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Immunocytochemical Localization of Secreted Transforming Growth Factor-β_1 to the Advancing Edges of Primary Tumors and to Lymph Node Metastases of Human Mammary Carcinoma

Bakul I. Dalal*, Paul A. Keown,*† and Arnold H. Greenberg‡

From the Division of Hematopathology, Department of Pathology and Department of Medicine,† Vancouver General Hospital; and the Manitoba Institute of Cell Biology,‡ University of Manitoba, Canada*

The level of expression and localization of transforming growth factor-β_1 (TGF-β_1) were analyzed by immunocytochemistry using antibodies that distinguished the sites of intracellular synthesis and extracellular secretion of TGF-β_1 in 28 cases of infiltrating duct carcinoma of breast, 12 of which had lymph node metastases. Twenty-seven of 28 primary tumors and all 12 lymph node metastases showed extracellular deposition of TGF-β_1. The extracellular TGF-β_1 staining was either confined to or more strongly expressed at the advancing edges of the tumor than in the center of the primary tumor. By contrast, 19 of 28 primary tumors and 11 of 12 metastases contained intracellular TGF-β_1, and no variation in the intensity was seen. The metastatic tumors were significantly more intensely stained for both intra- and extracellular TGF-β_1 than the primary tumor tissues. The preferential expression of secreted TGF-β_1 at the advancing tumor edges and in lymph node metastases suggests a role for TGF-β_1 in the malignant progression of breast carcinoma. (Am J Pathol 1993, 143:381–389)

The transforming growth factor-β (TGF-β) family are 25-kd homodimeric polypeptides that were initially identified because of their ability to induce mesenchymal cells to grow in soft agar.[1,2] Although the regulation of TGF-β_1 secretion is distinct from the two other mammalian isoforms TGF-β_2 and TGF-β_3, the mature peptides of all isoforms are structurally and functionally similar and can trigger a complex cascade of events both within cells and in the extracellular matrix.[3,4] These events include alterations that could have a considerable impact on the development and progression of malignancy, including effects on proliferative and differentiation processes, regulation of matrix protein and integrin synthesis that affects cell adhesion,[5–7] modulation of the immune response,[8–12] control of invasion and motility,[13] and metastasis formation.[14] TGF-β protein and mRNA have been demonstrated in neoplastic mesenchymal[15–17] and epithelial[18,19] cells. Several recent studies[20–24] have found TGF-β in mammary epithelial cells, both normal and neoplastic.

TGF-β_1 is reported to be an important regulator of the proliferation of breast and other types of epithelial cells. Most studies indicate that TGF-β_1 inhibits the growth of normal[25–27] and neoplastic mammary cells.[23,27–29] Mammary cancer cell lines lacking TGF-β receptors are resistant to its growth-inhibitory effect and addition of anti-TGF-β_1 antibody to quiescent breast cancer cell lines stimulates proliferation.[30] Although TGF-β_1 is antiproliferative for many mammary as well as other types of epithelial and mesenchymal tumor cells, it has been suggested that the growth inhibitory effects of TGF-β_1 are often lost on transformation and this may contribute to carcinogenesis.[27,31] Human mammary cells fully transformed by transfection with SV40-T and v-H-*ras* are less growth-inhibited by TGF-β_1 under anchorage-dependent and -independent conditions.[27]

In contrast to the antiproliferative effect of TGF-β_1, its effects on invasiveness and metastasis of mammary carcinoma and other tumor cells suggests that TGF-β may play a role in promoting the aggressive

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Address reprint requests to Dr. Arnold H. Greenberg, Manitoba Institute of Cell Biology, 100 Olivia Street, Winnipeg, Manitoba, Canada.

behavior of neoplasia. Some cell lines that constitutively secrete large amounts of TGF-β_1 are more metastatic than those that do not.[32] Transfection and overexpression of TGF-β_1 promotes tumor cell invasion and motility[13] and enhances *in vivo* tumor growth.[33]

