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Activated Oestrogen Receptors in Breast Cancer and Response to Endocrine Therapy

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Abstract—*The status of oestrogen and progesterone receptors has been measured in 147 primary breast tumours. In addition to the measurement of cytoplasmic oestrogen receptors, the ability of these receptors to bind to oligo(dT)-cellulose has been assessed. This indicates the capability for activation of cytoplasmic receptors to a form able to bind in the nuclear compartment* in vivo *and thus be part of a functional receptor pathway. All the receptor concentrations measured were increased in the postmenopausal group of patients. All nuclear oestrogen receptors in this group were available for labelling at 4°C, in contrast to the premenopausal group. The apparent functionality of the oestrogen receptor pathway could be equally assessed either by the co-presence of cytosol progesterone receptor with nuclear oestrogen receptor (30 or 4°C) or with activated cytosol oestrogen receptor. The presence of activated cytosol oestrogen receptor was as reliable (80%) as the presence of either nuclear oestrogen receptor at 30 (83%) or 4°C (81%) in predicting the response of breast tumours to endocrine therapy.*

INTRODUCTION

ANALYSES of the oestrogen receptor status of tumours from patients with breast cancer are currently being used to select for treatment those patients in whom endocrine therapy may be successful [1–3]. The presence of oestrogen receptor in a tumour biopsy is not, however, in itself a guarantee of the functionality of the receptor-mediated pathway of oestrogen action in that tissue. Obviously, qualitative differences in oestrogen receptors or the absence of other factors that participate in the receptor pathway may explain why only 55–60% of patients with cytoplasmic oestrogen receptors respond to endocrine therapy [3]. It is currently claimed that successful prediction of objective remission may be improved if the functionality of the oestrogen receptor system is assessed by the presence of both cytoplasmic and nuclear oestrogen receptors [4, 5] or by co-measurement of cytosol oestrogen and progesterone receptor [6–8], the latter being a product of oestrogen action in normal target tissue [9]. However, using these assessments, the objective remission rates to endocrine therapy are still only 60–75% [5, 10].

The ability of cytoplasmic oestrogen receptors to bind in the nuclear compartment *in vivo* and thus be part of a functional pathway can be assessed *in vitro* by measuring their degree of activation. The capability for binding to artificial nuclear acceptors, including oligo(dT)-cellulose [11], is thought to measure activation. Oestrogen receptor that binds to oligo(dT)-cellulose sediments as a 5S nuclear form [12]. This 5S form of receptor is thought to be essential to the initiation of a complete oestrogenic response [13]. Human breast tumours in which the 4S cytoplasmic receptor could not be transformed to a nuclear 5.5S form had a poor objective response to endocrine therapy [14].

In this study we have measured the extent of activation, i.e. the capacity of human breast tumour cytosol oestrogen receptor to bind to oligo(dT)-cellulose, and correlated it with the presence of cytosol progesterone and nuclear oestrogen receptors in those tumours. One hundred and forty-seven primary tumours have been studied and receptor parameters measured. Some patients receiving endocrine therapy have

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been evaluated after 6 months to correlate their clinical response with these receptor parameters.

MATERIALS AND METHODS

Patients

A series of 145 patients, aged 22–80 yr, presenting to Charing Cross Hospital with histologically confirmed primary carcinoma of the breast, has been studied. The total number of specimens amounted to 147.98 (67.6%) post-menopausal and 47 (32.4%) premenopausal patients. All the specimens were obtained at the time of biopsy and none of the patients had received any previous treatment.

Materials

[2,4,6,7-^{3}H]-oestradiol (sp. act. 85–110 Ci/mmol) and [1,2,6,7-^{3}H]-progesterone (sp. act. 80–110 Ci/mmol) were supplied by the Radiochemical Centre, Amersham, U.K.

Oligo(dT)-cellulose was obtained from Collaborative Research, Waltham, MA, U.S.A., supplied by Uniscience, Cambridge, U.K.

Cortisol, diethylstilboestrol and norethisterone were purchased from Sigma Chemical Company.

Methods

Tissue preparation. Tumour tissue was transported from the operating theatre in dry ice, fat, connective tissue and necrotic tissue dissected out and the tumour tissue then stored in liquid nitrogen for a period not more than 2 months. Tissues were pulverised in a Microdismembrator (Braun Instruments Ltd, W. Germany), the powder weighed and then resuspended in buffer (10 mM phosphate, 1.5 mM diKEDTA, 10 mM monothioglycerol, pH 7.4, containing 30% glycerol) to a concentration of 1:8 (w/v). All subsequent procedures were performed at 4°C. The suspension was stirred for 10 min followed by centrifugation at 2000 *g* for 15 min. The supernatant which contains the cytosol was used for the measurement of cytoplasmic oestrogen and progesterone receptors and the binding of the oestrogen receptor complexes to oligo(dT)-cellulose. Nuclear oestrogen receptors were measured in the resulting pellet.

