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Relationship of transforming growth factor β_1 to extracellular matrix and stromal infiltrates in invasive breast carcinoma

R.A. Walker, S.J. Dearing & B. Gallacher

Breast Cancer Research Unit, University of Leicester, Clinical Sciences, Glenfield General Hospital, Groby Road, Leicester LE3 9QP, UK.

Summary Transforming growth factor β (TGF-β) comprises a group of multifunctional regulatory proteins, whose effects include stimulation of extracellular matrix formation and modification of immune function. The presence of TGF-β_1 and TGF-β_2 in invasive breast carcinomas has been determined and related to pathological features, the presence of fibronectin and tenascin and lymphocyte/macrophage infiltration, using immunohistochemistry. Differences were observed in the extent of reactivity within the same carcinoma and between tumours stained with an antibody detecting TGF-β_1 and one detecting TGF-β_1 plus TGF-β_2, the latter having a higher level of reactivity. Prominent reactivity for TGF-β_1 was associated with lymph node metastasis, ($0.02 > P > 0.01$), increased detection of cellular fibronectin, fine stromal fibronectin staining, more prominent reactivity for tenascin ($0.02 > P > 0.01$), the presence of tumour-associated macrophage infiltration and altered ratios of CD4 and CD8 lymphocyte populations, with CD8 lymphocytes predominating. These associations were not observed for carcinomas showing prominent staining with antibody detecting TGF-β_2 as well as TGF-β_1. The findings indicate that TGF-β_1 may have a role in invasion and metastasis of breast carcinomas.

Transforming growth factor β (TGF-β) comprises a group of multifunctional regulatory proteins which have many effects on physiological and pathological processes (Roberts *et al.*, 1988). To date five TGF-β isotypes have been recognised: TGF-β_1, TGF-β_2 and TGF-β_3 are found in mammalian tissues (Derynck *et al.*, 1985, 1988; de Martin *et al.*, 1987), TGF-β_4 in avian (Jakowlew *et al.*, 1988) and TGF-β_5 in *Xenopus* (Kondaiah *et al.*, 1990). The mature forms of TGF-β_1, -β_2 and -β_3 show 70–80% homology at the amino acid level.

TGF-β can both stimulate and inhibit cell proliferation, the effect depending on the type of cell involved. It can block or effect entry into differentiation pathways. Extracellular matrix formation can be stimulated and cell migration either promoted or inhibited (Barnard *et al.*, 1990). The promotion of extracellular matrix formation is effected by several mechanisms: stimulation of synthesis of type I collagen and fibronectin (Ignotz & Massague, 1986) and tenascin (Pearson *et al.*, 1988); inhibition of proteinase synthesis and stimulation of proteinase inhibitor synthesis (Edwards *et al.*, 1987). TGF-β also has effects on immune function, suppressing the growth of T and B lymphocytes (Kehrl *et al.*, 1986*a* and *b*) and modifying the function of macrophages (Tsunawaki *et al.*, 1988).

The stroma of carcinomas differs from that of comparable normal organs and is believed to be an important factor in malignant growth (Van der Hooff, 1988). Abundant fibronectin can be identified in the stroma of many breast carcinomas, and the pattern of distribution correlates with metastatic potential (Christensen *et al.*, 1989). Tenascin is also highly expressed in the stroma of malignant but not benign breast tumours (Mackie *et al.*, 1987). The breast cancer cell line MCF-7 reacts to exogenous tenascin by adopting an invasive phenotype, losing cell–cell and cell–substrate contacts (Chiquet-Ehrismann *et al.*, 1989). The pattern and extent of lymphocyte and macrophage infiltrate in breast carcinomas may be of significance in relation to tumour behaviour (Vose & Moore, 1985). We have previously identified an association between high numbers of activated macrophages in breast cancers and lymph node metastasis (Zuk & Walker, 1987).

In a previous immunohistochemical study of TGF-β_1, in *in situ* and invasive breast carcinomas, a significant difference was noted, with fewer *in situ* carcinomas having detectable TGF-β_1 (Walker & Dearing, 1992). This suggested that TGF-β_1 may play a role in invasion. The present study has considered TGF-β in invasive carcinomas in relation to stromal components, lymphocyte/macrophage infiltrates and tumour characteristics to consider further the potential role of TGF-β in invasion and metastasis.

