Immunohistochemical Staining for Transforming Growth Factor \( \beta_1 \) Associates with Disease Progression in Human Breast Cancer

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Abstract

The transforming growth factor \( \beta_1 \) (TGF-\( \beta_1 \)) comprise a family of \( M_f \), 25,000 pluriportent growth factors which have been implicated in the development and progression of human breast cancer. Conflicting data suggest that TGF-\( \beta_1 \) 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 prevalence and distribution of immunoreactivity with antibodies specific for the three mammalian isoforms of TGF-\( \beta \) (\( \beta_1, \beta_2 \), and \( \beta_3 \)). We found that intense staining for TGF-\( \beta_1 \) 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-\( \beta \) is one such protein, and evidence suggests that it may play a central role in mammary neoplasia. The human TGF-\( \beta \) family consists of three highly homologous \( M_f \), 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-\( \beta \) 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-\( \beta \) by breast cancer cells has been documented to occur both in vivo and in vitro (3, 4). For the majority of human breast cancer cell lines, TGF-\( \beta \) inhibits proliferation (5). Reports that growth-inhibitory antiestrogens can increase the production of TGF-\( \beta_1 \) in hormonally responsive cell lines (3), and that estradiol (6) and norethindrone (7) can inhibit the expression of TGF-\( \beta_2 \) and \( \beta_3 \), concomitant with growth stimulation, have prompted speculation that TGF-\( \beta \) 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-\( \beta \) which favors net progression of the transformed phenotype. These studies demonstrate that TGF-\( \beta_1 \) mRNA is increased in transformed as opposed to normal breast epithelium (8), and that TGF-\( \beta \) increases the metastatic potential of mammary tumor cells (9). Possible mechanisms by which TGF-\( \beta \) 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-\( \beta \) 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-\( \beta \) 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-\( \beta \) isofrom 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-\( \beta_1 \) and TGF-\( \beta_2 \), and residues 9–20 of TGF-\( \beta_3 \). The specificity of these antibodies for the 3 isoforms of TGF-\( \beta \) was established by Western blot analysis utilizing intact native and recombinant human TGF-\( \beta_1 \), \( \beta_2 \), and \( \beta_3 \), 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-\( \beta \) isofrom-specific antibodies were at 5 \( \mu \)g/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.

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-\( \beta \).
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 corre-
spanding 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 differ-
ences as well as similarities in isoform-specific staining patterns
were noted. For instance, staining with the anti-TGF-β1 anti-
body was most evident in the cytoplasm of the ductal epithe-
lium, with prominence of the cytoplasmic membrane. By con-
trast, anti-TGF-β2, while having some affinity for epithelial
cells, manifested primarily by staining of stromal fibroblasts
and the luminal surfaces of ducts. Anti-TGF-β3 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-β1
and -β2 antisera. Compared to our small sample of benign
breast epithelium, malignant cells demonstrated greater vari-
ability in the intensity of immunoreactivity for the TGF-β iso-
forms. Staining of tumor cells tended to be cytoplasmic, al-
though TGF-β3 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-β2 and -β3 bore
no relation to patient outcome (log-rank P = 0.75 and 0.95,
respectively). Kaplan-Meier curves of progression-free survival
by staining intensity group (low versus high) are plotted for
anti-TGF-β2 and -β3 isoforms (Fig. 2). In contrast, an asso-
ciation was apparent between intensity of staining with the
anti-TGF-β1 antibody and disease progression, defined as tu-
mor 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-β1. 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 car-
cinomas. However, there were 3 colloid carcinomas, 2 tubular
carcinomas, 2 infiltrating lobular, and 1 poorly differentiated
mucin-producing adenocarcinoma. One specimen was classi-
fied as ductal atypia, but shortly thereafter this patient pre-
sented 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 immunoreac-
tivity for TGF-β1 versus patients with biopsy specimens that
exhibited minimal staining with this antibody (N = 57). A strik-
ing univariate association between these parameters was evi-
dent (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 be-
tween the intensity of TGF-β1 immunoreactivity and disease
progression was statistically significant and independent of es-
abled prognostic variables (P = 0.009; progression rate ra-
tio, 64,500; 95% confidence interval, 16 to 2 × 108). The ad-
justed rate ratios for TGF-β2 and -β3 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-em-
bedded histological sections obtained from primary breast can-
cer tissue provides an avenue for the exploration of the poten-
tial pathophysiological importance of TGF-β in mammary