Cytoplasmic oestrogen receptor assay. The method of King *et al.* [8] was used. Aliquots of supernatant (200 μl) were assayed in triplicate. Cytosol was incubated overnight at 4°C with 5 nM [^{3}H]-oestradiol in the presence or absence of a 200-fold excess of diethylstilboestrol in a final volume of 240 μl. The unbound [^{3}H]-oestradiol was removed by a 10-min incubation with 240 μl of 0.5% charcoal, 0.05% dextran T-70 in 10 mM Tris/1 mM EDTA buffer (pH 7.4). After centrifugation for 10 min at 2000 *g*, 250 μl of supernatant were counted in 2 ml of Pico Fluor-30 (Packard). Specific binding sites were estimated from the differences in radioactivity from incubations with or without diethylstilboestrol.

Cytoplasmic progesterone receptor assay. Again the method of King *et al.* [8] was used. Aliquots of supernatant (200 μl) were assayed in triplicate. Cytosol was incubated overnight at 4°C with 10 nM [^{3}H]-progesterone plus 1 μM cortisol in the presence or absence of a 100-fold excess of norethisterone in a final volume of 260 μl. The unbound [^{3}H]-progesterone was separated and counted as in the cytoplasmic oestrogen receptor assay but the incubation with dextran-coated charcoal was shortened to 5 min. Specific binding sites were estimated from the differences in radioactivity from incubations with or without norethisterone.

Oligo(dT)-cellulose chromatography. Aliquots of cytosol (200 μl) incubated overnight at 4°C with 5 nM [^{3}H]-oestradiol in the presence or absence of a 200-fold excess of diethylstilboestrol were chromatographed on columns containing 100 mg oligo(dT)-cellulose as described previously [15]. The cytosol was incubated for 45 min at 4°C with oligo(dT)-cellulose which had been previously equilibrated in buffer. Radioactivity and receptor complexes which had not bound to the matrix were then removed by washing with 15 ml buffer. Bound receptor complexes were then removed in 4 ml 1.2 M KCl in buffer, which was then placed in scintillation vials and radioactivity counted. Receptor complexes specifically bound to oligo-(dT)-cellulose were estimated from the differences in radioactivity eluted from incubations with or without diethylstilboestrol.

Nuclear oestrogen receptor assay. Crude nuclear pellets obtained from the first centrifugation step were resuspended in 5 ml of TEDG buffer (10 mM Tris/HCl, 1.5 mM EDTA, 1 mM dithiothreitol, containing 10% glycerol, pH 7.6) and centrifuged at 800 *g* for 10 min. The supernatant was discarded and the pellet washed by repeating this procedure twice more. The final pellet was resuspended in 9 vol. (original wet weight) of TEDG buffer. Aliquots of this suspension (200 μl) were incubated with 5 nM [^{3}H]-oestradiol in the presence or absence of a 200-fold excess of diethylstilboestrol in a final volume of 240 μl. Separate incubations were performed at 30°C (total receptor) and 4°C (available receptor) for 1 hr. The nuclei were washed by resuspension in 1 ml TEDG buffer containing 1% BSA, followed by centrifugation at 1500 *g* for 2.5 min. This procedure was repeated twice more, followed by a final wash in 1 ml of TEDG buffer. The radioactivity in the final pellet was extracted with ethanol (2 × 1 ml), transferred

into scintillation vials and counted. Specific binding sites were estimated from the differences in radioactivity from incubations with or without diethylstilboestrol.

Definition of positive receptor assay. Values of >0.1 pmol/g wet tissue were taken as positive for all parameters.

Clinical response. Thirty-two patients receiving endocrine therapy have been assessed after 6 months and their clinical condition at that date correlated with the receptor status of the primary tumour. Twenty of these patients received tamoxifen (40 mg/day), 3 received stilboestrol (15 mg/day) and 9 patients were oophorectomised, of whom one was treated with tamoxifen. Responders were those patients showing either complete or partial remission or no change.