Correspondence: R.A. Walker.
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Materials and methods

Tissues

Tissue from 86 invasive breast carcinomas were studied. All specimens had been received fresh immediately after surgery and samples frozen in liquid nitrogen with a parallel block fixed in 4% formaldehyde in saline for 18–36 h prior to processing through paraffin wax.

Antibodies

Two antibodies directed against TGF-β were used. One was an affinity-purified polyclonal antiserum to TGF-β_1 (a gift from Professor Marc Feldmann, Sunley Research Centre, Charing Cross, London, UK). It had been raised against human TGF-β_1, and specificity for TGF-β_1 but not for -β_2 or -β_3 had been confirmed by enzyme-linked immunoabsorbent assay (ELISA) immunoprecipitation and Western blotting (Chantry *et al.*, 1989). The other antibody was obtained from Genzyme and was a mouse monoclonal. It had been raised against bovine TGF-β_2 and recognised bovine and human TGF-β_1 and TGF-β_2 as well as *Xenopus* TGF-β_2 and chick TGF-β_3.

Monoclonal antibodies to human fibronectin (clone FN-15) and cellular fibronectin (FN-3E2) were from Sigma. The human fibronectin antibody was raised against fibronectin from human plasma; specificity had been confirmed by ELISA and Western blotting. The cellular fibronectin antibody was raised against fibronectin released from a breast cancer cell line and localised to the 240 kDa band of cellular fibronectin on Western blotting. The monoclonal antibody to tenascin (clone EB2) was raised against purified tenascin from fetal fibroblasts and was obtained from ICN. On Western blotting it reacts with tenascin polypeptides of 250 and 180 kDa.

Two CD68 monoclonal antibodies against human macrophages, PGM1 and EBM11 (Dako), were used. For T-lymphocyte detection the monoclonal antibodies (UCHTI (CD3) MT310 (CD4) and DK25 (CD8) (all Dako) were employed, with To15 (pan-B) (Dako) monoclonal antibody for B-lymphocyte detection.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were used for the detection of TGF-β, fibronectin and macrophages using PGM1. Frozen sections were used for the detection of tenascin, T and B lymphocytes and macrophages using EBM11.

The antiserum to TGF-β_1 was applied to 58 carcinomas at a concentration of 3.6 $\mu g\ ml^{-1}$ for 18 h at 4°C. After rinsing and washing in Tris-buffered saline, biotinylated swine anti-rabbit immunoglobulin serum was applied, followed by streptavidin–biotin–peroxidase complex. The peroxidase was developed using diaminobenzidene–hydrogen peroxide. Pre-immune rabbit serum was used as a control. The antibody against TGF-β_1 and TGF-β_2 was applied to 38 carcinomas, ten of which had been assessed with the TGF-β_1 antiserum. It was used at a 1:20 dilution with incubation for 18 h at 4°C. The same technique was used, but with a biotinylated rabbit anti-mouse immunoglobulin serum.

For the detection of fibronectin sections were digested with 0.025% pepsin (Sigma) in 0.01 M hydrochloric acid at 37°C for 45 min prior to the application of both antibodies. These were used at 1:50 dilution with the same technique as above. For the detection of macrophages in formalin-fixed, paraffin-embedded sections, digestion with 0.1% trypsin pH 7.8 for 20 min at 37°C was used with PGM1 at 1:100 dilution, and the streptavidin–biotin technique.

All frozen sections were fixed in cold acetone for 10 min prior to the application of the primary antibodies as described previously (Zuk & Walker, 1987; Jones *et al.*, 1992). Sufficient frozen material for the detection of CD4 and CD8 lymphocytes was available for 80 cases and for tenascin for 71 cases. The streptavidin–biotin complex technique was used throughout. Controls in all instances were the omission of the primary antibody.

Clinicopathological features

Haematoxylin and eosin-stained sections of all carcinomas were assessed for type and for histological grade, using the modified Bloom and Richardson system (Elston, 1987). Lymph node status was known for 75 cases.

Statistical analysis was by chi-square or Fisher's exact test.

