Eur J Cancer Clin Oncol, Vol. 18, No. 4, pp. 333–337, 1982.
Printed in Great Britain.

0277-5379/82/040333-05$03.00/0

Concentration of Endogenous Oestradiol as Related to Oestradiol Receptor Sites in Breast Tumor Cytosol

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Abstract—*Biopsies or mastectomy specimens from 69 malignant and 17 non-malignant human breast tumours have been examined with respect to cytoplasmic oestradiol receptors and endogenous oestradiol concentration. Of the malignant tumours, 45 (65%) had significant oestradiol receptor concentrations (136.7 fmol/mg protein ± 38.3 S.E.M.). Oestradiol values in the cytosol were not correlated to receptor levels. Cytosol oestradiol concentrations in the receptor-negative tumours were normally distributed about a mean value of 7.4 fmol/mg protein ± 0.9 (S.E.M.). Oestradiol levels were similar to the median concentration of receptor found in tumours from younger women, and consequently may influence receptor measurements in such tumours. In receptor-positive cytosols a much wider range (<1 to 69.7 fmol/mg protein) and a non-symmetrical distribution of oestradiol values were found which closely corresponded to the range of occupied receptor concentrations previously measured in tumour cytosol. When tumours having undetectable oestradiol values were excluded, receptor-positive cytosols had significantly higher oestradiol concentrations than those found in receptor-negative tumours ($P < 0.01$). No significant difference in cytosol oestradiol concentration was found in receptor-positive and negative pre- and postmenopausal women. This would indicate that factors other than plasma levels influence tissue availability of oestradiol.*

INTRODUCTION

A WIDE range of oestradiol receptor concentrations have been reported in human breast cancer, and generally lower concentrations and incidence of receptor have been found in premenopausal as compared to postmenopausal women[1–4]. One factor which may in part account for this is the variation in epithelial cell content of the tumours[5]. Another factor which must be considered is the endogenous tumour concentration of oestradiol, since most methods used for receptor assay measure unoccupied receptor only. High plasma concentrations of oestradiol is associated with low oestradiol receptor levels[6, 7]. This negative correlation could be explained by translocation of the steroid–receptor complex to the nucleus[8] or by occupation of receptor by extracellular oestradiol prior to incubation[9]. Theve *et al.*[7] have demonstrated that widely varying cytosol receptor concentrations were present at moderate to low peripheral oestradiol levels. This is an indication that tissue oestradiol content may not be a direct reflection of plasma levels[10]. Breast tumour tissue is able to synthesize or metabolize steroids[11–13], and tissue concentrations of oestradiol could be related to differences in the metabolic potential of the cells. Oestrone sulphate, a major plasma oestrogen in both pre- and postmenopausal women, has recently been shown to be converted to oestradiol with high efficiency in breast tumour cells[14, 15]. Consequently, it was reasoned that measurement of oestradiol in tumour cytosols might be more relevant to the question of receptor occupancy by endogenous oestradiol than plasma levels alone.

Previously published papers give data on cytosol oestradiol concentration and oestradiol receptor levels in human breast cancer[10, 16, 17]. Two of these papers[16, 17] conclude that endogenous oestradiol does not affect receptor measurements. However, since cytosols containing only occupied receptors will be classified as receptor-negative, and since oestradiol concentrations in receptor-negative cytosols are comparable to receptor concentrations in positive cytosols, it is difficult to see how such conclusions can be reached. Nagai *et al.* have stressed this point[10] and stated that receptor occupancy may incur false negative results.

Accepted 20 November 1981.

Since conclusions as to the effect of endogenous oestradiol on receptor measurements are at variance, further data seem to be needed to resolve this question.

MATERIALS AND METHODS

Preparation of tumour cytosols

Biopsies or mastectomy specimens were placed directly in liquid nitrogen and transported to the laboratory. They were transferred to a Revco Freezer and kept at −70°C until analysed. Following thawing at 4°C, approximately 500 mg of tissue was homogenized in 5 vol of phosphate buffer (5 mM, sodium phosphate, pH 7.4, 1 mM monothioglycerol and 10% glycerol). After centrifugation at 800*g* for 15 min in a refrigerated centrifuge, the cytosol fraction was obtained by centrifugation at 105,000 *g* for 60 min in an MSE 65 Superspeed centrifuge. Protein was quantitated in a 50 μl aliquot by the Coomassie blue absorption method [18].

