Endogenous Concentration and Subcellular Distribution of Estrogens in Normal and Malignant Human Breast Tissue

A. A. J. van Landeghem, J. Poortman, M. Nabuurs, and J. H. H. Thijssen

ABSTRACT

The endogenous concentrations and subcellular distribution of estrone and estradiol were measured in malignant and nonmalignant human breast tissue from pre- and postmenopausal women. The most striking finding was the significantly higher concentration of estradiol per g of tissue in the malignant tissues than in the nonmalignant tissues. The tissue concentrations of estradiol in pre- and postmenopausal women were similar despite the large differences in the peripheral plasma levels. No correlation was found between the estradiol receptor content and endogenous concentration and subcellular distribution of estradiol. No difference in the estrone tissue concentration was found between malignant and nonmalignant tissues. In comparison with human uterine tissues, which we have reported previously, human breast tissue “handles” estrogenic hormones differently from human uterine tissue. At equal concentrations of the estradiol receptor, concentrations and subcellular distribution of the estrogens are different in both tissues. It is concluded that the mechanism of action of estradiol via its receptor, a mechanism mainly based on studies in animal uterine tissue, applies only qualitatively to human breast cancer tissue.

INTRODUCTION

In view of the differences in urinary excretion, plasma levels, and metabolism of estrogens and androgens in breast cancer patients (10, 30, 46), we have investigated the possibility that a diminished production of androgens is related to the development or to the growth rate of mammary tumors (37). 17β-Estradiol (estradiol) exerts its biological activity by binding to a specific receptor protein in the cytosol prior to entering into the nucleus as a steroid-receptor complex (16) where it binds to an “acceptor site” on the chromatin (32), resulting in an increased synthesis of mRNA (22). Poortman et al. (29) reported that ADIOL, a metabolite of DHEA, is able to inhibit in vitro the binding of estradiol to its specific receptor, at a concentration within the physiological range. It was suggested that the lower plasma levels of ADIOL, as a result of a diminished production rate of DHEA 3-sulfate and DHEA, at unaltered levels of estradiol (i.e., an imbalance between estrogens and androgens) might favor an enhanced estrogenic stimulus. However, plasma levels of estradiol and ADIOL in mammary cancer patients were not different from women without breast cancer. Present evidence indicated that there is no simple relation between plasma levels and tissue concentrations of steroid hormones (8, 9, 26, 35, 39). Consequently, to determine the ratio between androgens and estrogens at the cellular level in the target organ in vivo, it is necessary to measure their tissue concentration. Measurements within the cell, in cytosol and nucleus, give additional information about the mechanism of action of estrogens and androgens. In order to test the hypothesis of an imbalance between androgens and estrogens occurring at the cellular level, we have measured the concentrations of estrone, estradiol, DHEA, DHEA 3-sulfate, and ADIOL in the cytosol and nuclear fraction of human malignant primary breast tumor tissues and of nonmalignant breast tissues of pre- and postmenopausal women.

This paper reports data on estrogens. The data on androgens and on the ratio between androgens and estrogens are reported separately (45).

MATERIALS AND METHODS

Tissue Specimens. Breast tissue specimens were obtained from 113 women. The tissues were classified in several subgroups, depending on the histology of the tissue as judged by the pathologist and on the menopausal status of the patient. “Normal” nonmalignant tissue was obtained from 3 groups of patients. It was excised from: Group a, mammary specimens from premenopausal women which contained only benign lesions at histological examination (n = 19); Group b, mammary specimens from postmenopausal women which contained a malignant tumor (n = 6); and Group c, specimens from premenopausal women which contained a benign tumor at histological examination (n = 5). Primary mammary tumor tissue was obtained from premenopausal (Group d, n = 21) as well as from postmenopausal (Group e, n = 34) patients. Hyperplastic tissue was obtained from premenopausal women undergoing a mamma correction (Group f, n = 16). In addition, benign tumor tissue (Group g, n = 6) and tissue containing fibrocystic lesions (Group h, n = 6), both from premenopausal women, were included in our study. After collection of all data and statistical analysis, we found no differences between Subgroups a and c. The individual data for the specimens of Subgroup c were normally distributed over Group a. In addition, we have combined the 3 subgroups of nonmalignant tissue from premenopausal women as Subgroup 1 (hypertrophic tissue), Subgroup g (benign tumor tissue), and Subgroup h (fibrocystic tissue). In the final analysis, we have used 5 groups: Group 1, Subgroups a plus c, premenopausal, normal tissue; Group 2, Subgroup d, premenopausal, malignant tissue; Group 3, Subgroup b, postmenopausal, normal tissue; Group 4, Subgroup e, postmenopausal, malignant tissue; Group 5, Subgroups f, g, and h, premenopausal, nonmalignant tissue. A summary of data on tissue specimens is given in Table 1.