Results

TGF-β reactivity

The staining of the carcinomas was classified as negative; having less than 10% positive cells; between 10 and 50% positive cells; and more than 50% positive cells. Differences were observed in the extent of reactivity with the two antibodies. The results are summarised in Table I. Staining of stroma with occasional staining of fibroblasts but without reactivity of tumour cells was only observed with the TGF-β_1 antiserum. The staining pattern with this antiserum differed in other respects, in that normal epithelium showed no staining or very weak reactivity, while tumour cell reactivity was prominent (Figure 1). This differed from the staining observed with the antibody detecting TGF-β_1 and TGF-β_2 in that normal epithelium was reactive, and the staining of tumour cells was generally of similar intensity. Of the ten carcinomas stained with both antibodies, four were negative with TGF-β_1 antibody but had 10–50% positive cells with the antibody against TGF-β_1 and TGF-β_2 (Figure 2), four

Table I Comparison of the extent of staining observed in breast carcinomas with the antibodies against TGF-β_1 and TGF-β_1 and -β_2

Reactivity	*TGF-β_1*	*TGF-β_1 and TGF-β_2*
Negative	20 (34.5%)	3 (11%)
Stromal only	7 (12%)	0
< 10% positive cells	10 (17.25%)	7 (25%)
10–50% positive cells	10 (17.25%)	9 (32%)
> 50% positive cells	11 (19%)	9 (32%)

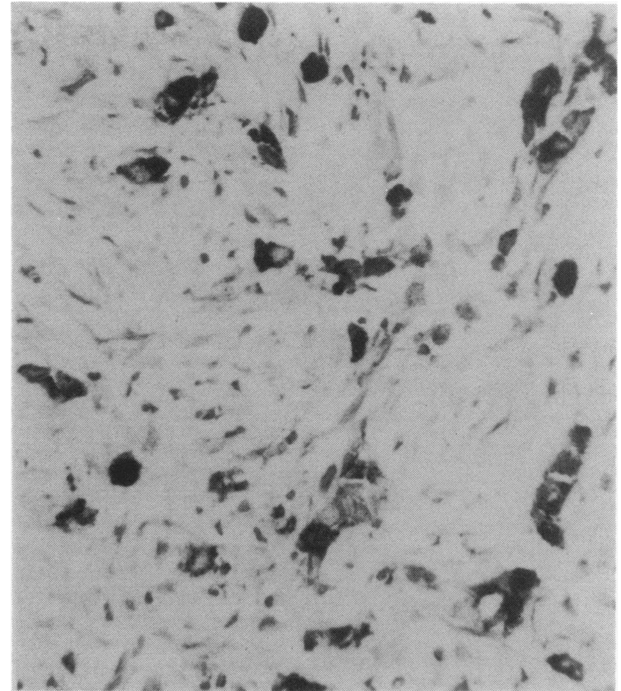

Figure 1 Infiltrating ductal carcinoma showing prominent staining for TGF-β_1 protein.

had less than 10% cells positive for TGF-β_1 but between 10 and 50% positive for TGF-β_1 and -β_2, and two had between 10 and 50% cells positive with TGF-β_1 antibody but more than 50% cells staining with the antibody against TGF-β_1 and TGF-β_2.

Apart from the small number of cases with stromal staining in which there was fibroblast reactivity, there was no staining of stromal cells such as macrophages.

Relationship to clinicopathological parameters

Seventy-five of the carcinomas were infiltrating ductal and 11 infiltrating lobular carcinomas. No differences were observed between the two categories with either antibody. There were ten well-differentiated carcinomas, 44 moderately differentiated and 32 poorly differentiated carcinomas. There was no relationship between staining and histological grade for either antibody.

Forty-five carcinomas had metastasised to lymph nodes and 30 had not. All of the carcinomas with > 50% of cells positive for TGF-β_1 had metastasised, which was significant ($0.02 > P > 0.01$). The distribution of node-positive and -negative cases for the other staining categories of TGF-β_1 was as expected, as was the distribution for all staining categories for TGF-β_1 and -β_2.

Relationship with stromal components

Cellular fibronectin was detected in tumour cells in 30 (35%) carcinomas (Figure 3). The extent of reactivity ranged from 10% to 80% of cells being positive, with associated lesser stromal reactivity. The other fibronectin antibody detected the stromal component, with cellular staining being seen much less frequently. The pattern of staining was predominantly of coarse bands, but in 13 carcinomas only fine irregular stromal staining was seen, and in a further 13 both coarse and fine stromal staining was observed. The comparison between fibronectin reactivity and staining for TGF-β is shown in Table II. The presence of cellular fibronectin was greater in those cases with more prominent reactivity for TGF-β_1 and TGF-β_1 plus TGF-β_2. A greater degree of fine stromal reactivity for fibronectin was seen in cases with more prominent reactivity for TGF-β_1.

