Estrogen Synthesis and Estradiol Binding by Human Mammary Tumors

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ABSTRACT

Breast tumor homogenate from 17 patients was incubated with androstenedione, and 13 tumors yielded estrone and/or estradiol as metabolites. To ensure proper identification of the metabolites, we used a dual-labeling technique consisting of incubating tumor homogenate with [3H]androstenedione and adding [14C]-estrogens after the reaction was stopped. The estrogen metabolites were isolated and purified, and the constancy of the [3H]:[14C] ratio was used as proof of estrogen formation. The level of estrogen receptor in these malignant tumors was determined by sucrose density gradient centrifugation analysis. No correlation was found between the ability of tumors to aromatize androstenedione and the presence or absence of estrogen receptors. It was found that, of the 13 tumors that metabolized androstenedione to estrogen, estrone was the major metabolite in six of six tumors from women who were postmenopausal for 10 or more years. In contrast, five of six tumors from premenopausal patients produced estradiol predominantly. A positive correlation was noted between the ability of the tumors to form estrone and menstrual status and increased age. Furthermore, the tumor incubations from premenopausal women yielded significantly higher levels of testosterone than those from postmenopausal patients, suggesting a difference in 17β-hydroxysteroid dehydrogenase activity. The data presented in this paper demonstrate for the first time the similarity between the function of normal endocrine tissue and neoplastic breast tissue.

INTRODUCTION

In recent years, work from several laboratories has demonstrated that human breast tumor is capable of transforming androgens to estrogens in vitro. Miller and Forrest (15) and Adams and Li (2) have reported the conversion of testosterone to estradiol by human breast cancer tissue, and Abul-Hajj (1) observed the synthesis of estradiol from testosterone to estradiol by human breast cancer tissue, and Adams and Li (2) have reported the conversion of androstenedione to estradiol and/or estradiol as metabolites. To ensure proper identification of the metabolites, we used a dual-labeling technique consisting of incubating tumor homogenate with [3H]androstenedione and adding [14C]-estrogens after the reaction was stopped. The estrogen metabolites were isolated and purified, and the constancy of the [3H]:[14C] ratio was used as proof of estrogen formation. The level of estrogen receptor in these malignant tumors was determined by sucrose density gradient centrifugation analysis. No correlation was found between the ability of tumors to aromatize androstenedione and the presence or absence of estrogen receptors. It was found that, of the 13 tumors that metabolized androstenedione to estrogen, estrone was the major metabolite in six of six tumors from women who were postmenopausal for 10 or more years. In contrast, five of six tumors from premenopausal patients produced estradiol predominantly. A positive correlation was noted between the ability of the tumors to form estrone and menstrual status and increased age. Furthermore, the tumor incubations from premenopausal women yielded significantly higher levels of testosterone than those from postmenopausal patients, suggesting a difference in 17β-hydroxysteroid dehydrogenase activity. The data presented in this paper demonstrate for the first time the similarity between the function of normal endocrine tissue and neoplastic breast tissue.

MATERIALS AND METHODS

Chemicals. [7α-3H]Androstenedione (11 Ci/mmol), 4-14C-estrogens, and [4-14C]testosterone were obtained from Amersham/Searle Corp., Arlington Heights, Ill., and [2,4,6,7-3H]estradiol (100 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. The purity of these chemicals was checked on TLC. In addition 10 μCi of [7α-3H]androstenedione was run through the procedure, as described below for the androstenedione metabolism experiment, to check for possible impurities in the phenolic fraction. Standard steroids were purchased from Steraloids, Wilton, N. H. All solvents were redistilled. Hepatone was treated with H2SO4 (2 changes) and washed with alkaline KMnO4 before distillation. The resin used for the ion-exchange column was Bio-Rad AG1-X2, 200 to 400 mesh, in the chloride form. TLC was done on Eastman Chromagram Sheets 6060.

Incubation and Extraction. At the time of surgery, the tumor was cleansed of fat and connective tissues and was frozen in liquid nitrogen. The tumor was weighed and pulverized, and a portion was removed for estrogen receptor assay. Approximately 1.0 g was homogenized in 4 ml of 0.25 M sucrose and 1 ml of 0.5 M Tris buffer (pH 7.6) by a ground-glass homogenizer with a motor-driven pestle. To the homogenate were added 6 mg NADP+*, 36 mg glucose 6-phosphate, 28 units glucose-6-phosphate dehydrogenase, and 10 μCi [7α-3H]androstenedione. The mixture was incubated for 3 hr at 37° in a Dubnoff metabolic shaking incubator. The reaction was stopped with 10 ml of hot acetone:ethanol (20:3), and the mixture was filtered through Whatman No. 1 paper. The filtrate was added 8000 dpm of [14C]estrone, 4000 dpm of [14C]estradiol, 2000 dpm of [14C]estradiol, and 500 μg of the 3 estrogens. The organic solvents were removed on a flash evaporator, and the radioactive metabolites were isolated by the method of Ryan and Smith (16). The steroids were extracted with

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3 The abbreviations used are: DHEA, dehydroepiandrosterone; TLC, thin-layer chromatography.