Pretreatment of mammary cancer cell lines with TGF-β increases their invasive and metastatic potential and this can be reversed by adding anti-TGF-β antibody.[14] Thus, TGF-β has the seemingly paradoxical effects of suppressing proliferation of some breast cancer cells, while increasing their capacity to invade and metastasize. If the primary effect of TGF-β *in vivo* is to inhibit cell proliferation, one would predict that malignant neoplasms and their metastases would produce relatively small amounts of this growth suppressive cytokine. However, if TGF-β has its main effect in promoting invasion and metastasis then enhanced expression would be expected in malignancies that have metastasized. To test this hypothesis, we have examined the expression of TGF-β_1 in paired primary tumors and lymph node metastases as well as nonmetastasizing primary tumors in patients with breast carcinoma. We report here that by immunohistochemistry most human breast cancer tissues contain a secreted form of TGF-β_1 at the advancing edge of the primary tumor and in very high levels in lymph node metastases, thus providing evidence consistent with a role for TGF-β_1 in invasion and metastasis in human breast cancer.

Material and Methods

Surgical Specimens

Surgical tissue from 28 patients with mammary carcinoma seen at the Vancouver General Hospital (Vancouver, BC, Canada) or the Health Science Center (Winnipeg, MB, Canada) were examined. Twelve of these had lymph node metastasis and tissue from both the primary tumor as well as the lymph node metastasis was tested in these cases. The mastectomy specimen was dissected, 3 mm slices of tissue promptly fixed in neutral buffered formalin for 4 to 6 hours and embedded in paraffin. Care was taken to include the whole primary tumor with as much surrounding tissue as possible in the section.

Immunohistochemistry

The antibodies to TGF-β_1 used in our study, anti-LC and anti-CC were generous gifts from Drs. Kathleen Flanders (National Institutes of Health, Bethesda, MD) and Larry Ellingsworth (Celtrix Corporation, Palo Alto, CA), respectively. The anti-LC and anti-CC antibodies to TGF-β_1 are polyclonal antibodies produced in rabbits against two different synthetic preparations of the same peptide sequence, corresponding to the first 30 amino acids of the N-terminal region of TGF-β_1.[34,35] Even though both of the antibodies were raised to the same peptide, they show a mutually exclusive staining pattern.[36] A distinctive region of reactivity against the 1–30 peptide has been mapped to the peptide 21–30, which is recognized by anti-LC but not anti-CC, whereas anti-CC is thought to react with discontinuous peptides in the N-terminus.[36] The TGF-β_1 epitope recognized by the anti-CC antibody seems to be a conformational change exposed after secretion of the polypeptide and interaction with extracellular matrix.[36] The antibody thus allows the detection of active secretion and areas of deposition of the TGF-β_1. Anti-LC antibody stains intracellular sites of TGF-β_1.[36] They show the same intracellular staining pattern as antibodies to amino acids 266–278 in the TGF-β_1 propeptide;[36] thus, the anti-LC reactive cells represent sites of active TGF-β_1 synthesis.

The immunocytochemical staining was performed using the streptavidin-biotin technique. Sections of 3 μM thickness were cut and placed on poly-l-lysine coated slides. The slides were dried in oven at 37 C overnight and then deparaffinized and hydrated in sequential gradients of ethanol. The endogenous peroxidase was blocked by immersing the slides in 0.6% hydrogen peroxide (Sigma Chemicals, St. Louis, MO) in methanol for 1 hour. For staining with anti-LC(1–30) the section was incubated with 1 mg/ml hyaluronidase (Sigma) in 0.1 M sodium acetate buffer, pH 5.5, at 37 C for 30 minutes. Hyaluronidase digestion was not performed for staining with anti-CC as it was found not to enhance the sensitivity of detection. Sections were washed in distilled water and then incubated with 5% skim milk powder for 30 minutes. The milk was then drained off and the sections covered with anti-LC or anti-CC at a final concentration of 14 μg/ml and 8.35 μg/ml respectively. Two negative controls were used: the primary antibody was replaced by either nonimmune serum or TGF-β_1 1–30 blocking peptide (generous gift from Dr. Ian Clarke-Lewis, Biomedical Research Center, Vancouver, BC), 15 μg/ml preincubated with anti-LC. The sections were layered with parafilm and incubated at 20 C for 2 hours. At the end of incubation the slides were gently rinsed in TRIS buffer and then incubated with biotinylated goat anti-rabbit secondary

antibody (Vector Laboratories, Burlingame, CA) at 20 C for 30 minutes. The sections were then rinsed and returned to the humid chamber for incubation with peroxidase-labeled streptavidin (Biocan Scientific, Westgrove, PA) at 20 C for 80 minutes. The slides were then stained with freshly prepared 3-amino-9-ethyl carbazole (Sigma) at 0.263 mg/ml for 10 minutes, counterstained with Meyer's hematoxylin, mounted with Crystal Mount (Biomeda Corp, Foster City, CA), and coverslipped with Entellan (Merck).