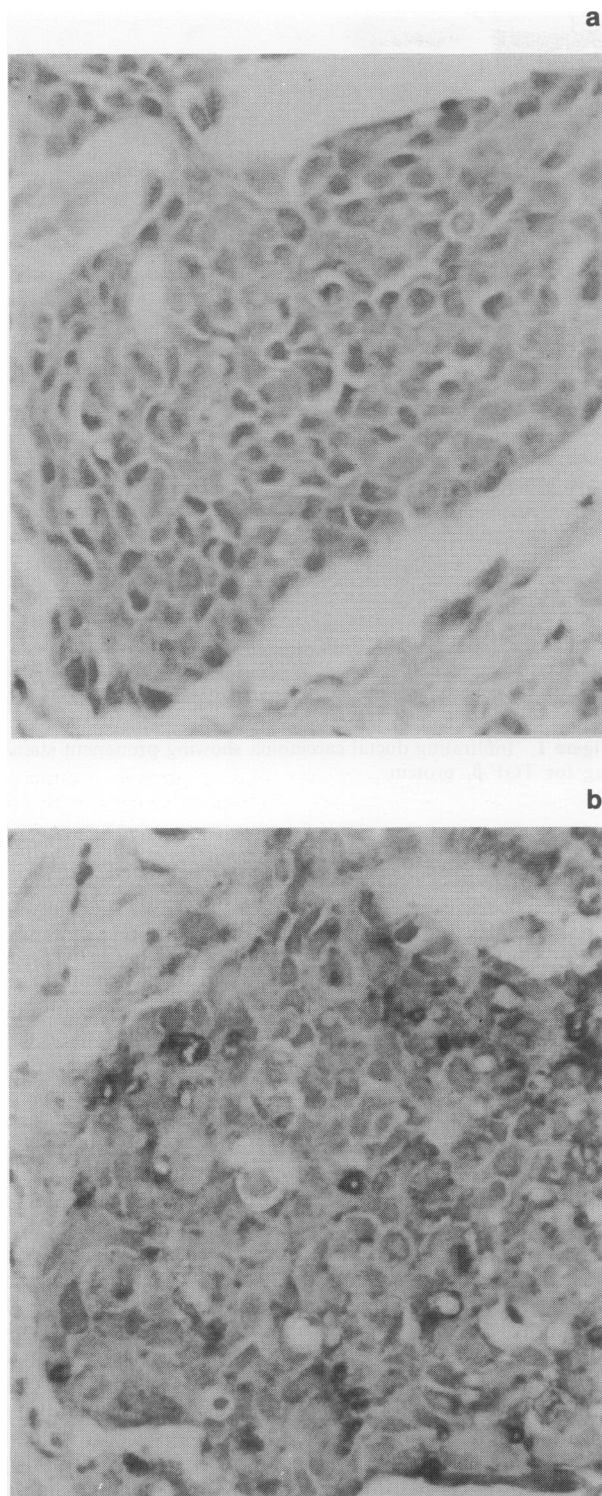

Figure 2 **a**, Infiltrating ductal carcinoma showing no reactivity with antibody against TGF-β_1. **b**, Serial section of the same carcinoma showing staining of tumour cells with antibody against TGF-β_1 and TGF-β_2.

The extent of staining for tenascin was subdivided into marked (+ + +), moderate (+ +) or scanty (+), as described previously (Jones *et al.*, 1992) (Figure 4). Marked reactivity was seen in 31 carcinomas, moderate in 29 and scanty in 11. The degree of staining in comparison with TGF-β reactivity is shown in Table III. Marked reactivity for tenascin was seen in almost all carcinomas with prominent staining for TGF-β_1, and was significant ($0.02 > P > 0.01$), but no relationship was observed for staining with the antibody against TGF-β_1 and TGF-β_2.

