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The Effect of Endocrine Therapy on the Levels of Oestrogen and Progesterone Receptor and Transforming Growth Factor-β1 in Metastatic Human Breast Cancer: an Immunocytochemical Study

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The levels of oestrogen receptor (ER), progesterone receptor (PR) and transforming growth factor-β_1 (TGF-β_1) were measured by immunocytochemistry in 19 patients prior to and 1 month after the start of endocrine therapy (tamoxifen 10 patients; aromatase inhibition 9 patients). A complete or partial response was observed in 10 patients. The proportion of cells showing ER staining was higher in responding patients, but there was no change observed with endocrine therapy in either responding or non-responding patients. In contrast, cells staining for PR in responding patients were significantly reduced following therapy (59 ± 9% to 24 ± 9%: $P < 0.05$). There was no reduction in immunocytochemical PR in non-responding patients, although the numbers of these patients with initially positive PR levels was small. Stromal tissue adjacent to tumour cells stained with the antibody to TGF-β_1, with particularly intense staining at the periphery of tumour cell aggregates. There was no correlation between the degree of TGF-β_1 staining and ER or PR status, and no evidence of a change with endocrine therapy. It is concluded that neither tamoxifen nor aromatase inhibitors produce a change in the ER content or TGF-β_1 content of breast tumours as detected immunocytochemically, but PR levels are significantly reduced after therapy in responding patients.

Key words: oestrogen receptor, progesterone receptor, transforming growth factor β-1, breast cancer
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INTRODUCTION

ENDOCRINE THERAPY is the mainstay of treatment in metastatic breast cancer. The likelihood of response to therapy is increased in the presence of both oestrogen receptors (ER) and progesterone receptors (PR) [1], although the exact mechanisms of the interaction of endocrine agents, such as tamoxifen or medroxyprogesterone acetate, with steroid receptors are not clearly understood. Changes in steroid receptor content of tumours following endocrine therapy are of interest in that they might provide insights into the mechanisms of response and relapse during sequential endocrine therapies. The effects of endocrine therapy on steroid receptor levels have been previously investigated *in vitro* using cell lines [2, 3], and *in vivo* using both animal models [4] and human tumours [5–10]. Our own study, published in 1982 [10], was carried out using the dextran-coated charcoal (DCC) assay, and suggested that therapy caused a reduction in ER, which recovered on regrowth. Other reported data on human tumours have produced conflicting results, and it has been suggested that this might be due to cellular heterogeneity between different tumour biopsies and/or tamoxifen interfering with the ligand binding assay. Use of more recently available immunocytochemical methods to measure the receptor content of samples might overcome both of these problems and allow analysis of small biopsies obtained after treatment.

Transforming growth factor β_1 (TGF-β_1) is one of several peptide growth regulators that are thought to be important in the growth regulation of both normal and malignant cells. TGF-β_1 is a 25-kD member of a family of growth factors first recognised for their ability to produce anchorage-independent

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growth in fibroblasts [11], and has been isolated from a variety of sources including breast cancer cell lines [12] and breast tumour biopsies [13].

TGF-β_1 is known to be an important multifunctional regulator of cell growth and differentiation, and is a potent inhibitor of epithelial cell proliferation, including breast cancer cells. We have previously demonstrated that human breast tumours invariably express TGF-β_1 and that high levels of TGF-β_1 mRNA might be associated with a longer relapse-free interval and node-negativity [14, 15]. Several investigators have suggested that the actions of endocrine agents in breast cancer might be mediated in part by the local regulation of growth factor production and secretion. Knabbe and colleagues showed that the anti-oestrogen, tamoxifen, produces growth inhibition and a profound increase in secretion of TGF-β from ER-positive oestrogen-sensitive MCF-7 cells, but not ER-positive oestrogen-resistant LYS-2 cells, implying that the growth inhibitory effects of tamoxifen might be mediated by this mechanism [16]. Colletta has reported that tamoxifen produces a 5–25-fold increase in TGF-β secretion from fibroblasts, and has suggested that an inhibitory paracrine action of TGF-β derived from stromal cells on the tumour epithelial cell is a possible mechanism of action of tamoxifen [17].

