, women at high risk, and healthy women, have shown no consistent differences between these groups.[3–5] Furthermore, if differences were observed, the investigators were not able to explain the large variations in incidence of the tumor between Western and non-Western countries,[6] the one possible exception being the very recently described changes in 2- and 16-hydroxylations as part of the metabolism of the estrogens.[7]

In the early reports of Bulbrook and coworkers, a decrease in the urinary excretion of androgen metabolites was described in women with metastatic or primary breast cancer[8]; later reports from a large prospective study[9] demonstrated a subnormal urinary excretion of the androgen metabolite etiocholanolone in women in whom mammary tumors were subsequently detected. It was shown that this subnormal excretion in postmenopausal women was associated with a decrease in the urinary production rate of dehydroepiandrosterone sulfate (DHEAS).[10]

On the basis of these data the possibility has been considered that the decrease in the production rate of DHEAS and, as a consequence, of dehydroepiandrosterone (DHEA) might be involved in the development or growth rate of mammary tumors in women.[11] Because steroid hormones exert their biological activity via interaction with specific intracellular binding proteins, called receptors, the hypothesis was put forward that a metabolite of DHEA was able to modulate the action of estrogens on target tissues. Indeed it was found that the metabolite 5-androstene-3β,17β-diol (5-Adiol) was able to inhibit *in vitro* the binding of estradiol to its receptor at concentration ratios within the observed physiological ranges of these steroids.[12]

It was postulated that a diminished concentration of 5-Adiol, at unchanged estradiol concentrations, could therefore be less able to counteract the stimulatory effects

of estradiol. However, measurements of plasma levels of 5-Adiol and estradiol in patients with mammary cancer did not show significant changes in either hormone.

Meanwhile a large number of *in vitro* studies,[13–22] supported by a few *in vivo* studies,[23–25] provided evidence that human breast tissue itself is capable of influencing its own endocrine environment. In addition, several investigators showed that there is no simple linear relation between plasma levels and tissue concentrations of steroid hormones.[26–28] Consequently it seemed more relevant to study the actual concentrations of the steroids inside the tissues, at the place where they exert their biological activities. According to the mechanism of action of steroid hormones, these substances not only have to enter the cells, but they must also be translocated as part of the steroid-receptor complex to the nuclei of target cells and have to remain there during a prolonged period of time for biological effects to occur.[29]

Using a recently developed technique[30] capable of extracting quantitatively the steroids from tissue compartments, we attempted to measure a number of the relevant steroids in the cytosol and the nuclear compartment of primary malignant breast cancer tissues and of nonmalignant and normal breast tissues of pre- and postmenopausal women. The androgens DHEAS, DHEA and 5-Adiol were measured together with estrone and estradiol.

MATERIAL AND METHODS

Tissue Specimens

Breast tissue specimens were obtained from 114 women. They were classified in five subgroups, depending on histology, as judged by the pathologist, and on the menopausal status of the patient. The subgroups were:

(1) "normal" tissue of premenopausal women, obtained from specimens with only benign lesions ($n = 19$) and from specimens with benign tumors ($n = 5$);

(2) premenopausal nonmalignant tissues (from women undergoing breast corrections), called hypertrophic tissue ($n = 16$); and from tissues from women with fibrocystic lesions ($n = 6$) and benign tumors ($n = 6$);

(3) malignant premenopausal tissues from specimens carrying a tumor ($n = 21$);

(4) "normal" tissue of postmenopausal women, excised from specimens of malignant tumors ($n = 7$);

(5) malignant tissues from postmenopausal women ($n = 34$).

All tissues were chilled immediately after operation and freed from fat and connective tissue by the pathologist, and a representative part was stored at −80°C.

Analytical Procedure

Portions of 0.8 to 1.0 gm of the tissues were homogenized and the cytosol and nuclear fractions were prepared by centrifugation at 100,000 × *g* for 30 minutes as described previously.[30] The steroids were extracted from both fractions with ethanol-acetone, the extracts were defatted with 70% methanol, and each extract was split

into three parts. From one part the estrogens were extracted, separated using Sephadex LH-20 columns, and measured by specific radioimmunoassays. The second part was extracted, and the androgens were separated using Celite-ethylene glycol columns, eluted with increasing amounts of ethylacetate in iso-octane, and quantitated with specific radioimmunoassays.[25] DHEAS was measured directly only in the cytosol without prior purification.