Relationship with macrophage/lymphocytic infiltration

There was generally a greater number of cells staining in the frozen sections incubated with EBM 11 than the fixed sections reacted with PGM1, and in all cases the higher level of macrophage staining was taken for comparisons. Macrophages were seen either within the stroma or within and closely abutting tumour cell groups, subsequently called tumour associated. The extent of macrophage infiltration and whether it was stromal and/or tumour associated were related to the degree of TGF-β reactivity within carcinomas. The extent of reactivity did not relate to TGF-β staining. Stromal macrophage reactivity only was seen in 35 carcinomas (41%), with stromal macrophage numbers being greater than tumour associated in 16 tumours (18.5%). In 25 carcinomas (29%) there was equal reactivity for stromal and tumour-associated macrophages. Only two tumours had

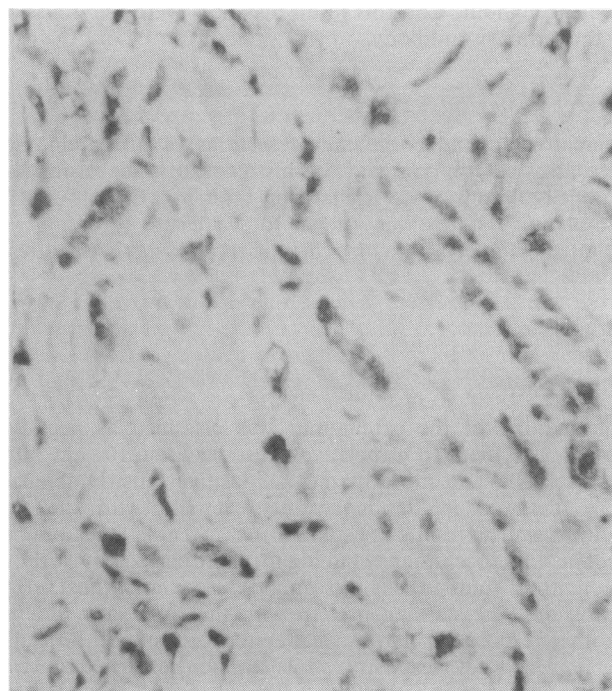

Figure 3 Infiltrating ductal carcinoma with many tumour cells showing staining for cellular fibronectin.

Table II The pattern and/or extent of reactivity of stromal components in relation to the extent of staining for TGF-β obtained with the two antibodies (n = number postive/total)

TGF-β reactivity	*Cellular fibronectin*		*Coarse stromal fibronectin*		*Fine stromal fibronectin*	
	TGF-β_1	*TGF-β_1 + TGF-β_2*	*TGF-β_1*	*TGF-β_1 + TGF-β_2*	*TGF-β_1*	*TGF-β_1 + TGF-β_2*
Negative	6/20	0/3	15/20	1/3	5/20	1/3
Stromal only	1/7	0	4/7	0	3/7	0
$<10\%$	2/10	0/7	3/10	4/7	3/10	1/7
10–50%	4/10	3/9	4/10	7/9	6/10	2/9
$>50\%$	8/11	6/9	4/11	7/9	6/11	0/9

Table III Extent of tenascin reactivity in comparison with TGF-β staining

TGF-β reactivity	Marked tenascin TGF-β_1	Marked tenascin TGF-β_1 + -β_2	Moderate tenascin TGF-β_1	Moderate tenascin TGF-β_1 + -β_2	Scanty tenascin TGF-β_1	Scanty tenascin TGF-β_1 + -β_2
Negative	5/17	1/3	6/17	1/3	6/17	1/3
Stromal only	3/6	0	3/6	0	0/6	0
$<$10%	1/8	3/6	5/8	3/6	2/8	0/6
10–50%	3/6	5/9	3/6	4/9	0/6	0/9
$>$50%	8/9	2/7	1/9	3/7	0/9	2/7

