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Transforming Growth Factor Beta$_1$ in Ductal Carcinoma *in situ* and Invasive Carcinomas of the Breast

Rosemary A. Walker and Sheila J. Dearing

Transforming growth factor beta (TGF-β) is a multi-functional regulatory protein which can affect growth, immune responses, angiogenesis and the formation of extracellular matrix. Its role in breast carcinomas has been investigated using an antiserum to TGF-β$_1$ and immunohistochemistry. 27 ductal carcinomas *in situ* and 54 invasive carcinomas were examined, employing formalin-fixed, paraffin-embedded material. There was no reactivity in 55.5% of *in situ* carcinomas in comparison with the invasive tumour where only a third were negative. Prominent reactivity was seen in 11% of *in situ* tumours, and 20% of invasive carcinomas. There was no correlation between detection of transforming growth factor β$_1$, and histological grade, oestrogen receptor status, epidermal growth factor receptor status and Ki-67 labelling for the invasive carcinomas. There was a significant relationship between prominent reactivity and node status, all carcinomas with this degree of staining having metastasised. This, along with the differences between *in situ* and invasive carcinomas, suggests that TGF-β$_1$ may be a determining factor for invasion and metastasis.
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INTRODUCTION

THE OBSERVATION that malignant cells in culture require less exogenous growth factors than their normal counterparts led to the proposal that malignant cells produce and respond to their own growth factors, i.e. autocrine control [1]. Transforming growth factor β (TGF-β) is one such factor [2] and this has been isolated from the culture medium of breast cancer cell lines [3]; messenger RNA has also been detected in the same cells [4]. However, studies of primary breast carcinomas are extremely limited.

TGF-β is of interest because of the many effects it has on both physiological and pathological processes [5]. Unlike other growth factors it can act as a stimulator or inhibitor of growth. Oestrogen receptor positive and oestrogen receptor negative breast cancer cell lines differ with regard to the effects of TGF-β although the findings are conflicting. Oestrogen receptor negative cell lines secrete large amounts of TGF-β [6,7], express high affinity TGF-β receptors and respond to very low concentrations of exogenous TGF-β, reducing DNA synthesis and cell proliferation. However, for oestrogen receptor positive cell lines findings differ, with some studies reporting growth inhibition [8,9] and others no effect [7].

TGF-β also has a role in the control of accumulation of matrix proteins [10] by increasing their synthesis and by controlling proteolytic degradation. The stroma of tumours differs from that of comparable normal tissues and is an important factor in malignant growth [11]. *In vitro* TGF-β secreted by MCF-7 cells can stimulate fibroblasts to produce the stromal protein tenascin and exogenous tenascin can result in MCF-7 cells losing cell-cell and cell-substrate contacts [12]. This raises the question as to the inter-relationship of TGF-β secreted by primary breast carcinomas and tumour stroma, and how this may relate to tumour growth and metastasis.

In this study the presence of TGF-β in *in situ* and invasive breast carcinomas has been examined, using immunohistochemistry with emphasis on its relationship to tumour behaviour.

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MATERIALS AND METHODS

Tissues

Tissue was available from 27 cases of ductal carcinoma *in situ* which had presented clinically, and from 54 unselected invasive carcinomas. The *in situ* cases were obtained by searching the files of the Leicestershire Histopathology Service and were from the time period 1985–1988. None showed evidence of invasion greater than 1mm. All material had been fixed in 4% formaldehyde in saline and processed through to paraffin wax. For all invasive carcinomas there was tissue which had been frozen and stored in liquid nitrogen, as well as parallel samples which had been formalin-fixed, paraffin-embedded, as for the *in situ* tumours.

Immunohistochemistry

An affinity purified polyclonal antiserum to TGF-β$_1$ was used (a gift from Professor M. Feldmann, Sunley Research Centre, Charing Cross). It had been raised against human rTGF-β$_1$ in rabbits, and specificity had been confirmed by enzyme linked immunosorbent assay and western blotting [13]. Formalin-fixed, paraffin-embedded material was used for all TGF-β$_1$ studies.

The antiserum was applied to tissue sections at a concentration of 3.6 μg/ml for 18 h at 4°C. After rinsing and washing in Tris buffered saline, biotinylated swine antirabbit immunoglobulin serum was applied, followed by streptavidin-biotin peroxidase complex. The peroxidase was developed using diaminobenzidine-hydrogen-perodixase. Pre-immune rabbit serum was used as a control.

