Abstract

The expression of transforming growth factor-α (TGF-α) has been evaluated in 51 breast cancers of known responsiveness to endocrine therapy using immunohistochemistry. High levels of TGF-α were observed in 65% of tumors and showed no relationship with tumor estrogen receptor or epidermal growth factor receptor status or Ki67 immunostaining. TGF-α levels did, however, relate to the endocrine sensitivity of the disease, with unresponsive tumors frequently showing high levels of TGF-α immunoreactivity. This relationship was observed in estrogen receptor-positive disease and was independent of the epidermal growth factor receptor status of the tumor. No quantitative association between TGF-α and Ki67 immunostaining was observed in any of the subcategories of tumors. These data infer a role for TGF-α in the development of endocrine insensitivity in estrogen receptor-positive breast cancer by mechanisms which appear independent of tumor growth fraction, as determined by Ki67 immunostaining.

Introduction

A central role for steroid hormones in the growth and development of breast cancer is well established. The dependence of the cancer cells on hormones is, however, transient and ultimately the majority of patients die with hormone-independent and/or endocrine-resistant tumors. There is, therefore, a need to understand the processes associated with these conditions and to determine whether features of tumor progression towards autonomy can be used as targets for new therapeutic initiatives.

Although many mechanisms have been proposed to explain the development of endocrine-insensitive states (1), recent experimental and clinical studies have linked growth factor signaling pathways to the process, with initial cooperativity between steroids and growth factors being disrupted by aberrations in signal transduction (2). The EGF-R+ appears central to these events, with its detection in clinical breast cancer specimens being frequently associated with high rates of cell proliferation (3) and endocrine insensitivity (3-6). Indeed, transfection studies in human breast cancer cells which elevate EGF-R have shown in some instances (7), but not others (8), that estrogen-dependent proliferation can be bypassed. Similarly, EGF can partially reverse the inhibitory effects of antiestrogens on the growth of human breast cancer cells (9). Since TGF-α is a ligand for the EGF-R (10, 11), inducing cell proliferation in several human breast cancer cell lines (12), and since its in vitro secretion has been shown to be enhanced by estrogens (12, 13), its levels in clinical breast cancer specimens are of considerable interest.

Previous studies examining TGF-α mRNA (14) or protein (15-19) levels and ER status in clinical samples have not established a consensus view, nor has the association between TGF-α, tumor growth fraction, and endocrine sensitivity been determined. The current paper seeks to address these issues.

The principal procedure used in these studies is immunohistochemistry. This has the advantage of directly monitoring proteins in individual tumor cells. Earlier investigations from our laboratory using this technique on breast cancers have established a relationship between the presence of ER (20), EGF-R (3, 4), and Ki67 labeling index (20) and the endocrine responsiveness of the disease. The Ki67 antibody reacts with a nuclear antigen that is present throughout the cell cycle of proliferating cells, but is absent in quiescent cells (21), and its expression in breast cancer specimens correlates with mitotic activity and their rate of recurrence after mastectomy (22, 23).

Patients and Methods

Fifty-one samples of primary tumor tissue from patients with histologically proven breast cancer presenting to Professor R. W. Blamey at the City Hospital, Nottingham, United Kingdom, during the period July 1984 to August 1987 were included in the study. No patient had received any form of adjuvant endocrine or cytotoxic therapy.

All patients received systemic endocrine therapy for locally advanced primary tumors (maximum diameter, 5 cm) or for local or distant recurrences. Pre- or perimenopausal patients received the luteinizing hormone-releasing hormone agonist goserelin (Zoladex, ICI 118630, 3.6 mg depot/28 days) alone (n = 3) or in combination with tamoxifen (20 mg twice daily, n = 12), while postmenopausal women received tamoxifen (n = 36).

Patients were assessed for complete or partial responses, static disease (no change), or progression at 2-3 monthly intervals by International Union Against Cancer criteria (24). As recommended by the British Breast Group, responsive and static diseases were only reported following a minimum duration of remission of 6 months (25).

Preparation of Tissue and Immunostaining Procedures. Samples of primary tumor tissue were rapidly frozen upon excision, stored at ~70°C, and transported on dry ice to the Tenovus Cancer Research Centre, Cardiff, United Kingdom, for analysis. A representative portion of the tissue was blocked for cryostat sectioning and immunostaining.

