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Oestrogen Receptors and Metabolism of Oestrone Sulphate in Human Mammary Carcinoma*

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Abstract—*The conversion of* [^{3}H] *oestrone sulphate into* [^{3}H] *oestrone and* [^{3}H] *oestradiol-17β by homogenates of mammary carcinoma tissue* in vitro *and the oestrogen receptor levels in the tumor cytosol was studied in* 23 *breast cancer patients with low peripheral serum levels of oestradiol-17β* (<150 pM). *Sixteen of the patients had cytosol oestrogen receptor values above* 0.3 fmole/μg *of DNA (receptor rich) and seven patients receptor values below this level (receptor poor).* [^{3}H] *oesterone sulphate was converted into* [^{3}H] *oestrone and* [^{3}H] *oestradiol-17β by all tumor homogenates. No significant difference between the receptor poor and receptor rich groups were found in the total hydrolysis of* [^{3}H] *oesterone sulphate. The transformation of* [^{3}H] *oesterone sulphate into* [^{3}H] *oestradiol-17β was significantly higher in the receptor poor group than in the receptor rich group* (35.95 ± 11.06 *vs* 5.33 ± 1.37 fmole [^{3}H] *oestradiol-17β formed per* min *and per* mg *of protein*, $P < 0.001$). *The mean age of the receptor poor group was significantly lower than that of the receptor rich group* (58.0 ± 5.5 *vs* 73.1 ± 2.1 yr, $P < 0.01$). *No significant difference was found in the peripheral serum levels of oestradiol-17β in the two groups.*

The results indicate that the ability of the tumour tissue to convert oestrone sulphate into oestradiol-17β significantly influences the cytosol oestrogen receptor level in patients with low peripheral oestrogen levels. An age-related decrease in the 17β-hydroxysteroid oxidoreductase activity in the tumour was also noted. We conclude that a high rate of formation of oestradiol-17β may lead to high intracellular levels of this steroid, resulting in blockade of the hormone binding site, increased nuclear translocation of the receptor and low cytosol oestrogen receptor values.

INTRODUCTION

OESTROGEN receptor rich tumours in human breast cancer are found more frequently among postmenopausal patients and this has been ascribed in part to the decrease in the peripheral oestrogen levels associated with the menopause [1]. In a previous study from this group [2] it was demonstrated that high peripheral oestrogen levels are strongly associated with low cytosol receptor values while low (postmenopausal) oestrogen levels can be associated both with low as well as with high receptor values. The low cytosol receptor values found at high peripheral oestrogen levels can be due to blockade of the hormone binding site by the endogenous oestrogen or nuclear translocation of the cytosol receptor. The reasons for the varying receptor values found at low peripheral oestrogen levels are more complex. Differences between tumour cells with regard to receptor levels and/or cellular heterogeneity might be one explanation [3]. Another reason may be differences in the oestrogen synthesis or metabolism by the tumours, especially in the intracellular formation of biologically active oestradiol-17β from other steroids. Different intracellular levels of oestradiol-17β may lead to different degrees of blockade of the hormone binding sites or nuclear translocation of the receptor and thus to differences in the cytosol receptor values.

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Formation of oestrogens from androgens and androgen precursors by human mammary tumour tissue *in vitro* has been described by some authors [4–7] while others have failed to demonstrate this type of conversion [8, 9]. Another peripheral precursor to intracellular formation of oestradiol-17β in the tumours is oestrone sulphate. Oestrone sulphate is by far the most abundant oestrogen in the peripheral circulation of non-pregnant women and considerable serum levels are present even after the menopause, i.e., about 1000–2000 pM, which are comparable with the levels of the two substrates for aromatization of the steroid A-ring, 4-androstene-3, 17-dione and testosterone [10–12]. Recently it had been shown by Vignon and co-workers [13] that oestrone sulphate is capable of entering intact MCF_7 breast tumour cells where it is converted into free oestrogens which are bound to the oestrogen receptor. Furthermore, other oestrogen target tissues, e.g., pituitary and uterus, convert oestrone sulphate into oestradiol-17β [14–18]. The entry and uptake of oestrone sulphate into intact rat uterus and human endometrium tissues is reported to be in the same order as for unconjugated oestogens [14, 16]

In view of these findings it was considered important to investigate if the ability of the breast cancer tissue to convert oestrone sulphate into oestradiol-17β could influence the cytosol oestrogen receptor values. The present communication describes the metabolism of oestrone sulphate *in vitro* by breast cancer tissues from patients with low peripheral oestrogen levels. A comparison is made between the *in vitro* metabolism and the oestrogen receptor value in the cytosol. We believe that these results, although they are based upon a limited number of patients, indicate a relationship between the oestrogen metabolism in the tumour and the cytosol receptor values and we hope that future research will conform our findings.