All results were corrected for losses during purification; and the results of the steroid measurements were recalculated to concentrations in pmol (estrogens) or nmol (androgens) and expressed per gram wet weight of each tissue. Because the data in each group were not normally distributed, differences between groups were tested statistically by the two-tailed Wilcoxon rank-sum test and correlations were calculated by the Spearman rank test.

RESULTS

Estrogen Concentrations in Breast Tissues

The median values of the concentrations of estrone and estradiol together with the 95% range of the individual samples are given in TABLE 1 for the cytosol, the nuclear compartment, and the total tissue in the five groups of patients studied. For both estrogens a large concentration range was observed.

The estrone levels in cytosol and total tissue were higher than the corresponding peripheral blood levels in approximately half of the tissues. Lower concentrations were seen in the nuclear fractions: on average, only one-fifth of the total tissue level was found in the nuclei. No significant differences in the estrone concentrations were found in any of the fractions studied between the malignant and the nonmalignant tissues of the corresponding age-matched groups. In accordance with lower estrone concentrations in the blood of postmenopausal women, the intratissue estrone levels were significantly lower in normal and malignant specimens compared with those from premenopausal women.

The concentration of estradiol in cytosol and total tissue was within the range of normal peripheral plasma levels in the majority of the nonmalignant samples from premenopausal patients; the nuclear concentrations were lower (approximately one-third of the total amount was found in the nuclei). Estradiol concentrations in cytosol, nuclear fractions, and total tissue of the malignant tissues were significantly higher ($p < 0.05$; $p < 0.05$ and $p < 0.01$, respectively) than in normal or nonmalignant tissues of premenopausal women. Despite the considerable differences in plasma estradiol levels between pre- and postmenopausal women, it is striking that the tissue levels are very similar, regardless of age, in normal as well as in tumor tissues. Also, for postmenopausal women, a significantly ($p < 0.05$) higher estradiol concentration was found in the malignant specimens.

In 51 of the malignant tumors (19 from premenopausal and 32 from postmenopausal patients) the estrogen receptor content had been measured. The estradiol concentration in the 41 receptor-positive specimens was significantly higher ($p < 0.05$) for cytosol, nuclear fraction, and total tissue than in the 10 receptor-negative tumors. No significant correlation could be calculated between the receptor content

TABLE 1. Estrone and Estradiol Levels in Normal Breast Tissue, Benign Lesions and Malignant Tumors

		Cytosol		Nuclear Fraction		Total Tissue	
	n	Median	95% Range	Median	95% Range	Median	95% Range
				Estrone			
Premenopausal samples							
Normal	24	795	370-3260	220	95-670	1035	480-3780
Benign	28	670	240-2550	185	20-520	1035	315-3040
Cancer	21	780	260-2000	165	75-350	1110	335-2590
Plasma	20					100	90-800
Postmenopausal samples							
Normal	7	350	220-815	95	35-295	500	335-1110
Cancer	34	465	110-2150	130	20-575	610	130-2260
Plasma	39					100	40-200
				Estradiol			
Premenopausal samples							
Normal	24	260	110-845	145	70-420	420	200-1190
Benign	28	220	110-570	110	35-385	330	200-880
Cancer	21	495	185-2110	260	90-3790	720	275-5880
Plasma	20					370	200-2000
Postmenopausal samples							
Normal	7	220	120-295	130	90-165	350	240-440
Cancer	34	480	130-7430	295	35-660	790	220-7720
Plasma	39					30	10-50

NOTE: The mean levels in cytosol, nuclear fraction, and total tissue are shown, together with the 95% range of the values in pre- and postmenopausal women. The values are expressed in fmol/g tissues (pmol/kg). The peripheral plasma levels indicated are given in pmol/L; the number of samples can be found under *n*.

(in fmol/mg protein) and the estradiol concentration in either compartment. The nuclei/cytosol ratio for estradiol was slightly higher in receptor-positive than in receptor-negative tumors, and the median ratios were 0.58 and 0.41, respectively.

Because of the increased estradiol concentrations in the malignant tumors, the ratio between estrone and estradiol was lower in the tumors than in normal and nonmalignant samples. Particularly in the nuclear compartment the ratio between the two estrogens was lower than in the cytosol because of the higher nuclear estradiol concentrations.