Tgfα Antiserum. Tgfα (Ab2) antisera (Oncogene Science, Inc.) is a mouse monoclonal IgG2a reacting with denatured and native Tgfα of human or rat origin, but showing no cross-reactivity with human or mouse EGF. A recombinant human Tgfα protein was used as the immunogen (26).

TGF-α Immunocytochemistry. Cryostat sections (5 μm) were thaw-mounted onto poly-L-lysine tissue adhesive-coated glass slides and immediately fixed in 3.7% formaldehyde in 0.01 M PBS (pH 7.2) at r/t for 15 min. Slides were then immersed in 2 baths of PBS for 5 min each and further fixed in absolute methanol (~20°C) for 5 min and then acetone (~20°C) for 3 min. Slides were finally immersed in PBS for at least 10 min before storage in sucrose/glycerol medium at ~20°C (27).

Sections fixed and stored in the above manner were taken for assay within 2 weeks of sectioning. An assay procedure was developed using the Oncogene Science immunohistochemistry system, an avidin-biotin complex-immunoperoxidase kit for TGF-α with a number of additional steps introduced to eradicate nonspecific reactivity of kit components.

Aliquots of neat kit primary antisera (Ab2), and of a matched control kit monoclonal antibody (Ab1), were incubated with 2 volumes of neat human serum for 30 min (r/t). PBS containing 0.1% BSA was added to primary antisera to a final dilution of 1/2000 and the solution vortexed and allowed to stand for a further 30 min before adding to the sections. This procedure improves the specificity of subsequent antibody reactions. During this incubation, sections were removed from the sucrose/glycerol solution and washed.
added dropwise to sections, from which the excess PBS had been wiped, for 20 min (r/t). Following blocking, the slides were wiped round, and primary or control antibodies (prepared as described above) were added to sections for 60 min (r/t). Slides were then washed (3 X 5 min) with PBS, and kit biotinylated anti-mouse secondary IgG (1 drop/ml 0.1% BSA/PBS) added for 30 min (r/t). Further washes (3 X 5 min) with PBS preceded the addition of the avidin-biotin horseradish peroxidase complex (avidin-biotin complex reagent, which was prepared 30 min in advance) for 30 min (r/t). Sections were washed (3 X 5 min) with PBS before the addition of the diaminobenzidine-hydrogen peroxide chromogen-substrate solution for 6 min (using reagents from the ER-immunocytochemical assay monoclonal kit; Abbott Diagnostics, Chicago, Illinois). Slides were washed with distilled water (5 min) and counterstained with 1% aqueous methyl green (10 min). A further distilled water wash (5 min) was followed by serial dehydration in ethanol, clearance in xylene, and mounting slides under coverslips in a xylene-soluble mountant.

In the initial phases of assay development, the primary antibody was preabsorbed with recombinant TGF-α. Inclusion of this step in the assay blocked Ab2-associated staining.

**ER, EGF-R, and Ki67 Immunostaining.** The assays for the immunohistochemical measurement of ER (28, 29), EGF-R (3, 4), and Ki67 labeling index (20) on frozen sections of breast cancer specimens have been fully described in previous publications from our group. The antibodies were obtained from Abbott Diagnostics; Amersham International, Amersham, United Kingdom; and Dakopatts, Denmark, respectively.

**Specimen Evaluation.** All specimen evaluation was performed on an Olympus microscope (BH-2) first using an ocular magnification of X10 in order to enable the localization and subsequent avoidance of normal and benign areas within the section. This initial examination also allows the heterogeneity of immunostaining within the tumor components to be assessed and thus ensures that adequate sampling of all areas is performed. All subsequent evaluations were carried out at a magnification of X40. TGF-α, ER, EGF-R, and Ki67 immunostaining patterns were evaluated by 2 personnel using a dual-viewing attachment to the microscope and a consensus interpretation agreed. Control slides were checked for nonspecific binding before assessing the percentage of tumor cells stained by the primary antibody (minimum of 2000 cells counted). For TGF-α, the predominant intensity of immunostaining was assessed semiquantitatively using a scoring system of 0, 1, 2, and 3 corresponding to negative, weak, intermediate, and strong staining, respectively. From this, a HScore value was calculated using the equation:

$$\text{HScore} = \left\{ \left( \% \text{ cells showing an intensity value } X \times 1 \right) + \left( \% \text{ cells showing intensity value } 2 \times 2 \right) + \left( \% \text{ cells showing intensity value } 3 \times 3 \right) \right\} / 100.$$  

For purposes of analysis, ER and EGF-R results are expressed as positive and negative, with >2% of tumor cells immunostaining denoting a positive value. Tumor nuclear staining using the Ki 67 antibody was subdivided into 3 categories, <10% cells, 11–29% cells, and >30% cells. The above cut off points for the ER (24), EGF-R (3,4), and Ki67 (20) assays have previously been established by our group to discriminate endocrine sensitivity and insensitivity in breast tumors.