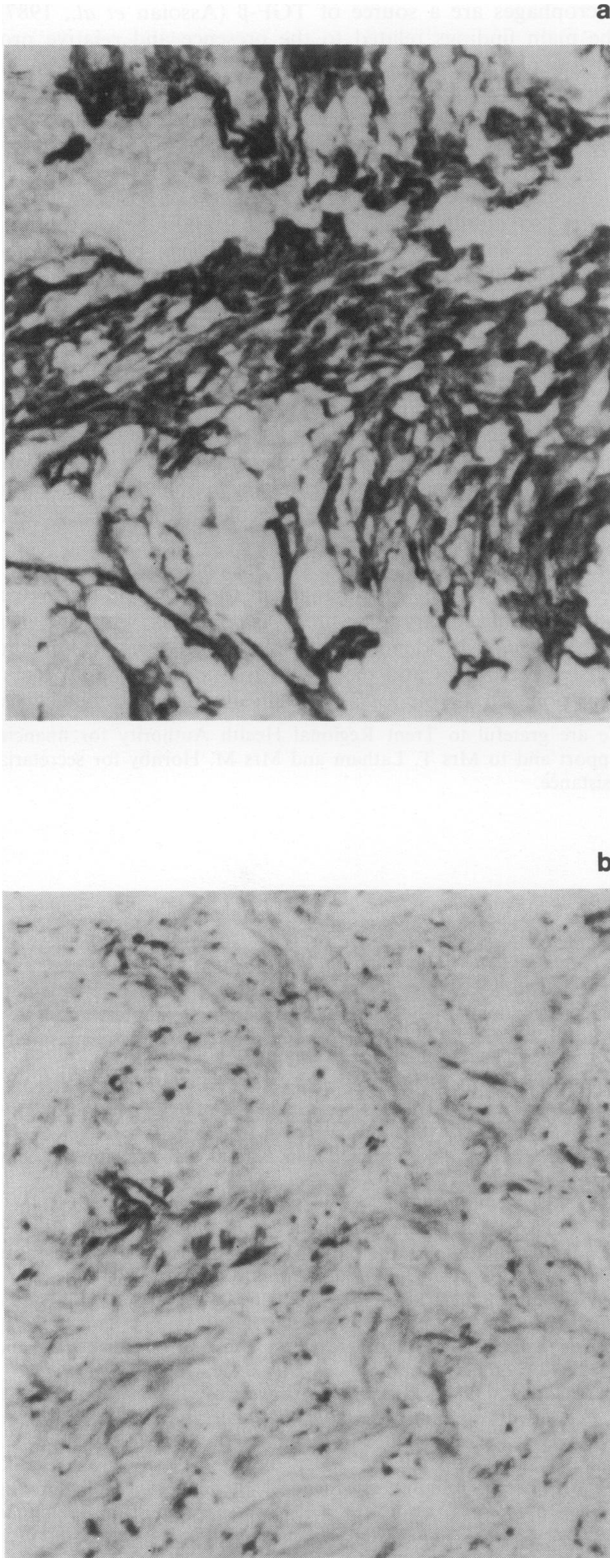

Figure 4 **a**, Frozen section of carcinoma showing prominent staining for tenascin. **b**, Minimal reactivity for tenascin in frozen section of another infiltrating ductal carcinoma.

more prominent tumour-associated macrophage staining, and eight carcinomas had this as the only pattern of macrophage staining. Stromal macrophage staining only or greater stromal macrophage reactivity was seen in over half the carcinomas in each TGF-β staining category apart from those carcinomas with prominent staining for TGF-β_1 in which two showed only stromal staining, two only tumour-associated macrophage staining and the remaining seven equal reactivity for stromal and tumour-associated macrophages.

B-lymphocyte reactivity was minimal in the majority of carcinomas studied, and showed no correlation with TGF-β reactivity. The numbers of T lymphocytes overall varied between the carcinomas, and this did not relate to TGF-β reactivity. The extent of the CD4- and CD8-positive lymphocytes did vary and the results are shown in Table IV. Two-thirds of the carcinomas had a greater number of CD4-positive lymphocytes than CD8-positive cells, with 16% having equal numbers and 20% a greater number of CD8-positive cells. Of those carcinomas with prominent TGF-β_1 reactivity, there were two-thirds with greater CD8 reactivity.

Discussion

In a previous immunohistochemical study of TGF-β (Walker & Dearing, 1992) we identified a difference in detection of TGF-β_1 between *in situ* and invasive carcinomas, indicating a role for TGF-β_1 in invasion. The present study has shown that any relationship between TGF-β and invasion and metastasis is only found for TGF-β_1 and not for TGF-β_2. This is in keeping with the findings of Gorsch *et al.* (1992), who identified a relationship between immunoreactivity for TGF-β_1 and disease progression in human breast carcinoma. It also reinforces the view of Arteaga and Coffey (1992), based on the study of McCune *et al.* (1992), that it is important to consider the different isoforms of TGF-β since they clearly do have different roles.

Because of the availability of antisera the number of cases which could be examined for TGF-β_1 was restricted. Comparison of staining in individual cases, and of the extent of reactivity in other cases, showed that there was greater reactivity using an antibody detecting both TGF-β_1 and TGF-β_2. Because the results obtained were clearly less significant, staining with this antibody was not pursued.