Preparation of Subcellular Fractions. All tissues were put directly on ice in the operation room. They were freed from surrounding fat and connective tissue by the pathologist. Aliquots were used for histological examination, and a representative part of the tissue was stored at -80°C until analysis. In general, 0.8 to 1.0 g of tissue was minced, pulverized in a Micro-Dismembrator (Braun, Melsungen, West Germany) and homogenized in 4 ml of cold Tris buffer (0.01 M Tris-HCl; 0.001 M EDTA; 0.003 M NaNO₃; pH 7.5). The cytosol and nuclear fraction were...
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Table 1

<table>
<thead>
<tr>
<th>Tissue groups</th>
<th>No. of patients</th>
<th>Mean age (yr)</th>
<th>Range of ages (yr)</th>
<th>Receptor status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tissue</td>
<td>24</td>
<td>38</td>
<td>15–55</td>
<td></td>
</tr>
<tr>
<td>Malignant tissue</td>
<td>21</td>
<td>46</td>
<td>30–58</td>
<td>14</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>6</td>
<td>65</td>
<td>56–82</td>
<td></td>
</tr>
<tr>
<td>Malignant tissue</td>
<td>34</td>
<td>69</td>
<td>49–95</td>
<td>27</td>
</tr>
<tr>
<td>Nonmalignant tissue</td>
<td>28</td>
<td>32</td>
<td>16–50</td>
<td></td>
</tr>
</tbody>
</table>

Prepared by centrifugation of the homogenate at 4°C for 30 min at 100,000 g in a Beckman Model L5-65 ultracentrifuge. Aliquots of this cytosol were used for quantitation of receptor sites and protein content. The remainder of the cytosol and the nuclear fractions were stored at −20°C until further analysis of estrogens and androgens.

Quantitation of Receptor Sites. Receptor sites were measured according to a modified European Organization for Research on the Treatment of Cancer procedure (1973) as described earlier (31, 43). Aliquots of cytosol were incubated overnight at 4°C with increasing concentrations of \(^{3}H\)estradiol (0.3 to 2 × 10^{-8} M). Values were corrected for nonspecific binding. The results are expressed as fmol/mg of cytosol protein. Tumors were classified as positive (ER+), borderline (ER±), and negative (ER−) according to the following limits: ER+ > 10, ER± 3 to 10, and ER− < 3 fmol/mg of cytosol protein (21). In addition, the Km should be < 10^{-8} M.

Extraction of Estrone from Cytosol and Nuclear Fraction. Estrone and estradiol were extracted from the cytosol and the nuclear fraction as described earlier (44). In brief, to both subcellular fractions, \(^{3}H\) tracers of the individual hormones were added to enable correction for procedural losses. Estrogens were extracted from the cytosol by precipitation in ethanol:acetone (1:1, v/v); the nuclear fraction was sonicated in ethanol:acetone (1:1, v/v). The ethanol:acetone extracts were defatted with 70% methanol and, after evaporation of the methanol, the residues were subsequently extracted with ether after resolution in Tris buffer. The extracts were purified on LH-20 columns on which both estrogens and androgens were subsequently extracted with ether after resolution in Tris buffer. The extracts were purified on LH-20 columns on which both estrogens and androgens were separated. In a previous paper, we have demonstrated that this extraction procedure removes more than 95% of the endogenous estrogens (44).