**RESULTS**

**TGF-α Immunostaining and Its Relationship to the ER, EGF-R, and Ki67 Assays.** Specific peptide-competable immunostaining for TGF-α was observed in the cytoplasm and plasma membranes of the majority of tumors (Fig. 1). Its expression, however, was highly variable, with, in some instances, almost all tumor cells strongly immunostaining. The average intensity staining value (HScore) was approximately 1, with 33 of 51 (65%) tumors staining at or above this value. Using a TGF-α HScore cut-off value of 1, no significant association was observed between TGF-α immunostaining and either tumor ER, EGF-R, or Ki67 status (Table 1). Highly Ki67-positive samples, however, often showed TGF-α levels of >1 (27 of 37).

**TGF-α and the Responsiveness to Endocrine Measures.** Fig. 2 shows that high TGF-α HScore values (1 or above) are frequently associated with either failures to respond to endocrine measures (23 of 29, 79%) or static disease (8 of 11, 73%). Conversely, the majority of tumors (9 of 11, 82%) which responded to the endocrine treatments had TGF-α values below 1. These differences were statistically significant ($\chi^2 = 13.44, P < 0.01$). Although the majority of objective remissions following endocrine therapy were seen in ER-positive disease, 12 patients with ER-positive tumors derived no clinical benefit from the endocrine measures and 10 women had static disease (Table 2). Significantly, 19 (86%) of these patients had tumor TGF-α HScore values of 1 and above (7 static and 12 progressive; Fig. 3). In patients with ER-positive endocrine-responsive disease, 9 of the 10 tumors had TGF-α HScore values below 1. These differences were statistically significant ($\chi^2 = 19.2, P < 0.01$).

Examination of Ki67 values and TGF-α HScore levels in the 3 response categories failed to demonstrate a convincing relationship between TGF-α and Ki67 in ER-positive disease (Fig. 4; $r^2 = 0.07$). Thus, although low TGF-α HScores and the percentage Ki67-positive cells were most frequently seen in patients responding to endocrine therapies, no further association between these parameters was seen between static and progressive tumors.

Further subdivision of the data derived from ER-positive tumors by

| Table 1 Relationship between TGF HScore and ER, EGF-R, and Ki67 status. |
| TGF-α |<1 |≥1 |
| ER⁺ ve | 12 | 20 |
| ER⁺ | 6 | 13 |
| EGF-R⁺ | 10 | 21 |
| EGF-R⁻ | 8 | 12 |
| Ki67 |<10 | 5 | 2 |
| 11–29 | 4 | 4 |
| ≥30 | 10 | 27 |
Fig. 2. Relationship between TGF-α levels and response to endocrine therapy. The results are analyzed using a $\chi^2$ statistic for contingency table analysis. Values, counts of patients with TGF-α HScore values $\geq 1$ and $<1$.

their EGF-R status revealed that the association between TGF-α and response to therapy was detected in both EGF-R-positive and -negative disease (Fig. 5, a and b). No overall relationship was observed between TGF-α and Ki67 immunostaining in either of these groups (data not shown).

In ER-negative EGF-R-positive breast cancers, 16 of 17 tumors (94%) were unresponsive to the endocrine measures used. Although these tumors were frequently highly positive for TGF-α and Ki67, no overall association was observed between these parameters (data not shown). The remaining 2 patients with ER-negative EGF-R-negative tumors had TGF-α HScore values of 1 and 1.6. These women had progressive and responsive diseases, respectively.