This pilot study was designed to investigate the effect of endocrine therapy on the ER, PR and TGF-β_1 content of human breast cancer by immunocytochemical staining of sequential biopsies from metastatic cutaneous nodules taken before and after the initiation of therapy.

PATIENTS AND METHODS

Patients' details

19 patients with metastatic skin nodules from breast carcinoma and evidence of disease progression who were about to start endocrine therapy were entered into the study. Informed consent was obtained prior to entry. The mean age of the population was 66 years (range 39–83). All but 2 patients were postmenopausal. 11 patients had received a single previous endocrine therapy (including adjuvant therapy in some cases). 3 patients had received two previous endocrine therapies. The treatment given was tamoxifen (10 patients) or an aromatase inhibitor (4-hydroxyandrostenedione 5 patients; aminoglutethamide 1 patient; pyridoglutethamide 1 patient; CG16949 2 patients). Previous endocrine therapy was discontinued for at least 4 weeks prior to entry into the study. An excisional biopsy was performed under local anaesthetic in the clinic immediately prior to endocrine therapy and 1 month after commencing endocrine therapy. Samples were stained for ER, PR and TGF-β_1 as described below. An adjacent section of the sample was stained with haematoxylin and eosin to confirm the presence of tumour. Patients were clinically assessed monthly and formally reassessed at 3 months with full biochemical and imaging investigations. Response was classified according to UICC criteria [18].

Immunocytochemical staining

Samples were immediately frozen after excision and stored in liquid nitrogen. Paired samples from each patient were always stained in the same assay. The immunocytochemical assay for ER and PR was performed using the Abbott ER-ICA, PR-ICA monoclonal antibody kits as described by the manufacturers (Abbott Laboratories, North Chicago, Illinois, U.S.A.). Briefly, 8-μm thick sections were cut on a cryostat and placed on poly-L-lysine-coated glass slides. The sections were fixed in 4% formaldehyde–phosphate-buffered saline (PBS) for 10 min and then transferred to PBS for 10 min. Slides were then placed in cold methanol at -10°C for 4 min, acetone at -30°C for 1 min and returned to PBS. Following a 15-min incubation with 2% normal goat serum in PBS, to reduce non-specific binding of primary antibody, the slides were incubated with the primary antibody (H222 rat monoclonal for ER, KD68 rat monoclonal for PR) for 30 min or normal rat immunoglobulin as a control. After PBS washing, the slides were incubated with bridging antibody, a goat anti-rat IgG, at a concentration of 1:100 for 30 min. After a 30-min incubation with PAP (horseradish peroxidase–anti-horseradish peroxidase) and PBS washing, the slides were incubated for 6 min with freshly-mixed chromogen, diaminobenzidine tetrahydrochloride and 0.06% hydrogen peroxidase in PBS. Sections were then counterstained with 1% Harris haematoxylin prior to dehydration and mounting in xylene-soluble mountant. The staining intensity was assessed by two separate observers blinded to the clinical details. The staining was recorded as the percentage of cells staining positive with the relevant antibody.

For TGF-β_1 staining, samples were thawed and fixed in 4% formalin overnight then post-fixed in Bouins solution for 4–6 h. Prior to paraffin embedding, dewaxed 4-μm sections were rehydrated and washed in 10 mM PBS pH 7.2 prior to pre-incubation with 1.5% normal goat serum for 1 h at room temperature. Sections were then incubated overnight at 4°C with either the IgG of the rabbit polyclonal antibody to TGF-β_1 or non-immune rabbit IgG at equivalent concentrations, diluted in PBS containing 5% bovine serum albumin. After PBS washing, the sections were incubated in biotinylated anti-rabbit IgG (Vector Labs, Peterborough, U.K.) for 30 min at room temperature followed by a 1-h incubation with an avidin–biotin–peroxidase complex (Vector Labs). Staining was visualised using 0.05% 3,3'-diaminobenzidine and sections were counterstained with Gill's haematoxylin. The antibody to TGF-β_1 was a gift from Dr M.B. Sporn (NIH, Bethesda, Maryland, U.S.A.) and the Collagen Corporation (Pallo Alto, U.S.A.). A staining score of + to + + + was assigned to the tumours, a more precise system was not possible because of the heterogeneity of the staining, the nature of the stromal staining and the small size of the tumour samples. Full details of the immunocytochemical methodology for ER, PR and TGF-β_1 staining have been previously published by our group [19–21]. Statistical analysis was performed using the Wilcoxon signed rank test for non-parametric data.