Fig. 1. A, specimen stained with TGF-β2
antisera demonstrating immunoreactivity of
the apical surface of ductal cells (curved ar-
row). B, this specimen stained for TGF-β3
shows prominent myoepithelial staining (blunt
arrow). C, specimen taken from a patient
with stage II breast cancer who developed pro-
gressive disease at 19 months, demonstrating
intense cytoplasmic reactivity for TGF-β1
(score = 2). By contrast, D shows light TGF-β1
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
(arrow). A, B, C, and D, × 1200, × 660,
× 660, and × 330, respectively.
A.

Fig. 2. Kaplan-Meier plots of disease progression in patients whose tumors exhibited minimal staining (score = 0 or 1, ) with anti-TGF-β2 (top; N = 23) or anti-TGF-β3 (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.

B.

Fig. 3. Kaplan-Meier plot of disease progression in patients whose tumors had little to no staining with anti-TGF-β1 antibody (A, score = 0, 1; N = 15) versus patients with tumors showing intense anti-TGF-β1 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

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>No. (%) of patients</th>
</tr>
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<tbody>
<tr>
<td>TGF-β1 staining intensity</td>
<td></td>
</tr>
<tr>
<td>Low (0, 1)</td>
<td>12 (30)</td>
</tr>
<tr>
<td>High (2)</td>
<td>28 (70)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;50 yr</td>
<td>12 (30)</td>
</tr>
<tr>
<td>≥50 yr</td>
<td>28 (70)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>16 (40)</td>
</tr>
<tr>
<td>II</td>
<td>15 (37)</td>
</tr>
<tr>
<td>III</td>
<td>7 (18)</td>
</tr>
<tr>
<td>IV</td>
<td>2 (5)</td>
</tr>
<tr>
<td>No. of positive nodes</td>
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</tr>
<tr>
<td>0</td>
<td>23 (58)</td>
</tr>
<tr>
<td>1–3</td>
<td>5 (13)</td>
</tr>
<tr>
<td>4–9</td>
<td>9 (22)</td>
</tr>
<tr>
<td>10+</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Estrogen receptor status</td>
<td></td>
</tr>
<tr>
<td>Negative (&lt;10 fm/mg protein)</td>
<td>8 (20)</td>
</tr>
<tr>
<td>Positive (&gt;10 fm/mg protein)</td>
<td>32 (80)</td>
</tr>
</tbody>
</table>

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-β1 expressed upon secretion was used, and which suggested that tamoxifen induces TGF-β1 secretion and stromal deposition.

Our data suggest that increased production of TGF-β1 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-β1 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 leishmaniasis (25). Various studies indicate that TGF-β1 is an important modulator of angiogenesis, with the potential for 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-β1. Interestingly, this association was not evident for TGF-β2 or β3 immunoreactivity. Since the three isoforms of TGF-β share a similar spectrum of biological activities, the suggestion that only TGF-β1 was associated with disease progression is of considerable interest. Possible explanations include the preferential activation of TGF-β1 from its latent complex, a phenomenon recently described for TGF-β2 (20), or an effect of TGF-β1 mediated by the β1 isoform with far greater potency, compared to the β2 and β3 isoforms.

Recently, other investigators have reported on the clinical correlates of anti-TGF-β1 immunohistochemical reactivity in breast cancer specimens. Walker and Dearing (21) observed that staining for TGF-β1 in primary breast cancer specimens was associated with metastatic spread to axillary lymph nodes. In contrast to our data suggesting an association between TGF-β1 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
promotion of neovascularization in both primary and metastatic breast cancer (12, 13, 26). Furthermore, TGF-β1 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-β1 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.

References


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