Table 2. Relationship between ER and response to endocrine therapy

<table>
<thead>
<tr>
<th>ER status</th>
<th>Respond</th>
<th>Static</th>
<th>Progress</th>
</tr>
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<tbody>
<tr>
<td>Negative</td>
<td>10</td>
<td>10</td>
<td>12</td>
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<tr>
<td>Positive</td>
<td>10</td>
<td>10</td>
<td>12</td>
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Subdivision of the data according to the menopausal status of the patients did not reveal any differences in response rates between pre- and postmenopausal women (data not shown). Insufficient patient numbers preclude further substratification of the data.

DISCUSSION

This study represents the first immunohistochemical analysis of the expression of TGF-α in breast cancers of known sensitivity to endocrine therapy. The rationale for the investigation was based on the known mitogenic activity of TGF-α on human breast cancer cells in culture (12), its estrogen inducability and suppression by antiestrogens (12, 13), the ability of TGF-α to partially reverse the inhibitory actions of antiestrogens (9), and the observations that endocrine-insensitive ER-positive breast tumors frequently show elevated growth fractions (20) and express the EGF-R (3–6). It was conceived, therefore, that in clinical breast cancer specimens TGF-α interactions with EGF-R might promote cell proliferation and act as the driving force subverting endocrine sensitivity.
Although the immunolocalization of TGF-α in breast cancer specimens has recently been reported by several groups (15–19), a wide range of TGF-α values have been recorded, and a diversity of associations with other biological endpoints observed. Thus, while Umekita et al. (17) have described TGF-α immunostaining in 28% of primary breast cancers and axillary lymph nodes, using the Ab2 mouse monoclonal antibody in paraffin-embedded material, and a relationship with either ER negativity or EGF-R positivity, Lundy et al. (16), using the same antibody in identically embedded material node-positive breast cancer, have found a 68% positivity rate and a positive relationship between TGF-α and ER. In our own previous study, using the Ab2 antibody, on unselected primary breast cancer specimens, we have described TGF-α staining in 79% of frozen sections, with a trend (nonsignificant) for high levels of TGF-α to be observed in ER-positive EGF-R-negative disease (19). Currently, using tumor specimens from patients with either locally advanced breast cancer or from women with relatively short disease-free intervals following mastectomy, we have observed almost all cancers to express immunodetectable TGF-α in frozen sections and, subsequently, the relationship between TGF-α and ER to be lost. Similarly, Dublin et al. (18) have reported TGF-α immunoreactivity in all but 9 of 195 primary breast cancers using the CIM1 polyclonal antibody, with no relationship between TGF-α and ER. TGF-α mRNA levels have been reported in 62% of 167 breast cancers, again with no obvious association with ER (14).

Within the above studies, no convincing associations have been observed between TGF-α levels and patient disease-free interval (15) and survival (18). In contrast, in the present study we report a strong relationship between high levels of TGF-α immunostaining (HScore 1 and above) and the loss of endocrine sensitivity in breast cancer (Fig. 2). Indeed, in ER-positive tumors, while TGF-α HScore values below 1 were regularly linked to objective tumor remissions, high values were most often associated with either static or progressive disease (Fig. 3). Significantly, however, although increasing Ki67 immunostaining levels are also linked to endocrine insensitivity (20), no convincing association between TGF-α and this parameter was observed (Fig. 4). Indeed, the relationship between TGF-α and loss of endocrine sensitivity was also independent of EGF-R status (Fig. 5) and within the various ER/EGF-R subgroups, no association was seen between TGF-α and Ki67.

These data raise 3 important issues regarding the potential of TGF-α to subvert endocrine sensitivity.

Firstly, given that TGF-α is thought to act through the EGF-R, why is the relationship between this parameter and response to therapy observed in both EGF-R-positive and -negative disease? As with the TGF-α assay, various proportions of tumors have been suggested to express significant amounts of the EGF-R, with values ranging from 14 to 91% (reviewed in Ref. 30). Although using our current immunohistochemical procedure on frozen breast cancer specimens we have established a clinical significance for our assay, including an association between EGF-R positivity and short disease-free interval...
and endocrine insensitivity (3, 4), we have more recently developed an immunocytochemical procedure for use on paraffin-embedded material which is more sensitive and which shows a much higher EGF-R positivity rate. In this light, it is of interest that ER-positive MCF-7 human breast cancer cells are also apparently negative for EGF-R using our frozen assay, yet MCF-7 cells have been shown to contain small numbers of EGF-R (~2400/cell) using a ligand binding assay (33). These cells are also responsive to EGF/TGF-α. Based on these observations, it may be more accurate to describe our EGF-R-negative tumors as receptor poor, but still potentially capable, therefore, of mediating the cellular actions of TGF-α.

