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Advances in Brief

Immunohistochemical Staining for Transforming Growth Factor β_1 Associates with Disease Progression in Human Breast Cancer[1]

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Abstract

The transforming growth factor βs (TGF-β) comprise a family of M_r 25,000 pluripotent growth factors which have been implicated in the development and progression of human breast cancer. Conflicting data suggest that TGF-β has the potential to either inhibit or promote the progression of mammary neoplasia. We therefore examined a pathological library of malignant breast biopsy specimens to determine the prevalance and distribution of immunoreactivity with antibodies specific for the three mammalian isoforms of TGF-β (β_1, β_2, and β_3). We found that intense staining for TGF-β_1 was positively associated with rate of disease progression, and that this was independent of age, stage, nodal status, or estrogen receptor status ($P = 0.009$).

Introduction

An accumulating body of evidence suggests that factors which regulate normal growth and cellular differentiation are fundamentally involved in the processes of malignant transformation and metastatic progression in human breast cancer (1). TGF-β is one such protein, and evidence suggests that it may play a central role in mammary neoplasia. The human TGF-β family consists of three highly homologous M_r 25,000 proteins which are usually secreted as part of a larger biologically inactive complex. Once activated by release from the latent complex, the three TGF-β isoforms exhibit similar effects, and have been implicated in the regulation of diverse processes, including cell proliferation, wound repair, angiogenesis, and immunosuppression (2). Synthesis and secretion of TGF-β by breast cancer cells has been documented to occur both *in vitro* and *in vivo* (3, 4). For the majority of human breast cancer cell lines, TGF-β inhibits proliferation (5). Reports that growth-inhibitory antiestrogens can increase the production of TGF-β_1 in hormonally responsive cell lines (3), and that estradiol (6) and norethindrone (7) can inhibit the expression of TGF-β_2 and -β_3, concomitant with growth stimulation, have prompted speculation that TGF-β may act as a negative autocrine growth factor which mediates the effects of hormonal therapy on breast cancer. On the other hand, several studies support a view of TGF-β which favors net progression of the transformed phenotype. These studies demonstrate that TGF-β_1 mRNA is increased in transformed as opposed to normal breast epithelium (8), and that TGF-β increases the metastatic potential of mammary tumor cells (9). Possible mechanisms by which TGF-β may facilitate the progression of tumor growth include immunosuppression, angiogenesis, and changes in the extracellular matrix (10–16).

Hitherto, the majority of studies examining the role of TGF-β in breast cancer have focused on tissue culture studies. Information gleaned from analysis of cell lines might not properly reflect the *in vivo* actions of TGF-β in the pathogenesis of breast cancer. We therefore undertook a retrospective immunohistochemical study of breast biopsy material in order to characterize the pattern of TGF-β isoform expression in malignant breast lesions and to determine whether expression of a particular isoform was related to disease progression.

Materials and Methods

Tissues. Tissues were obtained from the Dartmouth Hitchcock Medical Center pathology archive. Sequential breast biopsy specimens fixed in acetone-methyl benzoate-xylene, beginning in 1987 and continuing through the end of 1990 were selected for sectioning. The distribution of cases is weighted toward the latter part of this period as a reflection of the increased frequency with which breast tissue was processed by this method, beginning in 1989.

Immunohistochemistry. Isoform-specific polyclonal rabbit antisera were developed in the laboratory of Dr. L. Gold by immunization with synthetic peptides representing amino acid residues 4–19 of TGF-β_1 and TGF-β_2 and residues 9–20 of TGF-β_3. The specificity of these antibodies for the 3 isoforms of TGF-β was established by Western blot analysis utilizing intact native and recombinant human TGF-β_1, -β_2, and -β_3, and blocking of immunoreactivity by their respective immunizing peptides (17). Immunohistochemistry was performed in accordance with the directions supplied with the Vectostain Kit (Vector Laboratories). Briefly, tissue sections were deparaffinized in xylene, hydrated in phosphate buffered saline, and blocked with normal goat serum. Slides were incubated with primary antibody at 4°C for 16 h. The TGF-β isoform-specific antibodies were at 5 μg/ml. The following day, the tissue sections were incubated with biotinylated anti-rabbit antibody, followed by exposure to preformed avidin/biotinylated peroxidase complex. Sections were then developed with diaminobenzidine and hydrogen peroxide, which produces a brown precipitate. Sections were then counterstained with hematoxylin, dehydrated, and mounted. Primary antibody controls were negative.