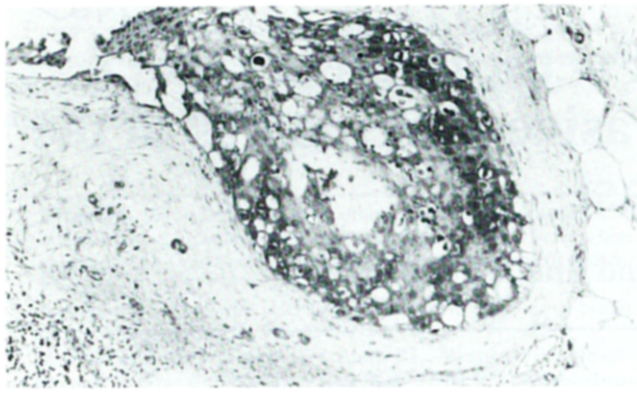

Fig. 1. Comedo type ductal carcinoma *in situ* with cytoplasmic staining for TGF-β_1 in many of the cells.

Oestrogen receptor was determined using the ERICA kit (Abbott) applied to frozen sections, following the fixation schedule recommended. Epidermal growth factor receptor (EGFR) was determined using the monoclonal antibody EGF-R1 [14] applied to frozen sections, followed by the streptavidin-biotin-peroxidase system. Ki-67 labelling was assessed to give an indication of cell proliferation using DAKO-PC antibody, followed by the same detection system as for EGFR.

Clinico pathological features

Haematoxylin and eosin stained sections of all cases of ductal carcinoma *in situ* were assessed for type; invasive carcinomas were determined for type using WHO criteria and from histological grade using a modification of the Bloom and Richardson system [15]. Lymph node status was known for 44 cases, and stage for 39 cases.

χ^2 statistical analysis was used.

RESULTS

Ductal carcinoma in situ

There were 13 cases of comedo ductal carcinoma *in situ*, 11 of cribriform type and three examples of papillary ductal carcinoma *in situ*.

Four patterns of reactivity were observed: no staining in areas of ductal carcinoma; staining of stroma around areas of *in situ* carcinoma with fine linear reactivity, but with no evidence of cellular reactivity; cytoplasmic staining of occasional cells in a small number of involved ducts; cytoplasmic staining of a third to all cells in half or more of the involved ducts (Fig. 1). Occasionally, normal breast acinar cells showed fine cytoplasmic staining in tissues with or without staining of associated ductal carcinoma *in situ*, but this reactivity was minimal.

Overall, 55.5% of the cases were negative. Two of the 27 carcinomas showed stromal staining only, 26% had only slight reactivity and three (11%) showed prominent staining. The relationship between pattern of staining and type of ductal carcinoma *in situ* is shown in Table 1. Over half of the comedo type showed some staining, whereas only three of 11 cases of the cribriform type were reactive. There were 6 cases with evidence of microinvasion; 1 showed slight reactivity and another more prominent staining.

Table 1. Relationship between pattern of staining for TGF-β_1 and the type of ductal carcinoma in situ

Type	TGF-β_1 Staining			
	Negative	Stromal only	Slight	Prominent
Comedo	6	1	5	1
Cribriform	8	0	1	2
Papillary	1	1	1	0

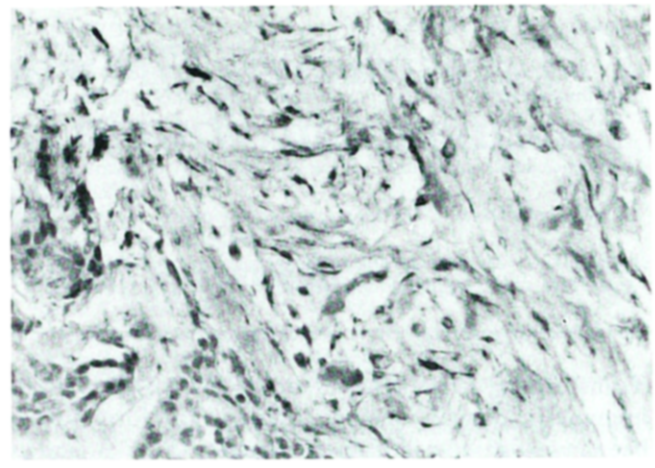

Fig. 2. Staining of stroma for TGF-β_1 around groups of tumour cells.