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chloroform and partitioned between heptane and 90% methanol.

**Isolation and Identification of Phenolic Steroids.** The methanol extract was run on an ion-exchange column prepared according to the directions of Eberlein (7). The neutral steroids were eluted with 25 ml of methanol, and the phenolic steroids were eluted with 35 ml of 80% methanol. The eluant was collected in 5-ml fractions, and a portion was counted to ensure proper separation. Tubes containing 14C were then combined and rerun on a second column to remove the contaminating neutral steroids.

The phenolic fraction was run on TLC in ethylene glycol monomethyl ether:benzene (8:92), and the zones corresponding to estrone, estradiol, and estriol were eluted. Approximately 12 mg of carrier steroid were added, and the estrogens were recrystallized to constant specific activity. The final precipitate was acetylated with acetic anhydride:pyridine. The acetates were run on TLC in ethyl acetate:cyclohexane (30:70). If sufficient material remained, the acetates were recrystallized. Aliquots of the previous samples were counted in duplicate in 10 ml liquid scintillation fluid (4 g PPO and 100 mg POPOP per liter toluene) to a S.E. of <5% on a Beckman LS-250 liquid scintillation counter with automatic quench compensation.

**Isolation and Identification of Testosterone.** [14C]Testosterone was added to the neutral fraction that was then chromatographed on paper by the method of Feher (8) with propylene glycol as the stationary phase and heptane:benzene:methanol (130:60:10) as the mobile phase. The area of testosterone was eluted, 12 mg of carrier steroid were added, and the sample was recrystallized to constant specific activity.

**Estrogen Receptor Assay.** Quantitative determination of estradiol receptor was carried out by a method described previously (18). The tumor cytosol was incubated with 5 nm [2,4,6,7-3H]estradiol for 4 hr at 0° in the presence and absence of nafoxidine. A dextran-coated charcoal pellet was used to remove excess steroid. The samples were layered on the top of 10 to 30% sucrose gradients and centrifuged for 16 hr at 50,000 rpm in a Beckman LS-50 centrifuge. The pmol of estradiol bound per mg of protein were determined in the 4S and 8S region of the gradient.

**RESULTS**

All of the results for the metabolism experiments are corrected for procedural losses. The recovery after each step is as follows: extraction, 90%; ion-exchange chromatography, 85%; and TLC, 60%. The amount of 14C-estradiol added was used to determine the amount of estrogen synthesized in each experiment.

The phenolic fraction of [7α-3H]androstenedione used for a purity check contained 5000 dpm 3H. The dpm 3H in each fraction after TLC were: estrone, 1000; estradiol, 800; and estriol, 200. The 3H:14C ratio for each steroid was <0.2. In the tumor incubations any estrogen fraction in which the ratio fell below 1.0 was considered negative. Therefore any impurities in the substrate were well below the sensitivity of this assay.

Chart 1 shows the separation and purification of the phenolic fraction by ion-exchange chromatography. Some of the neutral steroids trailed into the phenolic fraction on the first column, so it was necessary to use a second column for complete separation.

Table 1 discloses data for the identification of the estrogen metabolites of androstenedione formed by breast tumors from 13 patients. The 3 estrogen fractions from TLC were purified until the 3H:14C ratio was constant or fell below 1.0. Carrier recrystallization and derivative formation gave positive identification of these metabolites. No evidence for estriol formation was found in any of these tumors.

The actual 3H and 14C counts used to calculate the 3H:14C ratios for 1 tumor (from Patient LR) are given in Table 2. This tumor was selected because it had the lowest level of estrogen synthesized (0.3 pmol of estradiol). Virtually the entire sample of any mother liquor or final estrogen acetate precipitate that had low counts was used for counting to ensure accuracy.

The pmol of estrone and estradiol formed from 1 nmol of androstenedione by the tumors and the level of estrogen receptor are shown in Table 3. Of the 7 tumors that are estrogen receptor negative, 6 have aromatase activity. Ten tumors have estrogen receptors, and 7 of these also show the presence of aromatase activity. Thus the present data show a lack of correlation between estrogen synthesis by these tumors and the level of estrogen receptor.

When the age and menstrual status of the women whose tumors synthesized estrogen is taken into consideration, a pattern emerges. The first 6 patients listed in Table 3 were
premenopausal when breast cancer was detected. Five of 6 tumors from these premenopausal women synthesized estradiol exclusively. In contrast all 6 tumors from women who were postmenopausal for 10 or more years synthesized more estrone than estradiol. A $\chi^2$ test to compare the major metabolites formed in premenopausal and postmenopausal patients disclosed a $p$ value of <0.001, which is highly significant. Interestingly, all 4 tumors that failed to convert androstenedione to estrone were from women who were postmenopausal for <10 years.