RESULTS

10 of the 19 patients responded to endocrine therapy. The individual ER and PR staining is shown in Table 1 with the type of therapy and response.

ER staining

The change in oestrogen receptor staining with therapy is shown in Table 2. Although there was some degree of variation in ER levels, these were remarkably constant with no evidence of a change with therapy or a difference due to heterogeneity between tumour nodules. No change in oestrogen receptor levels was seen in either responders or non-responders. As expected, ER levels were significantly higher in responding patients ($P < 0.001$).

PR staining

Changes in PR before and after therapy are shown in Table 2. There was a significant difference in the PR level between

Table 1. TGF-β_1 and oestrogen receptor (ER)/progesterone receptor (PR) levels measured before and after endocrine therapy and tabulated for each patient against specific therapy and response

Case no.	Therapy	Response	ER (%) Pre	ER (%) Post	PR (%) Pre	PR (%) Post	TGF-β_1 Pre	TGF-β_1 Post
1	Tamoxifen	PR	70	70	90	20	+++	+++
2	Tamoxifen	PR	90	100	80	70	++	+++
3	Tamoxifen	PR	60	60	40	0	+++	++
4	Tamoxifen	CR	60	50	50	40	+++	++
5	Tamoxifen	PR	80	80	60	80	+++	++
6	Tamoxifen	PR	90	90	90	0	++	+++
7	4-OHA	PR	70	100	80	0	++	+++
8	AG	PR	60	40	60	20	++	++
9	CG16949	PR	80	80	40	0	+++	++
10	CG16949	PR	90	90	0	10	+	+++
11	Tamoxifen	NC	50	60	0	30	+++	+++
12	Tamoxifen	PD	0	0	0	0	+++	+++
13	Tamoxifen	PD	0	0	0	0	+++	+++
14	Tamoxifen	PD	0	0	0	0	++	++
15	4-OHA	NC	100	80	0	60	+	+++
16	4-OHA	PD	20	20	80	90	+++	++
17	4-OHA	PD	80	60	80	0	+++	+
18	4-OHA	PD	80	60	50	60	++	+++
19	PYG	NC	0	0	60	20	++	++

TGF-β_1, transforming growth factor-β_1; PR, partial response; CR, complete response; NC, no change; PD, progressive disease.

responders and non-responders ($P < 0.05$). In responding patients, there was a significant fall in PR following therapy. The mean staining prior to therapy was 59 ± 8.8% and following therapy was 24 ± 9.4% ($P < 0.05$). For non-responding patients, the PR levels before and after therapy were 30.0 ± 12.2% and 28.8 ± 11.2%.

TGF-β_1

The staining with the antibody to TGF-β_1 was predominantly extracellular and stromal, as noted in other tissues by other workers [22]. Although some weak stromal staining was seen in a heterogeneous distribution throughout the sections, the staining was particularly intense around infiltrating tumour cells. The staining was quite heterogeneous with areas of tumour showing intense stromal staining adjacent to tumour with absent stromal staining. Staining was particularly intense at the inferface between large aggregates of tumour cells and surrounding stroma. There was no correlation with ER positivity, PR positivity or response to therapy and no consistent change after endocrine therapy. Specifically, tamoxifen therapy did not appear to change the degree of TGF-β_1 staining.