Invasive carcinomas

The same four patterns of staining were seen. A third of the carcinomas (18) were negative. Seven (13%) showed stromal staining only (Fig. 2). A third had occasional cells with cytoplasmic staining, with or withour stromal staining and 11 carcinomas (20%) had prominent cytoplasmic staining of 50% or more cells (Fig. 3).

There were six infiltrating lobular carcinomas, the remainder being infiltrating ductal carcinomas. Two of the infiltrating lobular carcinomas had prominent staining, two showed slight reactivity and the other two were negative. There was no difference in the distribution of staining between the different types.

There was no significant relationship between histological differentiation and pattern of reactivity (Table 2), although there was a higher proportion of poorly differentiated carcinomas amongst the group showing prominent reactivity.

There was a significant relationship between marked staining for TGF-β_1 and lymph node status ($0.02 > P > 0.01$), all carcinomas with prominent reactivity (who were of known nodal status) having metastasised. The extent of staining was not related to advanced stage (Table 2).

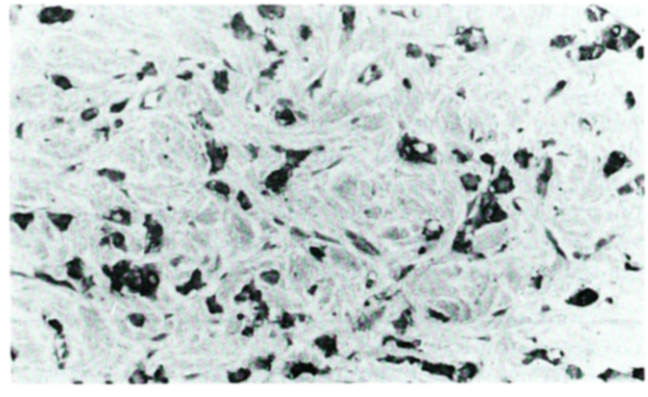

Fig. 3. Prominent cytoplasmic staining for TGF-β_1 of infiltrating tumour cells.

Table 2. Relationship between extent of staining for TGF-β_1 and grade, node status and stage

Grade	TGF-β_1 Staining Negative	Stromal only	Slight	Prominent
I	1	1	2	2
II	9	4	11	3
III	8	2	5	6
Node status				
Metastasis	9	4	7	9
Free from Disease	7	2	6	0
Stage				
I	5	2	4	0
II	4	3	6	7
III + IV	3	2	1	2

Thirty seven of the carcinomas (68.5%) were oestrogen receptor positive, and the distribution of positive cases was similar between the different staining categories for TGF-β_1. 25% of the carcinomas expressed EGFR and again the distribution was similar between the different staining categories for TGF-β_1. No relationship was found between Ki-67 labelling and TGF-β_1 reactivity, both TGF-β_1 negative and clearly positive tumours showing a range of Ki-67 labelling from 2 to 40%.

DISCUSSION

The majority of studies which some considered the role of TGF-β_1 in breast carcinoma have been confined to cell lines. These have examined the role which it may have in the control of growth, but in view of its multifunctional properties there are several aspects of the nature of breast carcinomas which could be affected by TGF-β_1.

Assessment of growth regulation of primary tumours cannot be as direct as with cell lines, but there was clearly no relationship between the detection of TGF-β_1 and the usual criteria for assessing growth, i.e. hormone and growth factor receptors and Ki-67 labelling. The ability to be able to use formalin-fixed, paraffin-embedded material meant that *in situ* and invasive carcinomas could be compared, giving a valuable dimension to the study. There were differences in the detection of TGF-β_1 both in relation to the incidence of any staining, and in the numbers of tumours with prominent reactivity. Staining of normal breast in relation to *in situ* and invasive lesions was infrequent, and confined to small numbers of cells. In thyroid TGF-β_1 is detectable in malignant but not benign or normal epithelium [16] and it was suggested that the increased synthesis may be linked to increased proliferation, and hence a growth advantage. The difference in staining frequency between *in situ* and invasive carcinomas suggests that TGF-β_1 synthesis is a factor in the progression of breast cancers from non-invasive to invasive. However, as with the findings for the infiltrating carcinomas, detection of TGF-β_1 did not appear to relate to growth advantage since prominent reactivity was observed in two cases of cribriform type, which are recognised as having a low proliferative activity [17].