Scoring. Stained sections were graded by two independent observers blinded to patient status on a scale of 0–2 *vis-à-vis* intensity of background staining (see Fig. 1). Differences were resolved by joint review and consultation with a third observer experienced in immunohistochemical pathology. Heterogeneity in the intensity of tumor cell staining within samples, although infrequently noted, was not included in the determination of the score.

Data Abstraction. The following data were obtained by chart review of corresponding patients: age and tumor stage at time of biopsy, quantitative estrogen and progesterone receptor status, nodal status, histological subtype, and time to progression in months (defined as tumor recurrence, progression, or cancer-related death) or disease-free survival for those who did not progress. Duration of disease-free survival was determined by using the date of the last clinic visit recorded in the chart.

Statistical Analysis. Kaplan-Meier survival curves (18) were computed for progression-free survival by staining intensity group (low *versus* high) for each of the three isoforms of TGF-β. We used the

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[3] The abbreviation used is: TGF-β, transforming growth factor β.

log-rank method to assess the univariate association between rate of disease progression and staining intensity. We used a Cox proportional-hazards model (19) to study the same relationship, adjusted for the known risk factors of patient age (pre- *versus* postmenopausal), stage of disease, estrogen receptor level (negative *versus* positive), and number of positive nodes. Point estimates of the disease progression rate ratios and confidence intervals were computed by transforming the corresponding regression estimates. The statistical package SAS procedure PHREG was used to perform all computations (SAS Institute, Inc., Cary, NC).

Results

Initially, 42 specimens were stained for immunoreactivity to all three TGF-β isoforms. A few biopsies with benign histology were examined as part of this group of specimens, and differences as well as similarities in isoform-specific staining patterns were noted. For instance, staining with the anti-TGF-β_1 antibody was most evident in the cytoplasm of the ductal epithelium, with prominence of the cytoplasmic membrane. By contrast, anti-TGF-β_2, while having some affinity for epithelial cells, manifested primarily by staining of stromal fibroblasts and the luminal surfaces of ducts. Anti-TGF-β_3 antibody was noted to stain myoepithelial supporting structures (Fig. 1) in addition to stromal fibroblasts. Vascular smooth muscle and endothelia demonstrated significant reactivity with TGF-β_1 and -β_2 antisera. Compared to our small sample of benign breast epithelium, malignant cells demonstrated greater variability in the intensity of immunoreactivity for the TGF-β isoforms. Staining of tumor cells tended to be cytoplasmic, although TGF-β_2 was occasionally noted to stain nuclei.

The association between intensity of immunoreactivity with each of the isoform-specific anti-TGF-β antibodies and clinical outcome was studied for those patients in whom the biopsy was interpreted as malignant (N = 30). Univariate analysis suggested that staining with antisera to TGF-β_2 and -β_3 bore no relation to patient outcome (log-rank P = 0.75 and 0.95, respectively). Kaplan-Meir curves of progression-free survival by staining intensity group (low *versus* high) are plotted for anti-TGF-β_2 and -β_3 isoforms (Fig. 2). In contrast, an association was apparent between intensity of staining with the anti-TGF-β_1 antibody and disease progression, defined as tumor recurrence, progression, or cancer-related death (plot not shown). Based on this observation, we proceeded to evaluate an additional 31 biopsy specimens for immunoreactivity with anti-TGF-β_1. Of a total of 73 cases, 16 were excluded from further analysis because 5 were pathologically benign, 4 had a second primary malignancy, 3 for inadequate or missing pathological specimen, 2 had no follow up information, and 2 were male. Fifty-seven remained available for study. The majority of these tumors were histologically classified as infiltrating ductal carcinomas. However, there were 3 colloid carcinomas, 2 tubular carcinomas, 2 infiltrating lobular, and 1 poorly differentiated mucin-producing adenocarcinoma. One specimen was classified as ductal atypia, but shortly thereafter this patient presented with frank carcinoma in the ipsilateral breast, and was included in the analysis.