Measurement of Estrogens. After separation of estrone and estradiol on LH-20 columns, both steroids were measured, in duplicate, by radioimmunoassay using highly specific antisera. All the assays met the requirements of specificity, accuracy, and precision as we described earlier (42, 44). Results for both cytosol and nuclear fraction are expressed as pmol/g, wet weight, of tissue.

Miscellaneous Techniques. Cytosol protein concentration was measured according to the method of Lowry et al. (18) using bovine serum albumin as the standard. Radioactivity was measured by adding 10 ml of a scintillator (Instagel; Packard Becker BV-Chemical Operations, Groningen, The Netherlands) to the samples. Liquid scintillation counting was performed with a Packard Model 2660 liquid scintillation counter with automatic correction for quenching.

Statistical Analysis. Because the individual data per group were found to be not normally distributed, differences between the groups were tested for significance by the Wilcoxon rank sum test for 2 samples.

RESULTS

The subcellular concentrations of estrone and estradiol were measured in 113 breast tissue specimens, divided into 5 groups based on the histology of the tissue and the menopausal status of the patient. Data on receptor status, menopausal status, mean age of the patients, range of ages, and cytosol protein content are given for each group in Table 1. Data on tissue concentrations of estrone and estradiol are given in pmol/g, wet weight, of tissue without correction for cellularity based on DNA or protein content due to different ploidy of tumor cells and because we found no significant differences in protein content between malignant and nonmalignant tissues.

Intracellular Concentrations in Relation to Normal Plasma Levels. The concentrations of estrone in the cytosol, in the nuclear fraction, and in total tissue (calculated as cytosol plus nuclear fraction) are shown in Chart 1. Tissue concentrations showed a large variation; 95% of the values ranged from about 0.2 to 3.3 pmol/g tissue. For each individual group, median values are indicated. In approximately one-half of the tissues, the concentration of estrone in cytosol and total tissue (pmol/g) is much higher than in peripheral plasma (pmol/ml); the range of plasma levels of normal healthy pre- and postmenopausal volunteers is indicated in Chart 1. Tissue levels in Groups 3 and 4 should be compared with the plasma levels in postmenopausal women and in Groups 1 and 2 with plasma levels in premenopausal women. Lower concentrations for estrone were found in the nuclear fraction. In the majority of tissues studied, the nuclear levels were within the range of normal plasma levels.

A large concentration range of the tissue concentrations of estradiol was found (Chart 2); 95% of the values ranges from about 0.2 to 2.6 pmol/g tissue. In the majority of the premenopausal nonmalignant specimens, the concentration of estradiol in cytosol and in total tissue is within the range of normal plasma levels. In most of these specimens, the concentration in the nuclear fraction is below the peripheral plasma level. The tissue-plasma gradient for estradiol, especially in the postmenopausal malignant group (Group 4), is very high. This gradient was found both in cytosol and in total tissue and, to a lesser extent, in the nuclear fraction. For both estrogens, the concentrations in the nuclear fraction are lower than in the cytosol.

Comparison of Estrogen Concentrations in the Different Groups. In premenopausal women (Group 2), the concentration of estradiol in cytosol, nuclear fraction, and total tissue is significantly higher in malignant than in normal tissue of premenopausal women (Group 1) (P <0.05, P <0.05, and P <0.01, respectively). No significant differences were found in the concentrations of estrone in malignant tissue (Group 2) and normal tissue (Group 1) of premenopausal women. For the comparison of normal and malignant tissue of postmenopausal women, normal tissue from 6 patients was available (Group 3). The concentration of estradiol was significantly higher in malignant than in normal tissue of postmenopausal women, in the cytosol and nuclear fractions (P <0.01, P <0.05, respectively). No significant difference was found in the tissue concentrations of estrone in pre- and postmenopausal women with breast cancer. Nevertheless, the tissue concentration of estrone tended to be higher in the premenopausal patients. Because of the considerable difference in peripheral plasma levels, especially of estradiol, between pre- and postmenopausal women, it is very striking that the concentration of estradiol in the mammary tissues were similar in pre- and postmenopausal breast cancer patients.