Further evidence for TGF-β_1 having a role in invasion rather than growth control in primary breast carcinomas comes from the findings of a relationship between prominent staining and the presence of node metastasis. Certainly, not all carcinomas with nodal metastases had detectable TGF-β_1, but then there are many factors which are involved.

For TGF-β_1 to have any effect it has to be biologically active, and this requires acidification [18], or protease activation [19] locally. The detection of TGF-β_1 by immuno-histochemistry cannot be used as a means of assessing whether TGF-β_1 will be effective at the cell level. However, studies using other antibodies to TGF-β_1 [20] have concluded that there is a good correlation between synthesis (i.e. detection) and secretion.

Another approach for determining the role of TGF-β_1 in breast carcinomas is the assessment of mRNA levels. Travers *et al.* [21] found that TGF-β_1 mRNA was more abundant in carcinomas than benign or normal breast tissue. In a further study [22] they found the converse to the present one, in that high levels of TGF-β_1 mRNA were associated with absence of lymph node metastases. What is not evident is whether there is a direct correlation between TGF-β_1 protein and mRNA levels in breast, since in other cells, e.g. macrophages there is a dissociation, with high mRNA levels but low protein levels. If this is the case then the differences between mRNA and immunohistochemical studies may be due to different groups of carcinomas being assessed. To examine this area further, we are extending our studies to include mRNA expression using *in situ* hybridisation, which has the value of identifying the nature of the cells expressing TGF-β_1, information that cannot be gained by northern analysis.

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Overexpression of the c-*erb*B-2 Oncoprotein: Why does this Occur More Frequently in Ductal Carcinoma *in situ* than in Invasive Mammary Carcinoma and is this of Prognostic Significance?

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Overexpression of c-*erb*B-2 occurs in 60% of *in situ* and 25% of infiltrating ductal carcinomas. We have previously found very strong associations between immunohistochemical staining for c-*erb*B-2 and histological pattern and nuclear size in ductal carcinoma *in situ* (DCIS) and less strong correlation with proliferative activity. In a further study of infiltrating ductal carcinomas we have found that, in addition to tumours arising from c-*erb*B-2 positive, large celled, rapidly proliferating, comedo carcinomas and c-*erb*B-2 negative small celled cribriform/micropapillary carcinomas with a low proliferative rate, there is a third group of c-*erb*B-2 negative tumours with large nuclei and variable proliferative activity. These latter tumours are not seen in pure DCIS suggesting that they have a very transient *in situ* stage. Therefore, although in pure DCIS c-*erb*B-2 positivity appears to be associated with tumours with a greater invasive potential, and c-*erb*B-2 negativity with tumours having a more favourable prognosis, the latter is not necessarily true in infiltrating disease.
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INTRODUCTION

AMPLIFICATION OF the c-*erb*B-2 oncogene and overexpression of the oncoprotein have been associated with poor prognosis in patients with infiltrating breast carcinoma [1]. Early papers reported a 35% difference in survival at 4 years for node positive patients with c-*erb*B-2 positive tumours [1]. This finding was emphasised in later studies with large numbers of patients [2–5]. Other smaller studies on fewer than 200 patients failed to show any significant difference, suggesting that any prognostic significance was weak [6]. Although the majority of studies which have included a large number of patients do find an association between c-*erb*B-2 oncogene enhancement and poor prognosis, a recent study by Clark and McGuire [7], of 362 patients failed to do so.

Other inconsistencies in the relationship between c-*erb*B-2 overexpression and mammary carcinoma are related to its correlation with tumour type. Whilst in studies of infiltrating carcinoma the proportion of tumours showing overexpression has ranged from 10 to 30% [8, 1] in carcinoma *in situ* the incidence of overexpression is much higher, in the region of 60% [9–11]. We have undertaken several studies of c-*erb*B-2 overexpression in mammary ductal carcinoma and have been particularly impressed by this latter finding [12, 13]. We, therefore, undertook the present study in order to investigate this further by reviewing our previous work, which concentrated on *in situ* tumours (studies 1 and 2), and by carrying out a new study of the detailed morphology of infiltrating ductal carcinomas (study 3).