Quantitation of receptor sites

Aliquots of cytosol (100 μl) were incubated with increasing quantities (6.25–50 pg) of [^{3}H]-oestradiol. Values were corrected for non-specific binding by carrying out control incubations containing a hundred-fold excess of non-radioactive oestradiol. Following incubation for 16 hr at 4°C, 500 μl of dextran/charcoal suspension was added (0.25% Norit A and 0.0025% dextran in 10 mM Tris/HCl, pH 8) and the mixture incubated for a further 15 min with intermittant stirring at 0–2°C. After centrifugation for 15 min at 2000 *g*, the supernatant containing bound [^{3}H]-oestradiol was counted in 10 ml of Diluene (Packard Instrument Comp). Results were calculated from the binding data corrected for non-specific binding on the basis of Scatchard plots [19].

Oestradiol assay

Oestradiol was measured in 2 ml of cytosol using an antiserum to oestrone-6-oxime-bovine serum albumin. All organic solvents were of analytical grade and were purified before use by distillation through a 90-cm fractionating column. After equilibration with 1000 counts/min of 2,4,6,7 [^{3}H]-oestradiol, the cytosol was extracted with 10 ml of hexane/ethyl acetate (75:25) and the extract washed with 1.5 ml of 0.01 N NaOH. The extract was evaporated and chromatographed on a microcolumn of Sephadex LH 20 (diameter 5 mm, height 70 mm). An aliquot of the appropriate eluate fraction was used for recovery and correction for procedural loss. Another aliquot was evaporated *in vacuo* and used for radioimmunossay. Antiserum was used in the dilution of 1:10,000. The antiserum solution contained 0.01% bovine serum albumin (Schwarz/Mann), 0.0025% bovine gamma globulin (Schwarz/Mann) and 10,000 counts/min of 2,4,6,7 [^{3}H]-oestradiol/ml. From this solution 250 μl was added to each tube containing dried eluate. The tubes were briefly mixed and incubated overnight at 4°C. After incubation, separation of antibody-bound and free steroid was performed by adding 250 μl of saturated $(NH_4)_2SO_4$ solution to each tube. The tubes were shaken briefly and centrifuged for 10 min at 2000 *g*. From the supernatant 0.4 ml was transferred to scintillation vials, mixed with 10 ml of Diluene and counted. Standard samples were made up with authentic oestradiol-17β (0, 6.25, 12.5, 25, 50 and 100 pg) and processed simultaneously for each set of samples analysed. All radioimmunoassays were performed in duplicate. The oestradiol content of each cytosol was determined from the standard curve and corrected for recovery and 'buffer blank'. The detection limit of the method was calculated from the 95% confidence limit of the zero standard and the slope of the standard curve below 6.25 pg, and was found to be 2 pg/sample. The 'buffer blank' value was obtained by carrying 2 ml of homogenization buffer through the complete procedure. Mean buffer blank was 3.4 pg ± 0.9 (S.D., $n = 8$). Since the antiserum was developed against oestrone-6-oxime conjugate, cross-reaction with oestrone was 100%. However, the Sephadex LH 20 column system gives a satisfactory separation between oestrone and oestradiol. Cross-reaction with other steroids was negligible (< 2%). Non-phenolic C-19 steroids, some of which may interact with the oestradiol receptor, were cleanly separated by the chromatographic procedure. The coefficients of variation calculated from 20 duplicate estimations each in the ranges above and below 10 fmol/mg protein were 13.1 and 17.3% respectively.

RESULTS

Material of biopsies or mastectomy specimens from patients admitted to the hospital for breast surgery was examined. Pathology diagnosis confirmed malignant tumours in 69 cases, and 17 benign tumours, mostly fibroadenomas.