We have also examined the neutral fraction for formation of testosterone. The amount of testosterone identified in tumor incubations from the premenopausal patients was significantly higher ($p < 0.05$) than that of postmenopausal women (Table 3). It should be noted that the difference in the formation of estrone and estradiol in tumors from pre-
Table 3

<table>
<thead>
<tr>
<th>Steroid synthesis by and the estrogen receptor level of human breast tumor</th>
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<tbody>
<tr>
<td>Steroid synthesized (pmol/g tumor)</td>
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<tr>
<td>-------------------------------------</td>
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<tr>
<td>GM 36 137 Premenopausal</td>
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<tr>
<td>DD 38 139 Premenopausal</td>
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<td>FM 42 125 Premenopausal</td>
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<td>EG 43 110 Premenopausal</td>
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<td>BS 45 106 Premenopausal</td>
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<td>EB 49 265 Premenopausal</td>
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<tr>
<td>PN 47 133 6 mos. postmenopausal</td>
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<tr>
<td>AW 50 142 2 yr postmenopausal</td>
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<tr>
<td>GF 53 117 7 yr postmenopausal</td>
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<tr>
<td>LT 55 88 2 yr postmenopausal</td>
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<tr>
<td>TL 55 137 9 yr postmenopausal</td>
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<tr>
<td>CT 59 103 15 yr postmenopausal</td>
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<tr>
<td>MC 62 122 20 yr postmenopausal</td>
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<tr>
<td>AZ 66 189 12 yr postmenopausal</td>
</tr>
<tr>
<td>MK 69 154 25 yr postmenopausal</td>
</tr>
<tr>
<td>LR 70 132 25 yr postmenopausal</td>
</tr>
<tr>
<td>CB 70 105 31 yr postmenopausal</td>
</tr>
</tbody>
</table>

* Tumor incubated with 1 nmol androstenedione.

** ND, not detected. The lowest level that could be measured was 0.4 pmol for estrone and 0.2 pmol for estradiol.

and postmenopausal women could be influenced by the presence or absence of 17β-hydroxysteroid dehydrogenase activity in these tumors. Although actual measurements of 17β-hydroxysteroid dehydrogenase activities in the tumors were not carried out, the data from the testosterone study give indirect evidence to suggest that tumors from premenopausal women favor the formation of 17β-hydroxylated steroids (testosterone and estradiol).

**DISCUSSION**

The use of a dual-labeling technique in our study ensures proper identification of the metabolites. Androstenedione was chosen as a precursor because it is more efficiently aromatized peripherally than the other 3 possible plasma-borne estrogen precursors (DHEA, DHEA sulfate, and testosterone) (14). We have now isolated and unequivocally identified for the first time both estrone and estradiol as metabolites of androstenedione in human breast cancer tissue.

In 1956, West et al. (21) isolated both estrone and estradiol from the urine of oophorectomized and adrenalectomized women with breast cancer, following the administration of testosterone. Chang and Dao (4) isolated 11β-hydroxy estrogens in urine from the same type of patient given cortisone acetate. The postulate that the tumor was the site of aromatization in these women was supported by the observation of Chang et al. (5) that human breast tumor slices could convert cortisone to 11β-hydroxyestrone. This has been followed by reports of the conversion of testosterone (2, 15) and DHEA (1) to estradiol, androstenedione to estrone (19), and cholesterol to androgens (6) by human breast tumors.

Altogether, these studies conclusively demonstrate that many human breast tumors have the enzyme necessary for the transformation of steroid precursors to estrogens. The question that remains to be answered is "What is the physiological significance of tumors synthesizing hormones?" Abul-Hajj (1) reported a correlation between the ability of breast tumors to aromatize DHEA and the lack of estrogen-binding capacity, suggesting hormone independence of the neoplasm. This observation, however, was not supported by work from other laboratories. Li et al. (12) reported a lack of correlation between the ability of breast tumors to aromatize testosterone and the level of estradiol receptors in these tumors. The results from our present investigation also fail to demonstrate a definite correlation between estrogen receptor level and aromatase activity.

The clinical implications of tumors synthesizing estrogens, particularly in patients with hormone-responsive neoplasms, is of practical importance. Further investigations are imperative to elucidate whether "extraglandular" production of steroid hormones in women with hormone-responsive breast cancers contributes to the continued growth of these neoplasms, in spite of the removal of adrenals and gonads.

Our present study disclosed an interesting and novel finding that breast tumors from young women formed estradiol predominately, whereas those from older women synthesized more estrone than estradiol (see Table 3). This observation shows that synthesis of estrogen by neoplastic breast tissue in young women is similar to that by the ovary of "normal" premenopausal women, which favors the production of estradiol over estrone (3), and androstenedione is believed to be an important intermediate in this synthesis (17). Aromatization of circulating androstenedione also
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occurs in normal premenopausal women although at a less efficient rate than in postmenopausal women (10). In normal women the extent of conversion of circulating androstenedione to estrone increases with age. Thus in postmenopausal women the principal estrogen formed is estrone. It was suggested that estrone was extraglandular via an aromatization of androstenedione since several authors have shown that little if any estrogen is secreted by the ovary in postmenopausal women (9, 13). This similarity in estrogen production by normal endocrine and neoplastic tissues has important therapeutic and theoretical significance. It would be reasonable to expect the therapeutic effect of agents that can effectively inhibit peripheral aromatization in breast cancer.

REFERENCES

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