Secondly, how might we envisage TGF-α preventing breast cancer cells clinically responding to endocrine therapies by mechanisms that do not involve TGF-α-induced cell proliferation? It has been suggested that the rate of growth of cancer is determined by the relationship between cell proliferation and active cell death (34). Only when the rate of cell proliferation exceeds cell death does tumor growth continue. Endocrine therapies, therefore, might act on responsive cells by either lowering the rate of cell proliferation of the cancer cells and/or by raising the rate of cell death. Experimental evidence exists for each of these actions with, for example, antiestrogenic drugs both antagonizing the growth-promoting activity of estradiol on human breast cancer cells in culture (35) and promoting ER-mediated cytotoxic effects (36). Indeed, since endocrine therapies appear most effective against slow growing tumors (37), which show low S-phase fractions (38) and low levels of Ki67 immunostaining (20), it may be inferred that the induction of cell death is an important element in the action of antihormonal agents.

In the above model, reduced sensitivity of breast tumors to endocrine measures could be promoted by aberrations in either the mechanisms governing the coordinated regulation of cell proliferation or those associated with cell death.

Although steroid hormones and peptide growth factors are known to regulate proliferation of some breast cancer cell lines in vitro, the elements which stimulate or inhibit active cell death have been less well studied. It is conceivable, therefore, that elevated TGF-α levels may contribute to maintaining breast cancer cell viability in the face of a reduced hormonal environment. A similar role has been suggested for the BCL2 gene product which, when transfected into mouse fibrosarcomatous cells, appears able to reduce the induction of active cell death by tumor necrosis factor (39). Interestingly, TGF-α, EGF, and basic fibroblast growth factor have been shown to inhibit the spontaneous onset of apoptotic DNA cleavage found during the culture of ovarian granulosa cells, with this inhibition being blocked by the inclusion of a tyrosine kinase inhibitor (40). Moreover, the overexpression of EGF in T-47D breast cancer cells, following the transfection of the EGF gene, reduces the sensitivity of the cells to the growth-inhibitory activity of antiestrogens and prostaglandins by mechanisms which are independent of the prevailing rate of cell proliferation (41). Although the authors of this paper suggested that the expression of growth factors might provide the cells with an alternative proliferation pathway in the presence of growth inhibitors, it is difficult to envisage why they would not also operate as growth stimulators in the absence of antihormonal drugs. Certainly, TGF-α has other in vivo biological functions in addition to its growth-stimulatory activity and may stimulate, for example, angiogenesis (42). Paracrine influences associated with the actions of TGF-α on the noncancerous components of the tumor may, in their turn, modulate the behavior of the epithelial cell population and alter their responsiveness to antihormonal agents. Clearly, the cellular response pathways associated with TGF-α signaling pathways remain to be clarified in breast cancer.

Thirdly, what governs the variable levels of TGF-α expression in breast cancers? The control of TGF-α expression in breast cancer cells has focused on ER-mediated events (12, 13), with estrogens inducing its production in human breast cancer cell lines (43) and differentiated rat mammary tumors (44). Loss of regulated function of TGF-α in breast cancer cells may, therefore, stem from aberrations in estrogen response pathways, leading to altered production rates. Certainly, ER variants have been described which may be of clinical significance and which appear in approximately 50% of tumors (45).

In conclusion, we have examined in clinical breast cancer specimens the relationship between TGF-α and the endocrine sensitivity of breast cancer. Our data are highly suggestive that TGF-α is not an important mitogen in breast cancer, rather, it may protect cells against anti-hormone-induced cell death. Such a role for TGF-α would offer new therapeutic initiatives and a mechanism for improving the anti-tumor efficacy of antihormonal treatments through combination with procedures directed towards the EGF-R pathway (46). Similarly, there is now a further imperative to establish and study: (a) what factors contribute to TGF-α levels in breast cancer; (b) why some breast tumors show high rates of cell proliferation; and (c) how these events may be modified to establish a phenotype which more closely resembles the hormone-sensitive breast.

REFERENCES


Transforming Growth Factor-α and Endocrine Sensitivity in Breast Cancer


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