Four sections from each of the tumors as well as metastatic lymph nodes were examined, and these included two negative controls of nonimmune serum and TGF-β_1 1–30 blocking peptide preincubated with anti-LC, plus the two experimental observations of sections stained with either anti-LC or anti-CC. Each section was stained three times on different days. Only those cases with absence of staining in both negative controls were analyzed. A positive control of known intensity of staining was included with every run and used for grading of the staining. These positive controls were highly reproducible. The tumor cells as well as the stroma and normal cells in their vicinity were evaluated. The intensity of staining was arbitrarily graded on a scale of 0–4 and the pattern of staining was recorded.

Table 1. *Intensity of Staining of Primary and Metastatic Mammary Carcinoma Tissues with TGF-β Antibodies*

	Anti-CC (1–30)*			Anti-LC (1–30)*†	
Patient Number	Tumor Center	Edge	Metastasis	Primary	Metastasis
A. With lymph node metastasis					
1	0	3	4	2	2
2	0	4	2	3	3
3	0	3	3	1	3
4	0	3	3	1	3
5	1	2	4	2	4
6	1	3	3	1	2
7	0	3	3	0	0
8	0	3	4	0	2
9	0	0	4	0	3
10	0	1	4	2	3
11	0	3	4	1	3
12	0	3	4	1	4
B. Without lymph node metastasis					
13	0	1	–	1	–
14	0	3	–	0	–
15	0	3	–	2	–
16	0	2	–	1	–
17	0	1	–	1	
18	0	1	–	1	–
19	0	1	–	1	–
20	0	2	–	2	–
21	0	1	–	0	–
22	0	3	–	0	–
23	0	3	–	1	–
24	0	3	–	0	–
25	0	3	–	0	–
26	2	1	–	2	–
27	2	1	–	0	–
28	1	3	–	1	–

* Intensity of staining was graded on a scale of 0–4+.
† Intensity of staining was equal in the center and at the advancing edges of tumor.

Results

Twenty-eight cases of mammary carcinoma were analyzed. Twelve of these cases had histologic metastases in the axillary lymph nodes, and in these, both the primary and the metastatic tissues were examined. Each tissue sample was evaluated in three separate assays and no significant variation was noted. Twenty-seven of 28 (97%) primary tumors and all 12 (100%) metastatic tumors were positive with anti-CC antibody (Tables 1 and 2). In comparison, 19 of 28 (62%) primary tumors and 11 of 12 (92%) metastatic tumors stained with anti-LC. The anti-CC staining was localized in the collagen stroma around the tumor cells (Figures 1A and 1B). The pattern of staining of anti-CC was either fibrillar or granular, and tended to follow the collagen tracks (Figure 1B). By contrast, the anti-LC staining was seen in the cytoplasm of the tumor cells (Figures 2A and 2B) and the pattern of staining was either granular or diffuse, whereas the stroma around the tumor cells was negative (Figure 2B). The intensity of staining was higher with anti-CC than with anti-LC in both the primary tumors as well as metastatic tissues (Figures 1 and 2). Sixteen of 28 primary tumors and 11 of 12 metastases showed ≥ 3+ staining for anti-CC, as compared with only one primary tumor and 8 of 12 metastases for anti-LC. One patient (No. 9) showed no staining of the primary tumor, yet had intensely positive lymph nodes with both antibodies (Table 1).