In premenopausal tissue samples (Group 1), the concentrations of estrone tended to be higher than in the postmenopausal
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Chart 1. Estrone (E1) levels in the cytosol, the nuclear fraction, and total tissue in the 5 groups studied. For each group, median values are shown. The range of plasma levels of normal healthy pre- and postmenopausal volunteers is indicated; F, L, and O, upper level in the follicular, luteal, and ovulatory phases, respectively, of the menstrual cycle. Tissue levels of Groups 3 and 4 are to be compared with the plasma levels in postmenopausal women; tissue levels of Groups 1 and 2 should be compared with plasma levels of premenopausal women. Group 5 represents 2 different subgroups: hypertrophic tissue (•); benign tumor and fibrocystic tissue (○).

Chart 2. Estradiol (E2) levels in the cytosol, in the nuclear fraction, and in total tissue in the 5 groups studied. Median values are shown for each group. The range of plasma levels of normal healthy pre- and postmenopausal volunteers is indicated; F, L, and O, upper level in the follicular, luteal, and ovulatory phases, respectively, of the menstrual cycle. Tissue levels of Groups 3 and 4 are to be compared with the plasma levels in postmenopausal women; tissue levels of the other groups should be compared with those of premenopausal women. Group 5 represents 2 different subgroups: hypertrophic tissue (•); benign tumor tissue and fibrocystic tissue (○).
tissue samples (Group 3) \((P < 0.1\) for the cytosol, the nuclear fraction, and total tissue). In normal tissue of pre- and postmenopausal women, similar estradiol levels were observed. Although Group 5 represents a heterogeneous group, analysis showed no difference between the 3 subgroups. Comparison of Group 5 with Group 1 (premenopausal normal) showed no statistical difference for estrone or estradiol. The subgroups in Group 5 more closely resemble the normal tissues in Group 1 than the malignant tissues in Group 2.

**Estrone:Estradiol Ratio.** Significantly different estrone:estradiol ratios were found in normal and malignant tissues of premenopausal women (Chart 3) \((P < 0.001\) for both subcellular fractions and total tissue). These differences are greater in the cytosol and in total tissue than in the nuclear fraction. Malignant tissues especially show ratios less than one. This can be explained by the higher estradiol concentrations in malignant tissue and not by a lower concentration of estrone. For all tissues, the ratio is lower in the nuclear than in the cytosol fraction, caused by higher nuclear concentrations of estradiol than of estrone. No differences between the subgroups in Group 5 were found. The estrone:estradiol ratios in the tissues in Group 5 were more comparable to those in the normal tissues of Group 1 than to those of the malignant tissues of Group 2.

**ER and Estradiol Tissue Concentration.** The receptor content was measured in the tumors of 19 premenopausal and 32 postmenopausal patients. From the premenopausal patients, 14 were classified receptor positive \((\geq 10 \text{ fmol} / \text{mg protein})\); in the postmenopausal group, 27 tumors were positive. The mean receptor concentrations in the pre- and postmenopausal patients were \(67 \pm 12.5\) (SE) and \(134 \pm 17.5\) fmol/mg protein, respectively. The difference in receptor content between pre- and postmenopausal tumor tissue was highly significant \((P < 0.01)\). There was no significant correlation between the receptor content and the concentration of estradiol in the cytosol, expressed in fmol/mg protein (Chart 4). The concentration of estradiol in the cytosol of receptor-positive tumors is significantly higher \((P < 0.05)\) than in the receptor-negative tumors (Chart 5). Also, in the nuclear fraction and in total tissue, the estradiol concentrations in the receptor-positive tissues are significantly higher \((P < 0.02)\) than in receptor-negative tissues.