Androgen Concentrations in Breast Tissues

The median values of the concentration of DHEA and 5-Adiol and the 95% range of these concentrations are shown in TABLE 2 for the cytosol, nuclear compartment, and total tissue in the five groups studied. In addition, DHEAS levels in the cytosol only are given. Also, very large variations were found in the tissue concentrations of these androgens.

In all groups the intratissue level of DHEA is higher than in peripheral plasma mainly because of the relatively high concentrations in the nuclear fractions. In four of the five groups studied the nucleus contained more DHEA than did the cytosol. Within the group of patients with nonmalignant lesions, the subgroup of specimens with benign tumors clearly can be distinguished, for there was a higher total tissue DHEA concentration ($p < 0.05$) than in the subgroup with only hypertrophy. The concentrations in the malignant tumors were statistically significantly lower ($p < 0.05$) than in the nonmalignant samples from premenopausal women.

The 5-Adiol concentrations in tissues were lower than those of DHEA in all groups; in the majority of samples they were comparable to or less than the 5-Adiol plasma levels. No significant differences were found between the normal and malignant tissues in either the pre- or the postmenopausal groups studied. Again in all groups the amount of 5-Adiol measured in the nuclear fractions was higher than in the cytosol. Additionally the subgroup of benign tumors showed the highest concentrations, similar to the observations on DHEA.

The concentrations of DHEAS were measured only in the cytosol; only occasionally were the levels in tissue higher than in plasma. In premenopausal women the subgroup with normal or benign tumor tissue had significantly ($p < 0.02$) higher concentrations than did those with malignant tissues.

For all three androgens, the median values of the tissue concentrations in samples from premenopausal women were higher than those of the samples from the corresponding postmenopausal patients, but these differences were not always significant in all fractions studied. The well-known age-dependency of the adrenal androgens in plasma was also reflected in the tissue levels; a highly significant weak negative correlation between age and the total tissue concentration was calculated for all patients for DHEAS ($r = -0.46$; $p < 0.001$; $n = 109$), DHEA ($r = -0.33$; $p < 0.001$; $n = 110$), and 5-Adiol ($r = -0.33$; $p < 0.001$; $n = 91$).

Since DHEAS can act as a precursor for DHEA and 5-Adiol, the correlations between the tissue androgens were calculated. In the total population, as well as in the individual subgroups, highly significant correlations existed between DHEAS and DHEA ($r = 0.75$; $p < 0.001$, total population) and between DHEA and 5-Adiol ($r = 0.88$; $p < 0.001$, total population).

TABLE 2. Androgen Values in Normal Breast Tissue, Benign Lesions, Hypertrophic Tissue, and Malignant Tumors

		Cytosol		Nuclear Fraction		Total Tissue	
	n	Median	95% Range	Median	95% Range	Median	95% Range
			Dehydroepiandrosterone				
Premenopausal samples							
Normal	24	38	7-210	94	10-345	130	14-700
Benign	12	42	17-200	128	10-345	195	28-520
Hypertrophic	16	14	3-180	24	7-310	42	10-450
Cancer	21	21	1-130	52	3-430	70	7-555
Plasma	25						10-30
Postmenopausal samples							
Normal	7	17	2-50	17	2-205	52	3-250
Cancer	32	10	1-45	31	3-240	38	7-240
Plasma	25						2-20
			5-Androstenediol				
Premenopausal samples							
Normal	22	2.4	0.5-13.1	3.5	0.5-27	5.5	0.9-28
Benign	12	2.1	1.2-13.8	4.1	0.4-14.1	6.6	1.6-21
Hypertrophic	16	1.2	0.3-2.6	1.3	0.3-14.5	2.8	0.6-17.2
Cancer	18	1.6	0.9-11.7	2.2	0.7-17.9	3.8	1.5-29
Plasma	25						1.5-5.5
Postmenopausal samples							
Normal	7	1.1	0.1-2.7	1.4	0.1-7.2	2.7	0.1-10.0
Cancer	27	1.2	0.3-3.2	1.1	0.2-7.2	2.5	0.7-10.7
Plasma	25						0.5-4.5
			Dehydroepiandrosterone Sulfate				
Premenopausal samples							
Normal	24	3450	600-25800				
Benign	12	3800	730-7000				
Hypertrophic	16	2450	600-15500				
Cancer	21	730	140-19800				
Plasma	25		2000-7000				
Postmenopausal samples							
Normal	7	1790	140-12000				
Cancer	29	430	110-2720				
Plasma	25		1000-6000				