Table 1. Frequency of receptor occurrence in breast tumour

Receptor	Premenopausal (% positive)	Postmenopausal (% positive)
RE_c	61.36	73.12
dT	26.47*	60.00
RE_{N30}	87.50	86.08
RE_{N4}	81.58*	38.75
RP_c	63.64**	40.43

Significant difference from postmenopausal (Fisher's exact test): $*P<0.003$; $**P<0.01$

RESULTS

Frequency of receptor occurrence and overall receptor status

The frequency of receptor-positive tumours in relation to menopausal status is presented in Table 1. There was no significant difference in the incidence of cytosol oestrogen receptor or nuclear oestrogen receptor (30°C) between the two groups. However, there was a significant decrease in the incidence of available nuclear oestrogen (4°C) receptor in the postmenopausal group. The incidence of progesterone receptor-positive tumours was significantly decreased in the postmenopausal group, while that of oestrogen receptor binding to oligo(dT)-cellulose was significantly increased.

Concentration of sex steroid receptors in breast tumours from pre- and postmenopausal patients

Receptor concentrations are expressed per mg wet weight. A comparison with expression per mg DNA gave a good correlation ($r=0.59$, $n=75$, $P<0.001$).

The concentration of oestrogen and progesterone receptors grouped according to menopausal status is shown in Table 2. All parameters in the postmenopausal group, except the concentration of cytosol progesterone receptor, were significantly increased compared to the premenopausal group. When a subset of receptor-positive tumours were considered (Table 2, figures in parentheses), all values including cytosol progesterone receptor were significantly increased in the postmenopausal compared to the premenopausal group. There was no significant difference in the ratio of total nuclear oestrogen receptor (30°C) to cytosol progesterone receptor between the two groups [RE_{N30}/RP_c: 2.083 ± 4.23 ($n=23$), pre-; 4.099 ± 6.01 ($n=44$), postmenopausal, $P<0.20$ (NS)], whereas the ratio of available nuclear oestrogen receptor (4°C) to cytosol progesterone receptor was increased in the postmenopausal group (RE_{N4}/RP_c: 1.138 ± 1.54 ($n=25$), pre-; 2.49 ± 2.71 ($n=36$), postmenopausal, $P<0.02$).

'Three-way' analysis of receptor status in individual tumours

Computer analysis of the frequency data allowed correlations to be made of the simultaneous occurrence of three separate receptor parameters in each tumour. In the premenopausal group all tumours that were positive for cytosol and nuclear oestrogen (30°C) receptor were positive for progesterone receptor (16–17).

Table 2. Breast tumour sex steroid receptor concentrations

	Premenopausal Mean ± S.E.M. (fmol/mg wet wt)	*n*	Postmenopausal Mean ± S.E.M. (fmol/mg wet wt)	*n*
RE_c	0.47 ± 0.11* (0.737 ± 0.182*)	47 (27)	3.93 ± 0.51 (5.139 ± 0.658)	100 (68)
dT	0.09 ± 0.02* (0.275 ± 0.050*)	34 (9)	0.71 ± 0.12 (1.171 ± 0.176)	80 (48)
RE_{N30}	0.56 ± 0.12** (0.637 ± 0.125*)	32 (28)	1.21 ± 0.16 (1.406 ± 0.171)	79 (68)
RE_{N4}	0.34 ± 0.04* (0.412 ± 0.041*)	38 (31)	1.44 ± 0.20 (1.583 ± 0.21)	80 (73)
RP_c	0.43 ± 0.11 (0.691 ± 0.165*)	47 (28)	0.87 ± 0.24 (2.148 ± 0.582)	98 (38)

Significant difference from corresponding postmenopausal value (Student's *t* test): $*P<0.001$; $**P<0.005$.

Figures in parentheses are receptor-positive values considered as a subset.

The corresponding analysis of the postmenopausal group of 58 tumours showed that only 32 were positive for progesterone receptor. However, in the postmenopausal group being negative for nuclear oestrogen (30°C) receptor correlated with being negative for progesterone receptor. There was an apparent correlation between cytosol oestrogen receptor, nuclear oestrogen receptor (4°C) and cytosol progesterone receptor in the premenopausal group.

In the premenopausal group all tumours that were positive for cytosol oestrogen receptor and binding to oligo(dT)-cellulose were also positive for cytosol progesterone receptor (9/9). No such correlation was apparent in the postmenopausal group.