**Subcellular Distribution.** Calculations of the ratio of the concentration in the nuclear fraction to the concentration in the cytosol for all groups revealed that the concentration of the estrogens was the highest in the cytosol. The following mean values were found: estrone, \(0.31 \pm 0.29\) (SD; \(n = 105\)); estradiol, \(0.62 \pm 0.39\) \((n = 108)\). For estrone, 95% of the values ranged between zero and 1.18, and the median values of the 5 groups ranged from 0.16 to 0.30. For estradiol, 95% of the values ranged from 0.02 to 1.52, and the median values ranged between 0.46 and 0.69. No significant differences were found between the groups. Although the mean receptor content in the receptor-positive postmenopausal patients was found to be twice the receptor content in premenopausal receptor-positive tissues, no significant difference was found in the ratio of estradiol in the nuclear fraction to estradiol in the cytosol in pre- and postmenopausal tumor tissues. The mean ratios were \(0.80 \pm 0.52\) \((n = 14)\) and \(0.62 \pm 0.35\) \((n = 27)\) for pre- and postmenopausal receptor-positive tumor tissues at median values of 0.65 and 0.53, respectively. This difference holds also for receptor-negative tumors with mean values of 0.61 \pm 0.43\) \((n = 5)\) for premen-
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3.50- p mol/gram tissue
3.00-
2.50-
2.00-
1.50-
1.00-
0.50-
0.00-

 Chart 4. Estradiol levels in the cytosol and the number of estradiol receptors in pre- and postmenopausal breast cancer patients.

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opausal and 0.31 ± 0.32 (n = 5) for postmenopausal breast tumor tissue at median values of 0.53 and 0.32, respectively.

The nucleus:cytosol ratio for estradiol was higher in receptor-positive (0.68 ± 0.42, n = 41) than in receptor-negative tumors (0.46 ± 0.39, n = 10) at median values of 0.58 and 0.41, respectively, but the difference was not significant. No correlation was observed between the receptor and the nucleus:cytosol ratio for estradiol, either in premenopausal or in postmenopausal patients.

DISCUSSION

In all groups, the tissue-plasma gradients for estrone were >1. The gradient was most pronounced in the cytosol and the total tissue in the postmenopausal breast cancer patients. In contrast, a distinct tissue-plasma gradient for estradiol was present only in the postmenopausal breast cancer group, and this gradient was much higher for estradiol than for estrone. The ratio of estrone to estradiol, calculated for each tissue specimen, clearly demonstrated the differences between tissue and plasma for these steroids. Peripheral plasma levels were therefore not reflected in endogenous tissue concentrations. This supports the findings of others who measured simultaneously plasma levels and breast tumor tissue concentrations (26, 39) or tissue concentrations in the human reproductive tract (8, 9, 35).

It should be noted that the steroid determinations were made after chromatographic purification using highly specific antisera for radioimmunoassay. The validation and reliability criteria of these assays were described previously (42, 44). In comparison with published data, the intracellular concentrations of estradiol and estrone in human breast tumor tissue are in good agreement with the literature (3, 13, 14, 19, 20, 26). Millington et al. (25), however, reported higher tissue concentrations of estrone and estradiol, while Vallent et al. (39) reported lower estrogen concentrations in tumor tissue but much higher concentrations in benign tumors. Only Maynard et al. (19, 20) measured estradiol in the nuclear fraction of breast tumor tissue; they reported concentrations similar to ours. In this study, the tissue concentrations have been compared to plasma levels of normal healthy volunteers. In agreement with the literature, the plasma levels in healthy women were not significantly different from plasma levels of breast cancer patients.

The most striking difference in tissue-plasma gradients was that between estrone and estradiol for pre- and postmenopausal breast cancer patients, while the gradient for estradiol was different between malignant and nonmalignant tissue. Several factors may affect the tissue concentrations and therefore the tissue-plasma gradients: (a) uptake of steroid hormones from the circulation; (b) tissue levels of specific binding proteins (receptors) or nonspecific binding proteins with low affinity but high capacity; (c) metabolic conversion from precursors to other steroids, not only in the tumor cell but also in the surrounding normal cells and fat cells. As far as we know, there are no differences in permeability for steroid hormones between normal and malignant breast cell membranes.