Seventy-one per cent of the malignant tumours contained detectable but widely varying concentrations of oestradiol receptor (range 1.1–1176 fmol/mg protein). Receptor

concentrations between 1 and 5 fmol/mg protein were considered 'borderline values'. In correspondence with results of other investigators [2, 20], the values were higher in postmenopausal than in premenopausal patients. Receptor concentrations (mean: 31.3 fmol/mg protein) in 18 patients below 55 years of age were significantly lower than in 33 patients aged 55 years or more (mean: 156.4 fmol/mg protein) using Wilcoxon's test for two samples ($P < 0.01$).

The results of oestradiol determinations in relation to the age of the patients are presented in Fig. 1. Assuming average protein concentration (3.9 mg/ml ± 1.2 S.D.), the sensitivity of the method is approximately 2 fmol/mg protein. This would mean that some of the values are not significantly different from zero. No significant difference was found by the Wilcoxon test between cytosol oestradiol concentration in receptor-positive and negative, pre- and post menopausal women. Figure 2 presents the distribution of oestradiol values in receptor-positive and negative cytosols. When tumours containing undetectable levels of oestradiol (< 2 fmol/mg protein) are excluded from the calculation, oestradiol concentrations in the receptor-positive group (mean: 12.8 fmol/mg protein ± 2.1 S.E.M.) was significantly higher than in receptor-negative tumours (mean: 7.6 fmol/mg protein ± 0.9 S.E.M.) using Wilcoxon's test for two samples ($P < 0.01$). A remarkably wide range of oestradiol concentrations was found in receptor-containing tumours. A high percentage of these tumours contained low to undetectable levels of oestradiol. On the other hand, oestradiol values

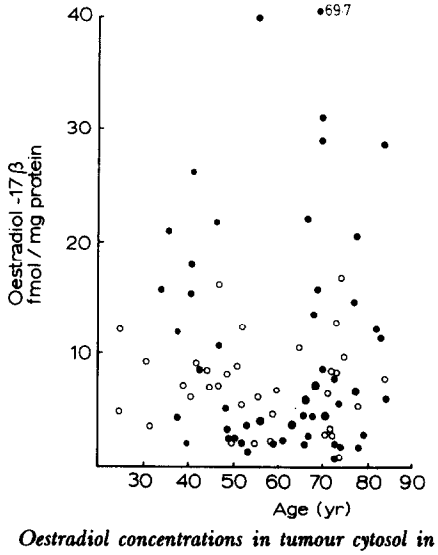

Fig. 1. Oestradiol concentrations in tumour cytosol in relation to the age of the patients. Closed circles: oestradiol receptor-positive tumours; open circles: oestradiol receptor-negative tumours.

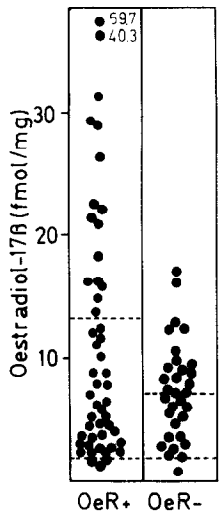

Fig. 2. Distribution of cytosol oestradiol concentrations in receptor-positive and negative tumours. Receptor-positive tumours had significantly higher oestradiol levels compared to receptor-negative ones (Wilcoson's test for two samples, P < 0.01).

were found in receptor-negative tumours comparable to or even higher than the median levels of receptor found in young women [4].

DISCUSSION

The level of oestradiol receptors, their relation to the age of the patients, and the proportion of receptor-positive samples found in the present work correspond well with results published by other investigators.

No significant difference in cytosol oestradiol concentration was found in tumours from pre- and postmenopausal women, whether receptor-positive or negative. This is in correspondence with results of Nagai *et al.* [10] and with concentrations of occupied receptor found in these groups [21, 22]. Since plasma concentrations generally are substantially higher in premenopausal women, these results would seem to emphasize the importance of local tissue processes in determining oestradiol levels in the cytosol. Cytosol oestradiol is influenced by a number of factors such as translocation to the nucleus, aromatization of steroid precursors [11, 23], hydrolysis of steroid sulphates [12, 14, 15], and oestrone and oestrone sulphate formation [13]. In addition, the cytosol (105,000 *g* supernatant) will contain extracellular oestrogens derived from interstitial fluid or plasma contamination, which can be bound during homogenization or handling of the samples prior to incubation [9]. The presence of considerable oestrogenic activity in postmenopausal breast cancers is substantiated by their high incidence and concentration of progesterone receptors and their excellent response to antioestrogen therapy.