Ratio of Androgens to Estrogens

In view of the hypothesis that 5-Adiol plays a role in the modulation of estradiol action, we were interested not only in its concentration, but also in the ratio of this androgen to estradiol in each sample. This ratio was calculated from the total tissue concentrations of both steroids. The ratio showed large variations: 95% of the values varied between < 1 and 40. Such a high ratio (> 20) would indicate a sample containing sufficient 5-Adiol to interfere with the binding of estradiol to its receptor. However, although there was a statistically significant difference in the ratio between the premenopausal normal and malignant samples, this difference was caused by the increased levels of estradiol in the malignant samples; the 5-Adiol levels themselves were not lower. For postmenopausal women no significant differences were found.

It is remarkable that the ratio was changed in the tissues from premenopausal women with benign mammary tumors. Because of the higher androgen content, the ratio was increased ($p < 0.05$) compared with that of all other tissue samples.

DISCUSSION

From our results on endogenous intratissue steroid levels in malignant, nonmalignant, and normal breast tissue, we can characterize the samples from *premenopausal* women as follows: There are (1) higher concentrations of DHEAS and DHEA in normal tissues when compared with malignant tumors; (2) no significant differences in 5-Adiol levels; (3) no significant differences in estrone levels; (4) significantly lower estradiol concentrations in normal and benign tissue samples compared with malignant ones; and (5) the tissue levels of the three androgens studied were significantly higher in the benign and normal tissue than in the tissue from the malignant tumors.

Although a lower tissue concentration of adrenal androgens was observed in the malignant samples, however, these findings do not support our hypothesis on the role of 5-Adiol because this steroid was found in very similar levels in all premenopausal groups studied.

In *postmenopausal* women the differences in androgen levels are much less pronounced. In the normal specimens there was a tendency for a decrease in the tissue concentration of androgens compared with premenopasual samples, and this runs parallel to the decreasing plasma levels with age. No significant differences were observed between normal and malignant samples from postmenopausal women, although the median tissue DHEAS levels were lower in the cancer patients ($p < 0.10$).

Regarding estrogens, our data on estradiol concentrations in total tissue are in agreement with those in the literature.[31-33] It is amazing that, despite the large decrease in plasma levels, the concentrations of estradiol in breast tissues are stable with age in normal as well as malignant samples. For estrone, however, in parallel with plasma levels, a significant decrease was found in postmenopausal compared with premenopausal women, again in malignant and nonmalignant samples. The discrepancies in the changes in plasma levels of estradiol before and after menopause point to the fact that simple accumulation of plasma estrogens in breast tissue cannot be the explanation for the tissue levels.

The differences in the estradiol concentrations between estradiol-receptor-positive and -negative tumors are in accordance with previously published data.[31-36] However,

the differences between the amounts of estradiol measured in the cytosol compared with the nuclear fraction cannot be explained by the presence of these proteins only since no correlation was found between the receptor and estradiol concentrations in the tumors and because the amounts of estradiol in nuclear fractions of uterine tissues were approximately 10 times higher at similar receptor levels.[37] In addition, the ratio between total tissue concentration of estrone and estradiol in the uterus is much lower than it is in breast tissue. Therefore it is more likely that additional mechanisms are involved in the uptake and accumulation of estrogens in target tissues. Factors like low-affinity binding proteins in the cytosol and the nuclei may be involved. The observed differences in the estradiol concentration between breast and uterine tissues at similar estradiol-receptor concentrations might be explained by the recent hypothesis that the receptor is localized mainly in the nuclear compartment,[38,39] and then the steroid entering the cell would be in a different compartment than the receptor.

An additional important factor may, of course, be the local synthesis of estradiol, a possibility suggested by many *in vitro* studies.[13–23] Insufficient data on the biosynthetic capacity of normal or malignant cells *in vivo* are available[23–25] to support or to exclude this possibility. In particular it is difficult to explain the very high levels of estradiol in breast tissue in normal and malignant cells of postmenopausal women.