MATERIALS AND METHODS

Clinical material

The clinical material consisted of 23 patients with histologically confirmed breast cancer and with low peripheral serum levels of oestradiol-17β (<150 pM). Twenty-two of the patients were postmenopausal (age 50–81 yr) and one premenopausal (H.T., age 34 yr). Surgical biopsies were performed and immediately transferred to the laboratory in ice cold saline. Receptor determinations were performed on fresh tissue or after freezing at −70°C. The samples for metabolic studies were frozen at −70°C until used.

Determination of oestrogen receptors

Cytosol oestrogen receptors in fresh or rapidly thawed carcinomas were determined by isoelectric focusing as described by Gustafsson *et al.* [19]. The results were expressed as fmole bound [^{3}H] oestradiol-17β per μg of DNA.

Determination of serum oestradiol-17β

The determination of oestradiol-17β in peripheral serum was performed after ether extraction by radioimmunoassay using anti-oestradiol-17β-6-(carboxy-methyl oxime) bovine serum albumin. This antibody cross reacts to 11% with oestrone [20]. Intra- and interassay variations were 11.7 and 13.1% respectively.

Metabolism of [^{3}H] oestrone sulphate by tumour tissue homogenate

One piece 5 × 5 mm of tissue was thawed, chopped into small pieces and homogenized into 2.5 ml of 0.06 M Tris-HCl pH 7.0 in a Polytron ST-10 homogenizer at full speed for 60 sec at 4°C. The incubation mixture consisted of 200 μl fresh homogenate diluted 1:10 with buffer, 20.9×10^{-12} mole [6, 7-^{3}H] oestrone sulphate (sp. act. 47.9 Ci/nmole, New England Nuclear Corporation, Boston, Mass.) in 100 μl of buffer and 100 μg each of NADPH and NADH respectively in 100 μl of buffer. Simultaneous incubations with buffer instead of homegenate were performed in order to correct for non enzymatic transformation of the substrate. All incubations were carried out in duplicates. After 50 min of incubation in air at 37°C in a shaking water bath, 200 μg each of non radioactive oestrone, oestradiol-17β and oestriol were added in 100 μl of ethanol together with 0.6 ml of 0.5 M sodium phosphate, pH 7.0.

The unconjugated steroids were extracted with 5 ml of chloroform, the chloroform extract was dried over Na_2SO_4, evaporated to dryness and dissolved in ethanol. Aliquots were taken for determination of the total ^{3}H radioactivity of the extracts and the rest of the samples were subjected to thin-layer chromatography on silica gel GF_{254} type 60 (E. Merck A.-G., Darmstadt, Germany) using 13% (v/w) ethanol in toluene as solvent. After visualization in u.v. light, the zones on the plate corresponding to the oestrogens as well as the non-fluorescent zones were scraped off

and eluted separately with ethanol. Aliquots were taken for determination of radioactivity while other aliquots from oestrone and oestradiol-17β fractions were pooled for identification as described below.

[^{3}H] Oestrone and [^{3}H] oestradiol-17β formed from [^{3}H] oestrone sulphate were identified by addition of the corresponding ^{14}C labelled oestrogens and measurement of the ^{3}H/^{14}C ratio after sequential chromatography and derivatization. [4-^{14}C] Oestrone and [4-^{14}C] oestradiol-17β (sp. act. 59 Ci/mole of both; New England Nuclear Corporation, Boston, Mass.) were purified before use by thin layer chromatography on silica gel GF_{254} with 13% (v/w) ethanol in toluene as solvent. The oestrone and oestradiol fractions obtained after thin layer chromatography of extracts from incubations were pooled separately and the appropriate ^{14}C-labelled oestrogen was added to give an initial ^{3}H/^{14}C ratio of 0.3–0.6 (step A). The fractions were rechromatographed on silica gel GF_{254} in 13% (v/w) ethanol in toluene (step B), and on Al_2O_3 GF_{254} type E (E. Merck A.G., Darmstadt, Germany) in the same solvent (step C), acetylated with acetic anhydride: pyridine 5:1 by vol at 60°C for 1 hr (step D) and finally rechromatographed as acetates on silica gel GF_{254} in 13% (v/w) ethanol in toluene (step E). The ^{3}H/^{14}C ratios of different fractions were measured after each step. R_F-values on silica gel GF_{254} system were for oestradiol-17β 0.34, for oestrone 0.44, for oestriol 0.17, for oestradiol-17β-diacetate 0.63 and for oestrone acetate 0.57. R_F-values on Al_2O_3 GF_{254} were for oestradiol-17β 0.36 and for oestrone 0.46.