Data on the endogenous concentration of oestradiol in mammary tumour cytosol have been published previously[10, 16, 17], and it has been claimed[16, 17] that receptor occupancy does not interfere with receptor assays. Sakai and Saez[22] maintained that receptor saturation by oestradiol does not occur in breast cancer cytosol. However, objections may be raised to the use of direct exchange assays at high temperature, due to varying tumour content of proteolytic activity[24, 25]. In agreement with results of Fishman *et al.*[17], no inverse correlation between receptor and oestradiol concentrations was found. The complexity of factors regulating tissue availability of oestradiol[14] makes the analysis of the relation between receptor and oestradiol difficult. However, we feel that the lack of an inverse relationship does not preclude the presence of fully occupied receptors.

In the present paper we report oestradiol concentrations in tumour cytosol which agree well with those found by Nagai *et al.*[10] and by Fishman *et al.*[17]. In a previous report[21], data on occupied oestradiol receptor in breast tumour cytosol were presented which fairly closely parallel oestradiol values found in the present material. Large variations in cytosol content of oestradiol have been demonstrated in this and previous work[10, 16, 17]. The very low to undetectable levels of oestradiol found in some receptor-positive tumours probably reflects translocation, possibly coupled with a low potential for cytosol receptor replenishment. In the oestrogen-stimulated MCF-7 cell, Horwitz and McGuire[26] found low to undetectable levels of occupied and unoccupied oestradiol receptors. In receptor-negative cytosols, oestradiol values were found which may significantly interfere in assays measuring unfilled receptor sites only. The median levels of receptor in younger women are approximately 10 fmol/mg protein[4], which is the same order of magnitude as the oestradiol levels present. This is in agreement with conclusions reached on a different basis by Theve *et al.*[7] and with our previous report that some tumours, particularly from younger women, contain only occupied receptor in the cytosol. Recently, exchange assays have been devised[21, 27–29] that measure total receptor conveniently and reliably. Such methods should help resolve the question of 'false negatives'.

REFERENCES

1. McGuire WL, Chamness GC, Costlow ME, Shepherd RE. Hormone dependence in breast cancer. *Metabolism* 1974, **23**, 75–100.
2. Wittliff LW. Specific receptors of steroid hormones in breast cancer. *Semin Oncol* 1974, **1**, 109–118.
3. Jensen EV, Smith S, DeSombre ER. Hormone dependency in breast cancer. *J Steroid Biochem* 1976, **7**, 911–917.
4. Elwood JM, Godolphin W. Oestrogen receptors in breast tumours: association with age, menopausal states and epidemiological and clinical features in 735 patients. *Br J Cancer* 1980, **42**, 635–644.
5. McGuire WL, Horwitz KB, Pearson OH, Segaloff A. Current status of estrogen and progesterone receptors in breast cancer. *Cancer* 1977, **39**, 2934–2947.
6. Maass H, Engels B, Hohmeister H, Lehmann F, Trams G. Estrogen receptors in human breast tissue. *Am J Obstet Gynecol* 1972, **113**, 377–382.
7. Theve NO, Carlstrøm K, Gustavson J-Å *et al.* Oestrogen receptors and peripheral serum levels of oestradiol-17β in patients with mammary carcinoma. *Eur J Cancer* 1978, **14**, 1337–1340.
8. Williams D, Gorski J. Equilibrium binding of estradiol by uterine cell suspensions and whole uteri *in vitro*. *Biochemistry* 1974, **13**, 5537–5542.
9. Williams D, Gorski J. A new assessment of cellular distribution of bound estrogen in the uterus. *Biochem Biophys Res Commun* 1971, **45**, 258–260.
10. Nagai R, Kataoka M, Kobayashi S *et al.* Estrogen and progesterone receptors in human breast cancer with concomitant assay of plasma 17β-oestradiol, progesterone and prolactin levels. *Cancer Res* 1979, **39**, 1835–1840.
11. Perel E, Wilkins D, Killinger DW. The conversion of androstenedione to oestrone, oestradiol and testosterone in breast tissue. *J Steroid Biochem* 1980, **13**, 89–94.
12. Vignon F, Terqui M, Wastley B, Derocq D, Rochefort H. Effect of plasma estrogen sulfates in mammary cells. *Endocrinology* 1980, **106**, 1079–1086.
13. Adams JW, Pewnim T, Chandra DP, Archibald L, Foo MS. A correlation between oestrogen sulphotransferase levels and oestrogen receptor status in human primary breast carcinoma. *Caner Res* 1979, **39**, 5124–5126.