In the primary tumors, the staining with anti-CC was significantly stronger ($P < 0.001$; Mann-Whitney U test) at the advancing edges than in the central areas (Figure 1 and Table 2). A comparison between the advancing edges of the primary tumor and the metastatic tumor from the same patients showed significantly stronger ($P < 0.02$) staining in the metastatic tumor than the primary tissue (Figure 3 and Table 2). Eight of 12 cases of metastatic tissue stained 4+ with anti-CC, compared with only one primary tumor. The anti-LC staining of primary tumors was uniform in all the cancer cells from all areas of the tumor in 15 of 19 positive cases (Figure 2A) and, in the remaining four cases, the variation in the staining intensity was random, with no preference for advancing edges noted. However, anti-LC

Table 2. *Summary of Immunocytochemical Staining of TGF-β_1 in Mammary Carcinoma*

Patient Category	*n*		Anti-CC (1–30) Staining			Anti-LC (1–30) Staining	
			Center	Advancing edge	Metastasis	Primary	Metastases
With metastases	12	Median	O_{ac}	3_{a*}	4_{c*}	1_d	3_d
		Frequency (%)	2/12 (17)	11/12 (92)	12/12 (100)	9/12 (75)	11/12 (92)
Without metastases	16	Median	O_b	2_b	- - -	1	- - -
		Frequency (%)	13/16 (19)	16/16 (100)	- - -	10/16 (63)	- - -

Note: abcd: medians with the same subscript letters are significantly different at $P = 0.001$ by a Mann-Whitney U test.
*: median comparison p-value = 0.02.