The subcellular distribution of estrone and estradiol in all breast samples studied is different from that in the endometrium or myometrium[28,37] measured with a comparable method. Again this points to the fact that estrogens are handled in a non-identical way in uterine and breast tissues. The supposition that the receptors in the cells are present in a different compartment than that of the steroid could explain these discrepancies.

Our results that show the tissue concentrations of DHEA to be higher than in peripheral plasma are similar to those reported by other authors[35,40]; our results show 5-Adiol levels to be comparable[35] or lower[40] than those found in previous investigations. No results on DHEAS have been reported so far. No accumulation of this sulfated steroid was observed, whereas the unconjugated androgens do show a positive tissue gradient. Because of the good correlation between DHEAS and the other two androgens in tissue, the intracellular metabolism of DHEAS may very well be responsible for at least part of the high concentrations of DHEA and 5-Adiol.

The intracellular concentration of the androgens is low in the subgroup of patients with hypertrophy of the breasts and is comparable to that in the malignant samples. No explanation is available for this phenomenom since only the androgen concentrations are similar, the endogenous estrogen levels being clearly lower in the hypertrophic than in the malignant samples.

The subcellular distribution of 5-Adiol, and especially of DHEA, shows a higher nuclear concentration in all tissue samples, but the mechanisms behind this unexpected distribution remain unknown. The presence of low-affinity binding proteins in the cytosol or the binding proteins in the nuclear matrix[41] may be involved.

The results on 5-Adiol do not support our original hypothesis on the inhibitory role of this steroid on the effects of estradiol via interference with the estradiol-receptor protein. Since the original publication on this hypothesis[11] we have already demonstrated that the postulated antiestrogenic activity of 5-Adiol is not very likely in view of the fact that this androgen clearly showed estrogenic activity on the immature rat uterus[42] and on the human breast cancer cell line MCF-7,[43] including the induction of the progesterone receptor. Although a possible role for adrenal androgens in the genesis of human breast cancer can be supported by our findings on lower endogenous DHEAS and DHEA levels in malignant tumors, the way in which these androgens express their influences remains unclear.

Recently it was demonstrated that DHEAS and DHEA can act as inhibitors of the 17 β-hydroxysteroid dehydrogenase in the endometrium,[44] leading to a decreased conversion of estradiol to estrone and thus to higher estradiol concentrations in the tissue. Because the concentrations of the androgens involved in our study are lower in the cancer samples, the higher estradiol levels can not be explained by this mechanism.

The local aromatization of androgens to estrogens has been demonstrated in malignant breast tissues, but an influence of the adrenal androgens on this enzymatic activity has not been observed. Therefore it is not possible at the moment to explain the role of the adrenal androgens in the etiology of human breast cancer.

SUMMARY

In order to exert their biological effects, steroid hormones must enter the cells of target tissues and after binding to specific receptor molecules must remain for a prolonged period of time in the nucleus. Therefore the endogenous levels and the subcellular distribution of estradiol, estrone, DHEAS, DHEA ad 5-Adiol were measured in normal breast tissues and in malignant and nonmalignant breast tumors from pre-and postmenopausal women. For estradiol the highest tissue levels were found in the malignant samples. No differences were seen in these levels between pre- and postmenopausal women despite the largely different peripheral blood levels. For estrone no differences were found between the tissues studied. Although the estradiol concentration was higher in the estradiol-receptor-positive than in the receptor-negative tumors, no correlation was calculated between the estradiol and the receptor concent. Striking differences were seen between the breast and uterine tissues for the total tissue concentration of estradiol, the ratio between estradiol and estrone, and the subcellular distribution of both estrogens. At similar receptor concentrations in the tissues these differences cannot easily be explained.

Regarding the androgens, the tissue/plasma gradient was higher for DHEA than for 5-Adiol, and for DHEAS there was very probably a much lower tissue gradient. The highly significant correlation between the androgens suggests an intracellular metabolism of DHEAS to DHEA and 5-Adiol. Lower concentrations of DHEAS and DHEA were observed in the malignant tissues compared with the normal ones and the benign lesions. For 5-Adiol no differences were found and therefore these data do not support our original hypothesis on the role of this androgen in the etiology of breast abnormalities. Hence the way in which adrenal androgens express their influence on the breast cells remains unclear.

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