Protein was measured by the biuret method using bovine serum albumin in Tris–HCl buffer as standard [21]. The rate of transformation was expressed as the amount of unconjugated oestrogen formed per minute and per mg of protein. The formation of oestradiol-17β was linear with respect to time for more than 90 min and to protein concentration up to 2900 μg/ml. In the samples used in this study, the protein concentration never exceeded 1740 μg/ml. The total hydrolysis rate was not linear in these respects but increased with time as well as with protein concentration. The variation between duplicates expressed as S.D. for 20 duplicate pairs was $\pm$6% for total hydrolysis and $\pm$15.0% for formation of oestradiol-17β.

Statistical methods

The individual values for the rates of transformation were found to have a lognormal distribution and Student's *t*-test was applied after logarithmic transformation. The values for the other parameters had a normal distribution allowing the use of Student's *t*-test without transformation of the individual values. The significance level was set at $P<0.05$. The values are given as mean $\pm$ S.E.M.

RESULTS

In previous reports from this group [2, 22] tumours with receptor values below 0.2 fmole/μg of DNA were considered as clinically receptor poor. However, in a recent summary of clinical material comparing different endocrine treatments of patients with breast cancer, it has been found that remission is not likely to occur in patients having tumours with receptor values below 0.3 fmole/μg of DNA as determined by the method described herein [23]. Thus the clinical material in the present study was divided into two groups; one with receptor values above (group A) and one with receptor values below 0.3 fmole/μg of DNA (group B). The sixteen patients in group A had a mean age of 73.1 $\pm$ 2.1 yr and a mean receptor concentration of 3.22 $\pm$ 0.71 fmole/μg of DNA. The corresponding values for the seven patients in group B was 58.0 $\pm$ 5.5 yr and 0.11 $\pm$ 0.04 fmole/μg of DNA, respectively.

The values for cytosol oestrogen receptor, serum oestradiol-17β, total hydrolysis and formation of oestradiol-17β from oestrone sulphate in the two groups are given in Table 1. The mean level of serum oestradiol-17β in group A(104 $\pm$ 12 pM) was somewhat higher than in group B(77 $\pm$ 12 pM), but this difference was not statistically significant. [^{3}H] Oestrone sulphate was enzymatically transformed into [^{3}H] oestrone and [^{3}H] oestradiol-17β by all tumour homogenates. The radiochemical homogenity of the oestrone and oestradiol-17β fractions isolated from the incubations is illustrated by the constant ^{3}H/^{14}C-ratios given in Table 2. There was no evidence for formation of [^{3}H] oestriol or other polar oestrogen metabolites during the incubations. As can be seen in Table 1 there was no significant difference between the two groups of patients with respect to the total enzymatic hydrolysis of oestrone sulphate by tumour homogenates *in vitro*, although the mean value for group B was somewhat higher (445.7 $\pm$ 106.7 fmole/min/mg protein) than that in group A(369.6 $\pm$ 56.3 fmole/min/mg protein). However, the mean rate of enzymatic conversion of oestrone sulphate into

Table 1. Serum levels of oestradiol-17β and enzymatic conversion of [^{3}H] oestrone sulphate by tumor homogenate in vitro in patients with high and low cytosol oestrogen receptor values. The values are given as mean ± S.E.M. and range

Patients	Age (yr)	Serum oestradiol-17β (pM)	Cytosol oestrogen receptor (fmole/μg of DNA)	Free oestrogens formed from [^{3}H] oestrone sulphate by tumour homogenate *in vitro* (fmole/min/mg protein)	
				Total enzymatic hydrolysis of oestrone sulphate	Oestradiol-17β formed from oestrone sulphate
Group A: receptor values above 0.3 fmole/μg of DNA ($N=16$)	73.1 ± 2.1 (50–81)	104 ± 12 (56–140)	3.22 ± 0.71 (0.77–10.13)	399.6 ± 56.5 (86.9–887.1)	5.33 ± 1.37 (0.43–22.85)
Group B: receptor values below 0.3 fmole/μg of DNA ($N=7$)	58.0 ± 5.5 (34–76)	77 ± 12 (50–91)	0.11 ± 0.04 (0.01–0.28)	445.7 ± 106.7 (159.7–945.7)	35.95 ± 11.6 (8.36–92.30)
Difference between group A and B, *P*-value	$P<0.01$	N.S.	—	N.S.	$P<0.001$

oestradiol-17β by tumour homogenates was found higher in patients with oestrogen receptor values below 0.3 fmole/μg of DNA (group B; 35.95 ± 11.06 fmole/min/mg protein) than in patients with oestrogen receptor values above 0.3 fmole/μg of DNA (group A: 5.33 ± 1.37 fmole/min/mg protein). This difference was statistically highly significant ($P<0.001$).