DISCUSSION

This study has demonstrated that the ER and the immunostainable TGF-β_1 content of metastatic skin nodules is unchanged following endocrine therapy with tamoxifen or aromatase inhibitors, but the PR content is significantly reduced in responding patients. In contrast, following oophorectomy of nitrosomethylurea-induced rat mammary tumour, ER levels as measured by ligand-binding assay were significantly lowered [4]. The situtation in humans *in vivo* is unclear. Several studies on human breast tumours have shown a fall in ER after endocrine therapy [8, 10, 23], while others have shown no change [9]. It has been suggested that previous studies that have demonstrated

Table 2. Change in receptor staining with endocrine therapy

	ER staining (%) ±S.E. Pre	ER staining (%) ±S.E. Post	PR staining (%) ±S.E. Pre	PR staining (%) ±S.E. Post
All patients ($n = 19$)	56 ± 8.4	55 ± 8.0	45 ± 7.9	26 ± 7.1
Responders ($n = 10$)	75 ± 4.0	76 ± 6.5	59* ± 8.8	24 ± 9.4
Non-responders ($n = 9$)	37 ± 13.7	31 ± 11.1	30 ± 12.2	28 ± 11.2

ER, oestrogen receptor; PR, progesterone receptor, *$P < 0.05$ comparing pre- and post treatment PR content.

a fall in ER following endocrine therapy have used the ligand-binding assay and it is likely that tamoxifen or its metabolites interfere with this assay [9]. Hull and colleagues [6] showed that ER levels are reduced by tamoxifen when measured by the ligand-binding assay and this reduction persists for 2 months. They attribute this to persistence of tamoxifen or its metabolites in the patient due to the long half-life of the drug. Hawkins and colleagues [9] reported no change in ER on sequential sampling of primary tumours undergoing a variety of endocrine manipulations except for a marked reduction in ER in 3 patients on tamoxifen. Noguchi and colleagues [24], using an enzyme immunoassay, which would overcome the problem of receptor binding to tamoxifen, found a significant reduction in ER in postmenopausal women following tamoxifen-medroxyprogesterone therapy compared to a control group, but no difference in premenopausal women. They have subsequently reported that medroxyprogesterone alone has no effect on ER levels with sequential sampling [25]. Cellular heterogeneity of small biopsy samples has also been suggested as a cause of discordant receptor levels on sequential sampling in previous studies. These used ligand-binding assays and methods of receptor level determination which do not take account of the relative proportion of stromal tissue in the specimen which can lead to inaccuracies [26]. It is likely that immunocytochemistry is a more reliable assay of receptor levels in small samples. In our study, there was a striking concordance between the paired samples in all except one case (case 11), negating the argument that heterogeneity may obscure any receptor level changes that may occur.

In vitro studies indicate that oestrogen regulates the expression of ER. In general, the evidence suggests that oestrogen causes a downregulation of ER in MCF-7 breast cancer cells [27], and this has been confirmed by our own group when we have examined the effects of an oestrogen and a pure anti-oestrogen on ER expression *in vitro* [28]. If this were the case *in vivo* we would observe an upregulation of ER with anti-oestrogen therapy. The reason for this discrepancy is unclear.

The fall in PR is noteworthy. In breast cancer cell lines oestrogen stimulates the synthesis of PR [2, 29], and in *N*-nitrosomethylurea-induced animal tumours, oestrogen deprivation produces virtual disappearance of PR [4]. Previous studies of endocrine therapy have shown a rise in PR after short-term (7-day) administration of tamoxifen, probably due to the early oestrogenic effect of low-dose tamoxifen, and prompted the rational behind sequential therapy with tamoxifen and a progestagen [23]. In a retrospective analysis of sequential biopsies, Gross and colleagues [7] found a significant reduction in PR if there had been intervening endocrine therapy, although the agents used were not specified and the time interval between biopsies was much longer than the present study. Our study shows that, after 1 month of therapy with tamoxifen or an aromatase inhibitor, there is a fall in PR in responding patients and, therefore, any benefit from sequential therapy with tamoxifen followed by a progestagen is not likely to be due to the induction of PR, which is transient. The demonstration of a fall in PR 1 month after commencing therapy without a corresponding fall in ER implies a direct effect on the regulatory mechanisms of the progesterone receptor rather than the persistence following therapy of a population of ER negative/PR negative cells as has been suggested.