14. WILKING N, CARLSTRØM K, GUSTAVSON S, SKJØLDEFORS H, TOLLBOM Ø. Oestrogen receptors and metabolism of oestrone sulphate in human mammary carcinoma. *Eur J Cancer* 1980, **16**, 1339–1342.
15. VIGNON F, DEROCQ D, TERQUI M. Interference of oestrogen sulphates with the evaluation of oestrogen binding and action in breast cancer cells. *Cancer Treat Rep* 1979 **63** (Abstract 142), 1173.
16. ABUL-HAJJ YJ. Relationship between estrogen receptors, 17β-hydroxy-steroid dehydrogenase and estrogen content in human breast cancer. *Steroids* 1979, **34**, 217–225.
17. FISHMAN J, NISSELBAUM JS, MENENDEZ-BOTET JC, SCHWARTZ KM. Estrone and estradiol content in human breast tumours. Relationship to estradiol receptors. *J Steroid Biochem* 1977, **8**, 893–896.
18. SEDMAK JJ, GROSSBERG SE. A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G 250. *Anal Biochem* 1977, **48**, 544–552.
19. SCATCHARD G. The attraction of protein for small molecules and ions. *Ann NY Acad Sci* 1949, **51**, 600–672.
20. KIANG DT, KENNEDY B. Factors affecting estrogen receptors in breast cancer. *Cancer* 1977, **40**, 1571–1576.
21. THORSEN T. Occupied and unoccupied oestradiol receptors in human breast tumour cytosol. *J Steroid Biochem* 1980, **13**, 405–408.
22. SAKAI F, SAEZ S. Existence of receptors bound to endogenous estradiol in breast cancers of premenopausal and postmenopausal women. *Steroids* 1976, **27**, 99–110.
23. MCINDOE HJ. Estradiol formation from testosterone by continuously cultured human breast cancer cells. *J Clin Endocr Metab* 1979, **49**, 272–277.
24. GAROLA G, MCGUIRE WL. Estrogen receptors and proteolytic activity in human breast tumour nuclei. *Cancer Res* 1977, **37**, 3329–3332.
25. SHERMAN MR, TUAZON FB, MILLER LK. Estrogen receptor cleavage and plasminogen activation by enzymes in breast tumour cytosol. *Endocrinology* 1980, **106**, 1715–1727.
26. HORWITZ KB, MCGUIRE WL. Estrogen control of progesterone receptor in human breast cancer. *J Biol Chem* 1978, **253**, 2223–2228.
27. CHAMNESS GC, HUFF K, MCGUIRE WL. Protamine-precipitated estrogen receptor: a solid phase exchange assay. *Steroids* 1975, **25**, 211–218.
28. GAROLA G, MCGUIRE WL. An improved assay for nuclear estrogen receptor in exeperimental and human breast cancer. *Cancer Res* 1977, **37**, 3333–3337.
29. THORSEN T. Occupied and unoccupied nuclear oestradiol receptor in human breast cancer: relation to oestradiol and progesterone cytosol receptors. *J Steroid Biochem* 1979, **10**, 661–668.