Whether steroid hormones enter the cell by passive diffusion or by a membrane-mediated process is still unknown. In a recent review on this subject, Rao (33) concluded that passive diffusion alone was unlikely.

Our data confirm the higher estradiol concentrations in the...
cytosol of receptor-positive tumors than in the cytosol of receptor-negative tumors (13, 14, 19, 20, 38). Also, the significantly higher estradiol concentration in the nuclear fraction of receptor-positive tumors is in agreement with the data of Maynard et al. (20). Furthermore, no differences in tissue estradiol concentrations occurred between pre- and postmenopausal patients, although the receptor content in postmenopausal patients was twice the concentration in premenopausal women. These results are in agreement with published data (13, 19, 20, 26, 34, 36, 38). It is suggested that the differences in estradiol tissue concentrations cannot be explained solely by the level of the receptor proteins because: (a) no correlation existed between the estradiol and the receptor concentrations; (b) with the exception of about one-half of the breast tumor tissues, the concentration of estrone was higher than the estradiol concentration and showed no relation with the receptor content; (c) the estradiol concentration in human endometrium and myometrium has been reported to be about 10 times higher than in breast tumors (7) at similar receptor concentrations (40). Thus, besides the involvement of the estrogen receptor, it is possible that proteins with a lower binding affinity and higher capacity, so-called nonspecific binding proteins, play a role in the uptake and concentrating mechanism for both estrone and estradiol.

Many in vitro studies have demonstrated that breast tissue contains a variety of enzymes capable of converting precursors to biologically active steroid hormones (41). Some differences in these conversions between pre- and postmenopausal patients and between receptor-positive and receptor-negative tumors have been reported (1–6, 17, 23, 24, 28, 46). No specific metabolic pathway for either estrone or estradiol biosynthesis or association between receptor status and aromatization has been reported for in vitro studies in human breast tumors (4, 17, 23, 46). These investigations therefore add little to our understanding of the tissue-plasma gradients of the estrogens. No consensus of opinion on whether normal tissue is able to convert androgens to estrogens is available (4, 24, 27). Our findings of a higher estradiol concentration in the tumors than that in normal tissue suggest that local aromatization might be involved. Human breast tumor tissue is able to metabolize steroid hormones in vivo (11, 12, 15, 42); however, data on differences in metabolic pathways between pre- and postmenopausal women and between receptor-positive and receptor-negative tumors are not available.

Consequently, it is concluded that the tissue concentrations of estrone and estradiol in tumors, compared to malignant tissues from both pre- and postmenopausal women, cannot be explained by selective uptake of estrone and estradiol or by differences in receptor status. Previously, after a 12-h infusion of estrogens, we reported the uptake, metabolism, and subcellular distribution of estrogens in human endometrium, myometrium, and vagina (48). From that study, we concluded that these tissues preferentially accumulate estradiol and that in these tissues the concentration of estrone largely results from intracellular conversion from estradiol. After infusion of [3H]estradiol, most of the radioactivity was localized in the nuclear fraction, while after infusion of [3H]estrone, the majority of the activity was present in the cytosol, and only a minor amount was present in the nuclear fraction. These tracer studies were confirmed by measurement of the subcellular distribution of endogenous estrogens in human uterine tissues using the same technique as in the present study (7). In contrast to the observed subcellular distribution of both estrogens in human uterine tissue, the concentration of estradiol in human breast tissue was highest in the cytosol at a receptor concentration equivalent to that in uterine tissue. Moreover, a considerable amount of estrone was present in the nuclear fraction. Although the nuclear:cytosol ratio of estradiol was higher in receptor-positive than in receptor-negative tumors, no correlation was found between receptor content and estradiol concentration. It is therefore concluded that in human breast tumor tissue the receptor plays only a minor role in the subcellular distribution and that, in addition to an active transport of estradiol into the nucleus, there is a free distribution between cytosol and nucleus. Whether the intracellular concentrations and subcellular distribution of androgens play an additional role, at the cellular level, in the etiology of human breast cancer remains to be seen.

REFERENCES


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