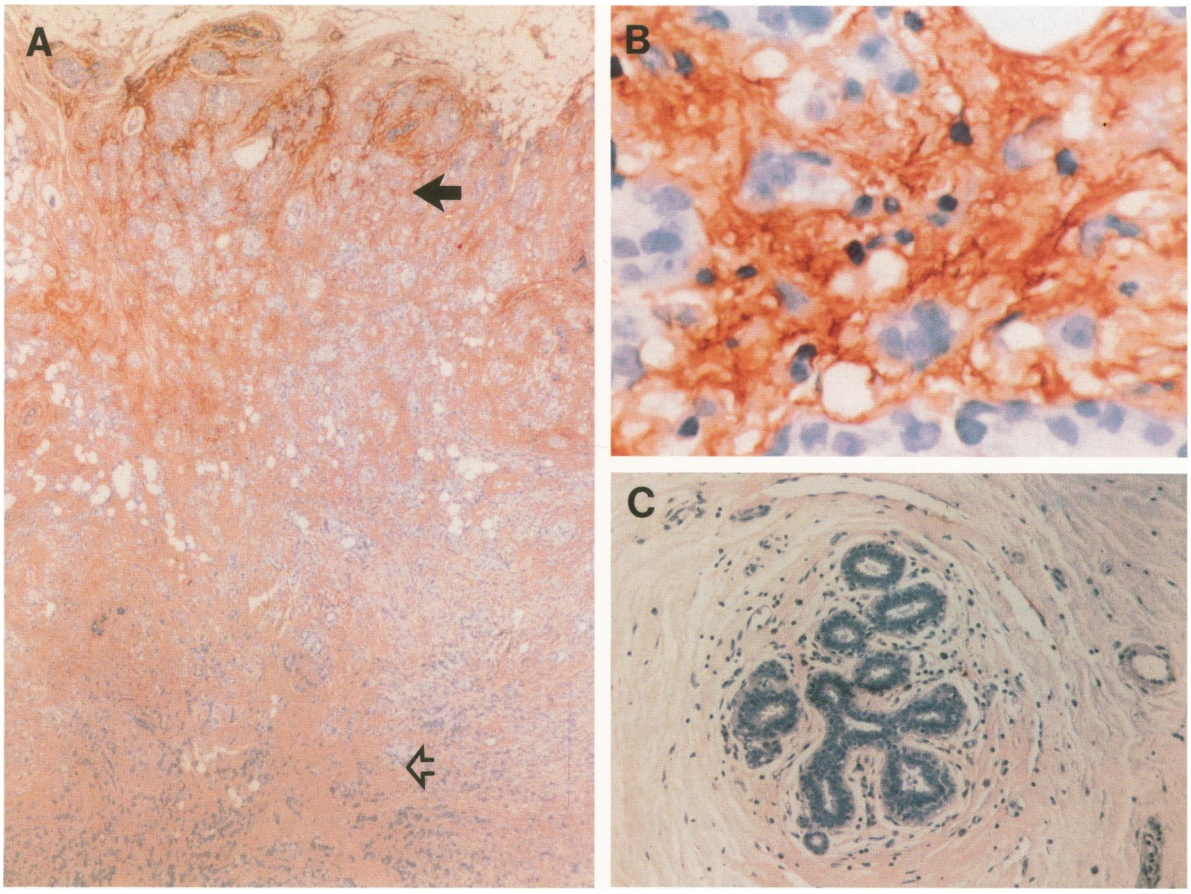


Figure 1. *Immunolocalization of extracellular TGF-β_1 in human mammary carcinoma. 1A: Anti-CC TGF-β_1^{1-30} antibody reacted strongly with extracellular TGF-β_1 (4+) at the advancing edges (closed arrow) of a primary mammary carcinoma when compared with the center of the tumor^{0-1+}(open arrow). The streptavidin-biotin technique with biotinylated goat anti-rabbit second antibody and peroxidase-conjugated streptavidin was used. Slides were reacted with 3-amino-9-ethy-carbazole, which appears dark red-brown, and counter stained with Meyer's hematoxylin. (Magnification ×10). 1B: Higher magnification of extracellular localization of the anti-CC antibody to the collagen matrix at the tumor margin (Magnification ×160). 1C: Normal breast tissue stained with anti-CC antibody. No evidence of extracellular TGF-β1 was detected (×63).*

staining of lymph node metastases was also significantly greater ($P < 0.001$) than in the primary tumors (Table 2). No significant difference in anti-CC or anti-LC staining intensity was seen between primary tumors that were lymph node positive and negative.

Discussion

Our observations on the detection of TGF-β_1 in cells of patients with mammary carcinoma confirmed those of several groups.[23,24,37,38] The unique finding of this study was the identification of high