Table 2. ^{3}H/^{14}C-ratios of oestrone and oestradiol-17β fractions after addition of ^{14}C-labelled standards followed by sequential chromatography and acetylation. The different steps are described in Materials and Methods

	^{3}H/^{14}C-ratio following step				
	A	B	C	D	E
Oestradiol-17β	0.598	0.596	0.546	0.569	0.565
Oestrone	0.396	0.385	0.381	0.381	0.381

DISCUSSION

Determination of the peripheral serum levels of oestradiol-17β is performed routinely in all of our breast cancer patients in which cytosol oestrogen receptors are determined. In another clinical material from our departments a highly significant correlation was found between the serum levels of oestradiol-17β and oestrone sulphate at oestradiol-17β levels below 150 pM ($t=5.131$, $r=0.433$, $N=116$, $P<0.001$). Thus low peripheral serum levels of oestradiol-17β are invariably associated with low levels of oestrone sulphate and this makes the more complicated assay of oestrone sulphate superfluous in these patients.

The results from the present investigation strongly indicate a connection between the oestrogen metabolism of the tumour and its cytosol oestrogen receptor content in patients with low peripheral oestrogen levels. Thus a high capacity of the tumour tissue to convert oestrone sulphate into oestradiol-17β could lead to high intracellular levels of the latter steroid, in turn leading to a higher degree of blockade of the hormone binding sites, accompanied by increased nuclear receptor levels [24]. Together this could result in low cytosol receptor values. In tumours with low metabolic activity the intracellular levels of oestradiol-17β will be low, provided that low peripheral levels of this steroid are present. In this situation mainly high cytosol receptor values are found. This hypothesis is further supported by the recent demonstration by Vignon and co-workers [13] that oestrone sulphate is capable of entering intact MCF_7 breast tumour cells where it is converted into free oestrogens which are bound to the oestrogen receptor.

No significant correlation between sulphatase activity and oestrogen receptor values were found in the present study although this can in part be attributed to the fact that the assay system was not optimized for measuring the total hydrolysis of oestrone sulphate. This indicates that the reduction of oestrone will be the rate limiting step in the formation of oestradiol-17β in this system. Reduction of oestrone to oestradiol-17β by breast tumours has previously been demonstrated by Willcox and Thomas [25]. No formation of oestriol or other polar metabolites was found in the

present study which is in accordance with previous findings concerning oestrogen metabolism in mammary carcinoma *in vitro* [8, 26, 27].

It is not yet settled if peripheral oestrone sulphate (and oestrone) or androgens are the main substrates for the intracellular formation of oestradiol-17β in breast tumours. However, most neoplastic tissues *in vivo* have a restricted oxygen supply in which reductive conditions predominate [27, 28]. The hydrolysis of steroid sulphates is independent of oxygen supply and the reductive conditions may favour 17β-reduction rather than the oxygen dependent aromatization of androgens.

An interesting observation in the present study is that the group of patients which showed the higher mean enzymatic conversion of oestrone sulphate into oestradiol-17β in the tumour was significantly younger than those with a low enzymatic conversion (mean ages 58.0 ± 5.5 and 73.1 ± 2.1 yr respectively, $P < 0.01$). It is of interest to compare these findings with the strong positive correlation between age, peripheral serum levels of oestradiol-17β and oestrogen receptor content previously reported by us [2]. When the data from the present study were combined with the data from the subjects who had serum oestradiol-17β levels below 150 pM from the previous investigation, the resulting clinical material could be divided into 22 patients with oestrogen receptor levels below 0.3 fmole/μg of DNA and 50 patients with oestrogen receptor values above this level. The mean age for the 22 receptor-poor patients was 59.5 ± 2.8 yr as compared to 67.6 ± 1.4 yr for the 50 receptor-rich patients. This difference was statistically significant ($P < 0.01$). The mean serum levels of oestradiol-17β were almost identical for the two groups, 77 ± 6 pM and 78 ± 4 pM respectively. Thus it may be speculated that the difference in tumour oestrogen metabolism between the two groups in the present study, which is significantly associated with differences in oestradiol receptor values, could reflect an age dependent decrease in the 17β-hydroxysteroid oxidoreductase in the tumour. This hypothesis is further supported by the results from a recent investigation by Varela and Dao [7]. They incubated breast tumour homogenate from pre- and postmenopausal patients with [^{3}H] 4-androstene-3, 17-dione and studied the formation of testosterone and oestrogens. The formation of [^{3}H] testosterone as well as the ratio between [^{3}H] oestradiol-17β and [^{3}H] oestrone was significantly higher in the premenopausal group and they suggested a difference in the 17β-hydroxy-steroid oxidoreductase activity in the tumour tissue between the younger and the older woman.

The results from the present investigation indicate that the oestrogen metabolic pattern in the tumour tissue influences the cytosol receptor values in patients with low peripheral oestrogen levels. The conversion of oestrone sulphate into oestradiol-17β by the tumour may directly stimulate tumour growth by providing biologically active oestrogen. Thus factors which affect this transformation may also affect tumour growth in patients with low peripheral oestrogen levels. Studies on this subject are in progress at our departments.

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