What could be responsible for the lack of change in ER positivity and reduction in PR? One possible explanation is that, at this point in time, a reduction in oestrogen-induced transcription is taking place; *in vitro* studies by other groups [27] and our own [28] suggest that by depriving cells of oestrogen an upregulation of ER eventually occurs.

It is not possible to say with any certainty whether the reduction in PR is confined to responding patients as suggested by this study. As the number of non-responding patients with positive PR levels was low, it is possible that a reduction would be seen with larger numbers. In one non-responder (case 17), there was a profound fall in PR. It is interesting, however, to speculate that an observed fall in PR could indicate a functional ER, and those non-responding, ER-positive tumours might have mutated or non-functional ER.

It might be possible, by studying changes in PR levels in PR-positive patients, to select second-line endocrine therapy; more rationally in those patients whose PR levels fall without response, further endocrine therapy is unlikely to be helpful, while those patients whose PR levels are unchanged might respond to therapy with progestagens or anti-progestagens. Further studies are required to answer this important clinical question.

The antibody to TGF-β_1 in this study was raised to a synthetic (1–30) peptide sequence of TGF-β_1 [30] and stains extracellular TGF-β_1 in various tissues [22]. Other TGF-β_1 antibodies, raised to the same peptide sequence, recognise intracellular TGF-β_1. Presumably, different epitopes are recognised due to different tertiary structures of the two peptides. In the original report of the localisation of the TGF-β_1 staining, this antibody stained extracellular sites thought to be of TGF-β_1 associated with extracellular matrix [22].

We have previously demonstrated the distribution of TGF-β_1 staining in primary breast tumours to be localised predominantly within the stroma, and suggested that TGF-β_1 is associated closely with extracellular matrix proteins [21]. The distribution seen in metastatic disease in this study is similar to our first report. The enhanced intensity of staining at the interface of tumour deposits and surrounding stroma might be explained by the influence of several cell types regulating the behaviour of TGF-β, as suggested by the co-culture data of Antonelli-Orlidge and colleagues, who showed that TGF-β activation was enhanced in the presence of endothelial cells and pericytes compared to TGF-β activity in the presence of either single cell type alone [31]. No correlation of TGF-β_1 staining was found in relation to ER or PR staining. This is in contrast to the data reported by King and colleagues who have shown in a small series of patients that TGF-β_1 levels, as measured by western blotting, were inversely related to ER levels [13]. Arteaga and colleagues found that ER-positive breast cancer cells were unaffected by and produced low levels of TGF-β, while ER-negative cells secreted significant amounts of TGF-β and were growth inhibited by exogenous TGF-β [12]. No detectable change in staining occurred with a variety of endocrine therapies, including tamoxifen. It remains possible that a change in TGF-β activation occurs with endocrine therapy, and that the antibody used in this study does not detect the difference between latent and active TGF-β_1. Another possibility is that other members of the TGF-β family might be involved in the mechanism of endocrine therapy. Recently, it has been shown that TGF-β_2 and TGF-β3, but not TGF-β_1, mRNA levels are downregulated by oestrogen therapy in breast cancer cell lines [32].

The results of this study suggest that TGF-β_1 might play a role in the growth control of metastatic breast carcinoma, but do not support the hypothesis that TGF-β_1 levels *in vivo* are regulated by endocrine therapy.

The total number of patients studied was small, reflecting the difficulty of accruing sufficient numbers of patients in such a

study. Further work on a larger cohort of patients is required to substantiate these results, and more precise methods of assessing TGF-β_1 activity *in vivo* are required to assess its possible role as a mediator of the response to endocrine therapy in human breast cancer.

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