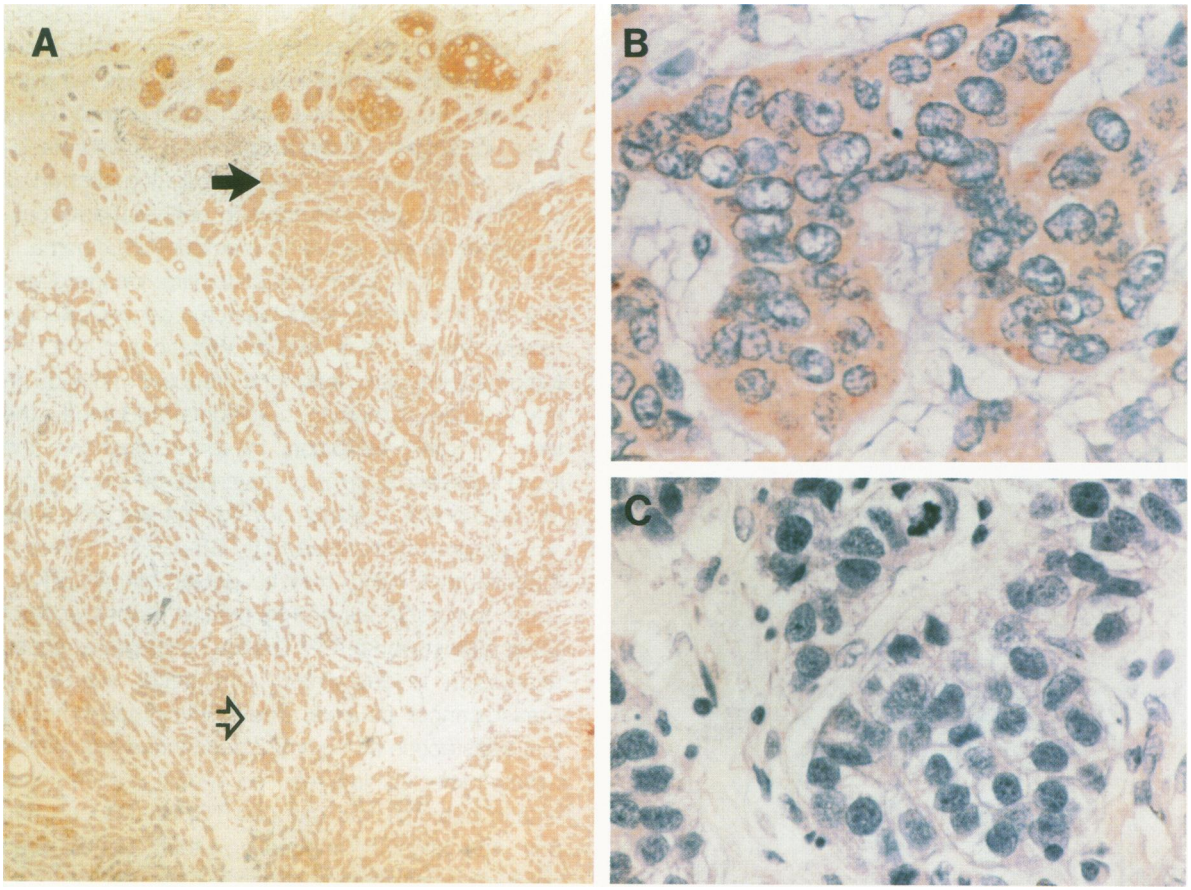


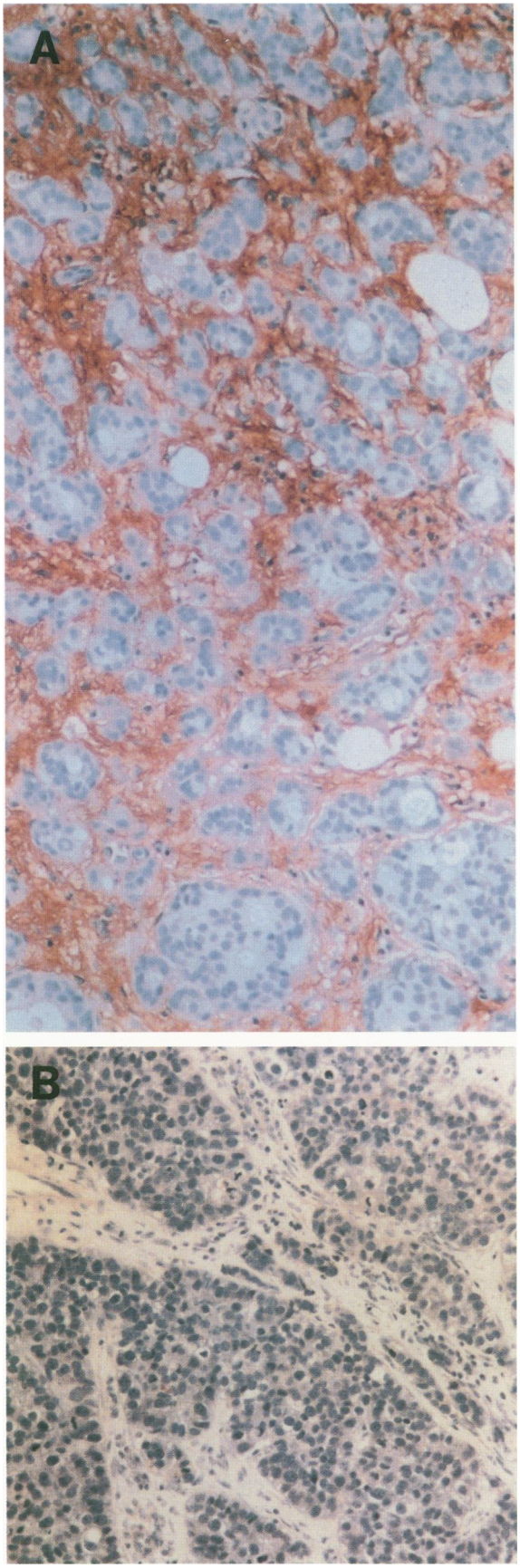
Figure 2. *Immunolocalization of intracellular TGF-β_1 in a mammary carcinoma. 2A: Mammary carcinoma tissue shown in 1A was incubated with anti-LC TGF-β_1 (1–30) antibody, which reacts with intracellular TGF-β_1. Uniform staining was found in all parts of the tumor including the advancing edge (closed arrow) and central tumor areas (open arrow). (Magnification ×10). 2B: Higher magnification of the anti-LC staining, illustrating diffuse TGF-β_1 distribution in the cytoplasm with no extracellular staining detected (Magnification ×160). 2C: Inhibition of anti-LC staining of mammary carcinoma with excess TGF-β1 N-terminal peptide$^{1-30}$ (×160).*

intensity staining of extracellular TGF-β_1 at the edges of the primary tumor, in contrast to the stroma in the center of the primary tumor that was either negative or stained much more weakly than did the periphery. In addition, both intra- and extracellular TGF-β_1 were identified at significantly higher levels in lymph node metastases.