Table 3. Frequency of steroid receptor combinations

	RE_c	RE_{N30}	RP_c (No. of patients)	
			+	-
Premenopausal	+	+	16	1
	+	-	4	4
Postmenopausal	+	+	32	26
	+	-	1	7

	RE_c	RE_{N4}	RP_c (No. of patients)	
			+	-
Premenopausal	+	+	17	3
	+	-	3	2
Postmenopausal	+	+	31	30
	+	-	2	3

	RE_c	dT	RP_c (No of patients)	
			+	-
Premenopausal	+	+	9	0
	+	-	7	5
Postmenopausal	+	+	26	20
	+	-	5	11

Table 4. Incidence of objective response in 32 patients receiving endocrine therapy for 6 months

	Patients	Responders	%
RE_c			
+	22	15	68.0
-	10	2	20.0
RE_c RP_c			
+ +	15	11	73.3
- -	7	1	14.3
RE_c RE_{N4}			
+ +	17	13	76.4
- -	3	0	0.0
RE_c dT			
+ +	15	11	70.0
- -	6	1	16.7
RE_c RP_c RE_{N30}			
+ + +	12	10	83.3
- - -	4	0	0.0
RE_c RP_c RE_{N4}			
+ + +	11	9	80.9
- - -	2	0	0.0
RE_c RP_c dT			
+ + +	10	8	80.0
- - -	5	1	20.0

Clinical response

The incidence of objective response in the 32 patients who received endocrine therapy for at least 6 months is presented in Table 4. The criteria for response were described by Hayward *et al.* [16]. Responders were those patients showing either complete or partial remission or no change. The rate of response (68%) observed in patients whose tumours contained cytoplasmic receptors for oestrogen was improved when either progesterone receptor (73.3%), nuclear receptor (30 or 4°C) (76.4%) or binding to oligo(dT)-cellulose (70%) were also present. There was further improvement when, apart from the presence of cytoplasmic oestrogen and progesterone receptors, nuclear 30°C (83.3%) or nuclear 4°C (80.9%) receptors were present in tumours. The presence of binding to oligo(dT)-cellulose in tumours with cytoplasmic oestrogen and progesterone receptors gave a response rate of 80%. The majority of tumours that were negative for 3 parameters did not show response to endocrine treatment.

DISCUSSION

We have measured receptor parameters which are clinically useful in predicting the response of breast tumours to hormonal therapy and, in addition, have included quantitation of binding to oligo(dT)-cellulose, which mimics the process of nuclear binding of oestrogen receptor, and its conversion to the 5S form *in vivo* [11, 12].

The concentration of each receptor parameter studied was significantly higher in the postmenopausal group than in the premenopausal group of patients (Table 2). Differences in cytosol oestrogen receptor content between the two groups may be due to the higher content of endogenous oestradiol in the premenopausal patient which occupies and hence conceals the receptor from detection [17]. However, the increase in total nuclear and cytosol progesterone receptor content in the postmenopausal group implies a lack of cyclical progesterone influence which, in the premenopausal group, would lead to a decrease in oestrogen and progesterone receptor content [18].

The increase in the mean content of available nuclear oestrogen receptors (4°C) in the postmenopausal group is in agreement with Thorsen [19]. This may be explained by occupation of the nuclear receptor by oestrone, which has a lower affinity for receptor than oestradiol [20] and is the predominant plasma oestrogen in the postmenopausal woman [21]. Breast tissue can form oestrogens *in situ* from precursors in plasma [22], so that a simplistic interpretation of the occupancy of nuclear receptors based on plasma oestrogen may not be entirely valid.

The simultaneous measurement of various receptor parameters to improve the prediction rate to hormone therapy [7, 10, 23–25] is based on the rationale that each parameter represented a functional component of the receptor system. In premenopausal patients who were positive for oestrogen cytosol receptor, the positivity for nuclear oestrogen receptor (30°C) was associated with positivity for progesterone receptor (Table 3). The smaller incidence of such cases in the postmenopausal group suggests a less functional receptor pathway. Similar findings are seen for nuclear 4°C receptor.

In the premenopausal group all patients that were positive for cytosol oestrogen receptor and activating factor were also positive for progesterone receptor, but the corresponding figures in the postmenopausal group were 26 positive out of 46 tumours. In both groups of patients the presence or absence of activated receptor was as reliable as that of nuclear oestrogen receptor in assessing receptor functionality determined by the presence or absence of progesterone receptor respectively. Using this criterion, premenopausal tumours appeared to be more functional than those of postmenopausal patients.

Our assessment shows that predictibility of responsiveness to endocrine manipulation in a tumour improves when two or three parameters are estimated simultaneously. We find, in agreement with King *et al.* [8] and Leake *et al.* [5], that the co-presence of cytoplasmic oestrogen and progesterone receptors, or the simultaneous presence of nuclear and cytosol oestrogen receptors, improves the predictibility made by the presence of cytosol oestrogen receptor alone. We also find that tumours which have cytoplasmic oestrogen receptors and are able to bind to oligo(dT)-cellulose are likely to respond to endocrine therapy.

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