Fig. 3 displays a comparison of progression-free survival of patients whose specimens possessed intense immunoreactivity for TGF-β_1 *versus* patients with biopsy specimens that exhibited minimal staining with this antibody (N = 57). A striking univariate association between these parameters was evident (log-rank P = 0.07). A multivariate analysis, restricted to 40 patients for whom all risk factor data were complete, was performed adjusting for age, stage, estrogen receptor status, and involvement of axillary lymph nodes. Table 1 reports the clinical characteristics of these study patients. Patients ranged in age from 34 to 86 years (mean = 60). The association between the intensity of TGF-β_1 immunoreactivity and disease progression was statistically significant and independent of established prognostic variables (P = 0.009; progression rate ratio, 64,500; 95% confidence interval, 16 to 2×10^8). The adjusted rate ratios for TGF-β_2 and -β_3 were not significant (P = 0.28 and 0.24, respectively).

Discussion

Despite the abundance of data implicating TGF-β in the growth and regulation of breast cancer, few studies have directly addressed its role *in vivo*. The ability to examine paraffin-embedded histological sections obtained from primary breast cancer tissue provides an avenue for the exploration of the potential pathophysiological importance of TGF-β in mammary

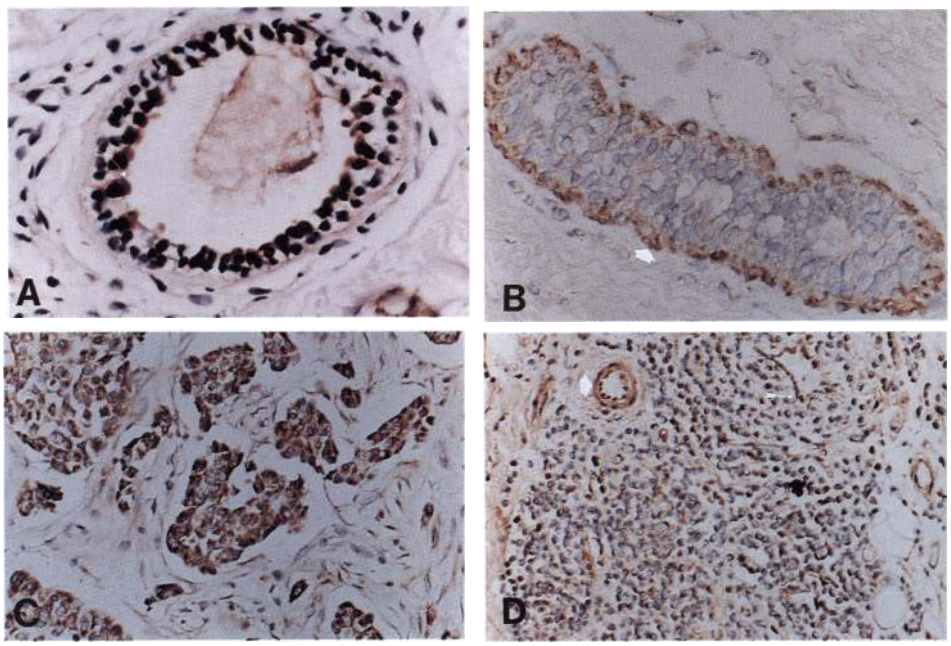

Fig. 1. *A*, specimen stained with TGF-β_2 antisera demonstrating immunoreactivity of the apical surface of ductal cells (*curved arrow*). *B*, this specimen stained for TGF-β_3 shows prominent myoepithelial staining (*blunt arrow*). *C*, specimen taken from a patient with stage II breast cancer who developed progressive disease at 19 months, demonstrating intense cytoplasmic reactivity for TGF-β_1 (score = 2). By contrast, *D* shows light TGF-β_1 reactivity (score = 1) in a patient with stage I breast cancer who remains disease-free at 23 months. Note the staining of the arteriolar smooth muscle in the *upper left* (*blunt arrow*), and venular endothelium in the *upper right* (*tailed arrow*). *A*, *B*, *C*, and *D*, × 1200, × 660, × 660, and × 330, respectively.