The source of secreted TGF-β_1 detected in our study is likely the breast carcinoma cells. However, the intracellular staining for TGF-β_1 with anti-LC did not duplicate the distribution of the secreted form detected by anti-CC. Most patients exhibited intracellular TGF-β_1 throughout the tumor, yet abundant extracellular TGF-β_1 was found only at the tumor edges. This localization may be a result of an increased rate of TGF-β_1 posttranslational processing and secretion by the tumor cells at the advancing edge of the primary tumor, as compared with the cells in the center of the tumor. Alternately, it may be explained by the differential expression of the matrix proteins that bind secreted TGF-β_1 at the tumor edges. The possibility that the TGF-β_1 is being secreted by normal cells in these areas is unlikely as no staining was detected in other cells at, or adjacent to, these sites.

Some patients with no detectable anti-LC reaction stained with high intensity for the CC antibody in both primary tumor and metastases. The anti-LC antibody is relatively less sensitive than anti-CC on the basis of peptide inhibition studies,[36] suggesting that some cells with very low levels of intracellular production were not detectable by the anti-LC antibody and only once the TGF-β_1 was secreted was it stained by anti-CC antibody. We[39] and others[36,40] have observed this differential staining pattern in other tissues producing TGF-β_1.

Many reports indicate that TGF-β inhibits the proliferation of mammary carcinoma cells,[23,25,26,28,29] and it has been suggested that the regulation of proliferation involves a negative autocrine loop, in which the carcinoma cells that secrete TGF-β are inhibited from further proliferation.[25,26] Our findings



of high levels of TGF-β_1 in lymph node metastases and around most proliferative zones of breast cancer growth implies that this proposed negative autocrine loop has either been interrupted or that TGF-β does not act as a growth suppressor *in vivo*. Resistance to the negative growth-regulating properties of TGF-β has been observed in epithelial and mesenchymal tumors including mammary carcinoma *in vitro*.[4] This has been linked to viral oncoproteins that interact with the retinoblastoma gene product in rodent tumors[41,42] as well as mutations of the p53 tumor suppressor gene,[43] a frequent mutation in many types of human carcinoma.[44] Besides the loss of growth inhibition, in some instances, the more aggressive forms of mammary carcinoma, colon carcinoma, melanoma, and fibrosarcoma were actually growth stimulated by TGF-β.[32,45–48] The hypothesis that TGF-β may be growth stimulatory for some tumors is supported by the recent work of Steeg and coworkers[49,50] on the nm23 gene, whose expression is associated with better survival in breast cancer patients. These investigators found that transfection of the nm23 gene into a highly metastatic tumor cell line resulted in both reduced metastatic potential and reduced TGF-β_1-induced colony formation,[51] suggesting that rather than produce growth suppression, TGF-β_1 may be used by metastatic cells to achieve anchorage-independent growth.

The interplay between the tumor cell and the stroma is an important determinant of the invasiveness of neoplastic cells. In normal cells, TGF-β_1 generally enhances adhesion through increased matrix production and decreased proteolysis.[52] On the other hand, tumor cells use proteolysis coupled with motility to achieve invasion,[53] and highly malignant and invasive cells show increased protease activity when compared with normal or poorly invasive cells.[54,55] Although collagenases, cathepsin L and transin/stromolysin are suppressed by TGF-β1 in normal cells,[13,56,57] the synthesis and activity of these and other proteases are often enhanced by TGF-β in tumor cells.[13,14,58] TGF-β_1 secreted by tumor cells may act in an autocrine manner to enhance invasion and motility of fibrosarcomas and promote the degradation of collagen through increased protease production.[13] Furthermore, treat-

Figure 3. *Lymph node metastasis of mammary carcinoma. 3A: Lymph node metastases stained intensely and uniformly throughout the tumor mass with anti-CC TGF-β_1^{1-30} antibody. (Magnification ×63). 3B: Control normal IgG staining of lymph node metastasis (×63).*

ment of the murine mammary carcinomas with TGF-β_1 enhances their invasion and rate of pulmonary metastasis.[14]

In conclusion, the immunohistochemical observation that TGF-β_1 is secreted and localized at the advancing tumor edges in primary mammary carcinoma and in lymph node metastases is consistent with the hypothesis that this polypeptide is not acting as an inhibitor of breast tumor progression *in vivo*, and may play a role in promoting invasion and metastasis.

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