Prominent reactivity for TGF-β_1 was associated with nodal metastasis, higher frequency of detection of cellular fibronectin, different patterns of reactivity of stromal fibronectin, marked tenascin reactivity, higher frequency of macrophage infiltration being tumour associated and different levels of CD8 lymphocyte infiltrates in comparison with CD4. The various correlates were not restricted to tumours with prominent TGF-β_1, and not all tumours having that pattern of TGF-β_1 reactivity showed them, but there were obvious associations. Further studies using a monospecific reagent are needed to consolidate these findings.

Studies of rat mammary adenocarcinoma cells have shown that exogenous TGF-β_1 may modulate the metastatic potential of mammary tumour cells by controlling their ability to break down and penetrate basement membrane barriers (Welch *et al.*, 1990). The TGF-β_1 secreted from tumour cells could have the same effect, providing it is biologically active. This can only be determined by *in vitro* assays. Mizoi *et al.*

Table IV The extent of CD4 and CD8 reactivity in carcinomas in comparison with TGF-β staining, with both antibodies

TGF-β *reactivity*	CD4 > CD8 *TGF-β_1*	*TGF-β_1 + -β_2*	CD4 = CD8 *TGF-β_1*	*TGF-β_1 + -β_2*	CD8 > CD4 *TGF-β_1*	*TGF-β_1 + -β_2*
Negative	13	2	2	0	3	1
Stromal only	3	0	3	0	0	0
$<10\%$	8	3	0	4	1	0
10–50%	6	5	2	2	1	2
$>50\%$	4	7	0	1	7	1

(1993) have demonstrated in gastrointestinal carcinomas that the precursor form of TGF-β_1 is within the cytosol of tumour cells, which may suggest blocked transport. Further studies, preferably dynamic, would be required to determine whether this is the situation in breast carcinomas.

Differences in stromal and cellular fibronectin were observed relating to TGF-β_1. Previous immunohistochemical studies of stromal fibronectin have described pericellular reactivity, particularly at the invasive border, as well as a diffuse staining pattern (Christensen *et al.*, 1989). Pericellular staining was rarely seen, the more striking difference in the present study being the presence of fine stromal staining. Cytoplasmic fibronectin has previously been reported to be related to the degree of anaplasia, and more striking in independently growing breast cancer cells (Christensen *et al.*, 1985). The other extracellular matrix protein studied, tenascin, was readily identified in the stroma of the breast carcinomas, as previously reported (Mackie *et al.*, 1987; Natali *et al.*, 1991; Jones *et al.*, 1992). Tenascin is induced by TGF-β *in vitro* (Pearson *et al.*, 1988). Tenascin can block the action of fibronectin (Chiquet-Ehrismann *et al.*, 1988), inhibiting cell attachment. *In vitro* addition of tenascin to MCF-7 breast cancer cell lines results in their loss of cell–cell and cell–substrate contacts (Chiquet-Ehrismann *et al.*, 1989). If the same occurs in primary breast carcinomas *in vivo*, it could be proposed that the overexpression of TGF-β_1 stimulates synthesis of tenascin, which aids invasion and hence metastasis.

We were unable to detect TGF-β in macrophages within the breast carcinomas, although in other sites, such as lung, macrophages are a source of TGF-β (Assoian *et al.*, 1987). The main findings related to the presence and relative proportion of tumour-associated macrophages, which were increased in relation to greater TGF-β_1 expression. In other tissues TGF-β is a potent chemoattractant for macrophages. The function of macrophages within breast carcinomas could be as a host defence mechanism or the converse owing to release of enzymes involved in destruction of basement membranes, so aiding invasion. An association between nodal metastasis and macrophage infiltration has been observed (Zuk & Walker, 1987).

No differences were found in the numbers of B and T lymphocytes in relation to TGF-β reactivity, but an alteration in the ratio of CD4 to CD8 cells was seen. As in a previous study (Zuk & Walker, 1987) CD4 lymphocytes predominated in many of the carcinomas, apart from those with prominent TGF-β_1 reactivity. Naukkarinen and Syrjanen (1990) identified an association between CD8 lymphocytic infiltration and post-capillary venule endothelium in breast carcinomas. TGF-β_1 has a role in angiogenesis and may account for this association.

Prominent expression of TGF-β_1 but not TGF-β_2 is therefore associated with changes in the extracellular matrix and in stromal infiltrates in breast carcinomas, which in view of the previously identified differences between *in situ* and invasive carcinoma and the higher frequency of nodal metastasis points to a role for TGF-β_1 in invasion and metastasis.

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