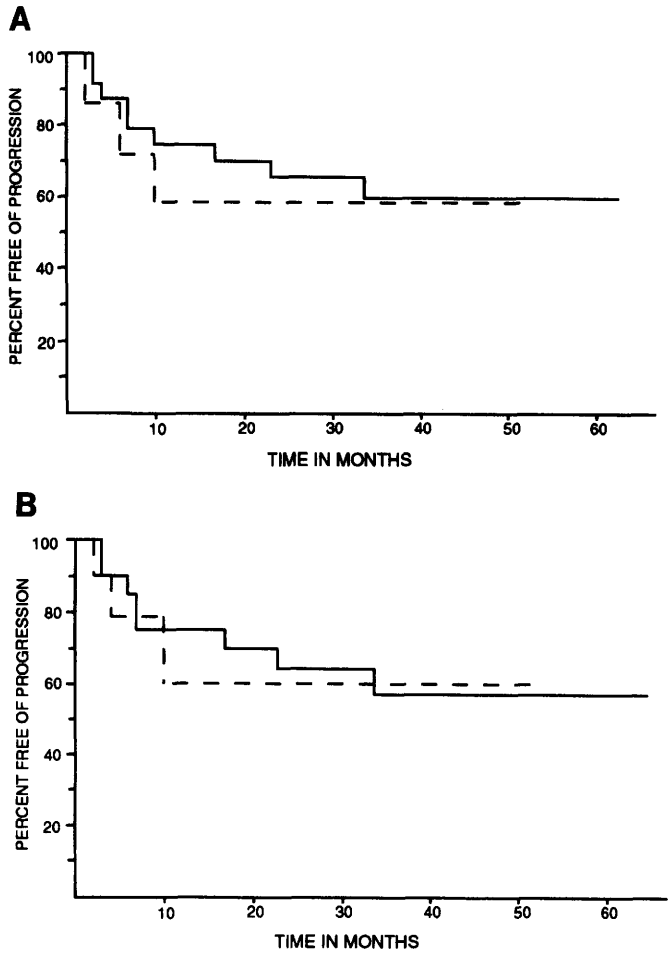

Fig. 2. Kaplan-Meier plots of disease progression in patients whose tumors exhibited minimal staining (score = 0 or 1, ——) with anti-TGF-β_2 (*top*; $N = 23$) or anti-TGF-β_3 (*bottom*; $N = 20$) antibody *versus* patients with tumors showing intense staining with those antibodies (score = 2, – – –; $N = 7$ and 10, respectively). *P* values are not significant. The median follow-up is currently 32 months.

neoplasia. We have evaluated a pathological library of breast biopsy specimens for immunoreactivity to each of the three known isoforms of TGF-β. Our analysis has identified a striking association between the presence of aggressive disease and intense staining for TGF-β_1. Interestingly, this association was not evident for TGF-β_2 or -β_3 immunoreactivity. Since the three isoforms of TGF-β share a similar spectrum of biological activities, the suggestion that only TGF-β_1 was associated with disease progression is of considerable interest. Possible explanations include the preferential activation of TGF-β_1 from its latent complex, a phenomenon recently described for TGF-β_2 (20), or an effect of TGF-β mediated by the β_1 isoform with far greater potency, compared to the β_2 and β_3 isoforms.

Recently, other investigators have reported on the clinical correlates of anti-TGF-β_1 immunohistochemical reactivity in breast cancer specimens. Walker and Dearing (21) observed that staining for TGF-β_1 in primary breast cancer specimens was associated with metastatic spread to axillary lymph nodes. In contrast to our data suggesting an association between TGF-β_1 expression and aggressive disease, Mizukami *et al.* (22) have reported that a higher proportion of patients with TGF-β-positive breast tumors exhibited relapse-free survival at 2 years (97 *versus* 81%). Some of these discrepancies may derive from the nature of the antibodies used. We have used antibodies which are isoform specific, whereas the above-mentioned studies used either a commercially available antibody known to cross-react with other TGF-β isoforms (22), or an antibody preparation with undocumented isoform specificity (21). Clearly, if expression of different isoforms of TGF-β has biological significance in this setting, as suggested by our data, the use of non-cross-reactive antibodies becomes imperative for future studies. Epitope specificity may also be important. In this context, it is unclear how to relate our data to those of a recent study by Butta *et al.* (23) wherein an antibody which recognizes an epitope of TGF-β_1 expressed upon secretion was used, and which suggested that tamoxifen induces TGF-β_1 secretion and stromal deposition.

Our data suggest that increased production of TGF-β_1 by tumor cells may augment certain aspects of their malignant phenotype. A similar conclusion was reached in a recently published study of a cell line which became more tumorigenic upon transfection with a TGF-β_1 expression plasmid (24). Given the wide spectrum of biological activities exhibited by TGF-β, a number of possible mechanisms by which TGF-β may facilitate the progression of tumor growth can be postulated. TGF-β is a potent inhibitor of both humoral and cellular immunity, an effect which may underlie the ability of locally produced TGF-β to alter the immune response and clinical course of cutaneous leshmaniasis (25). Various studies indicate that TGF-β_1 is an important modulator of angiogenesis, with the potential for

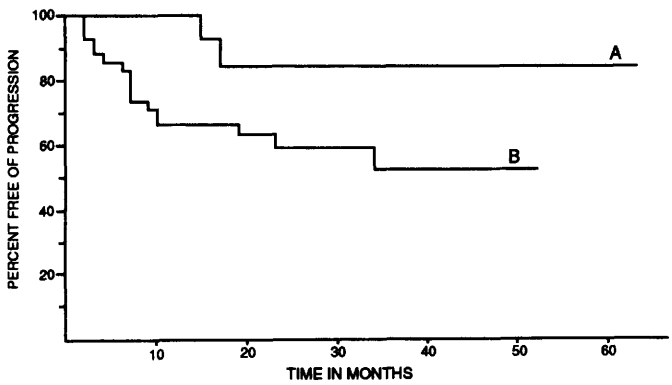

Fig. 3. Kaplan-Meier plot of disease progression in patients whose tumors had little to no staining with anti-TGF-β_1 antibody (*A*, score = 0, 1; $N = 15$) *versus* patients with tumors showing intense anti-TGF-β_1 staining (*B*, score = 2; $N = 42$). Time is measured from biopsy to tumor recurrence, progression, or last follow-up. The median follow-up is currently 20 months.

Table 1 *Characteristics of patients in multivariate analysis*

Patient characteristics	No. (%) of patients
TGF-β_1 staining intensity	
Low (0, 1)	12 (30)
High (2)	28 (70)
Age	
<50 yr	12 (30)
≥50 yr	28 (70)
Stage	
I	16 (40)
II	15 (37)
III	7 (18)
IV	2 (5)
No. of positive nodes	
0	23 (58)
1–3	5 (13)
4–9	9 (22)
10+	3 (7)
Estrogen receptor status	
Negative (<10 fm/mg protien)	8 (20)
Positive (>10 fm/mg protien)	32 (80)

promotion of neovascularization in both primary and metastatic breast cancer (12, 13, 26). Furthermore, TGF-β_1 regulates production of some components of the extracellular matrix, and increases production of basement membrane-degrading enzymes, which may thereby alter the cellular milieu in such a way as to favor metastatic spread (14–16).

In summary, we have observed an association between anti-TGF-β_1 immunoreactivity and disease progression in breast cancer. The rate ratio we observed was imprecise because our findings are based on a small sample size consisting of a heterogeneous population; expansion of our analysis to a larger cohort of patients is indicated. Our data suggest that future immunohistochemical studies of TGF-β expression must take into account the isoform specificity of the antibodies used. Information obtained from such analyses may help clarify some of the differences inherent in studying growth factors in tissue culture rather than *in vivo*.

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