Transforming Growth Factor-β1: Histochemical Localization with Antibodies to Different Epitopes

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Abstract. We have localized transforming growth factor-β (TGF-β) in many cells and tissues with immunohistochemical methods, using two polyclonal antisera raised to different synthetic preparations of a peptide corresponding to the amino-terminal 30 amino acids of TGF-β1. These two antibodies give distinct staining patterns; the staining by anti–CC(1–30) is principally extracellular, while that by anti–LC(1–30) is intracellular. This differential staining pattern is consistently observed in several systems, including cultured tumor cells; mouse embryonic, neonatal, and adult tissues; bovine fibropapillomas; and human co-

lon carcinomas. The extracellular staining by anti–CC(1–30) partially resembles that seen with an antibody to fibronectin, suggesting that extracellular TGF-β may be bound to matrix proteins. The intracellular staining by anti–LC(1–30) is similar to that seen with two other antibodies raised to peptides corresponding to either amino acids 266–278 of the TGF-β1 precursor sequence or to amino acids 50–75 of mature TGF-β1, suggesting that anti–LC(1–30) stains sites of TGF-β synthesis. Results from RIA and ELISAs indicate that anti–LC(1–30) and anti–CC(1–30) recognize different epitopes of this peptide and of TGF-β1 itself.

TRANSFORMING growth factor-β (TGF-β),[1] a 25-kD homodimeric peptide, is a multifunctional regulator of both cell growth and differentiation and has been shown to influence extracellular matrix production by a number of mechanisms (for reviews see Roberts and Sporn, 1988; Sporn et al., 1987). While in vitro studies have provided valuable information concerning the nature of TGF-β action, their relevance to in vivo systems is only beginning to be examined. To understand TGF-β action in vivo, it is essential to be able to localize the protein in various target tissues.

As a first approach to this problem, we have developed antibodies against peptides corresponding to several regions of the TGF-β1 molecule for use in immunohistochemical studies (Ellingsworth et al., 1986; Flanders et al., 1988). In this article, we present evidence of two conformations of TGF-β1 in several systems. When tissue sections are stained with two antibodies raised to different synthetic preparations of a peptide corresponding to the amino-terminal 30 amino acids of TGF-β1, two distinct staining patterns are observed. One antibody stains principally extracellular TGF-β, while with the other, staining is intracellular.

Since the two different antibody preparations are now being widely used, it is essential to define the basis and consistency of their use as reagents for staining. In the present study, we have deliberately examined a wide and diverse set of cells and tissues from three species of animals, including normal embryonic and adult, as well as malignant, phenotypes. A consistent pattern of staining has been observed in all situations. As shown in the following paper (Thompson et al., 1989), combined use of the two antibodies provides a useful tool for localizing TGF-β in many tissues of a given species.

Materials and Methods

Production of Antisera

1. *Abbreviation used in this paper:* TGF-β, transforming growth factor-β.

All polyclonal antibodies to TGF-β were made in rabbits using synthetic peptides as immunogens. Anti–CC(1–30) was produced as described by Ellingsworth et al. (1986) to a peptide corresponding to the first 30 amino acids of mature TGF-β1 (Monsanto Co., St. Louis, MO). Anti–LC(1–30) was made to another synthetic preparation (Frederick Cancer Research Facility, Frederick, MD) of the identical peptide. This second preparation of the peptide was injected, also uncoupled, into New Zealand white rabbits as described (Flanders et al., 1988). Anti–P(50–75) was made to the peptide corresponding to amino acids 50–75 of mature TGF-β1, with an added car-

boxyl-terminal tyrosine (Peninsula Laboratories, Inc., Belmont, CA). This peptide was coupled to ovalbumin and injected as described (Flanders et al., 1988). Anti-pre(266-278) and anti-pre(46-56) were made as described (Wakefield et al., 1988). Rabbit anti-human fibronectin was from Collaborative Research Inc. (Waltham, MA).

Preparation of Cells and Tissues for Immunostaining

Malignant cells from tissue cultures were trypsinized, resuspended in media containing 10% calf serum, washed three times with PBS, fixed overnight in 10% neutral-buffered formalin, treated in Bouin's solution for 4-6 h at room temperature, and finally stored in 70% ethanol before embedding in paraffin and sectioning at 5 μm. Tumors arising in nude mice 5 wk after injection of cultured cells were fixed and sectioned in a similar manner, as were human colon carcinomas, bovine fibropapillomas, and mouse embryos.

Immunohistochemical Staining

TGF-β was localized in sections as described by Heine et al. (1987), using either avidin–biotin–peroxidase or avidin–biotin–glucose oxidase kits (Vector Laboratories Inc., Burlingame, CA). After deparaffinization, blocking of endogenous peroxidase in hydrogen peroxide/methanol, and permeabilization with hyaluronidase, the sections were blocked with 1.5% normal goat serum/0.5% BSA, incubated overnight at 4°C with IgG fractions of antibody prepared by protein A-Sepharose chromatography (Flanders et al., 1988) at 25 μg/ml, washed extensively, and then incubated with biotinylated goat anti-rabbit IgG and avidin-enzyme complex. Sections were stained with 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and hydrogen peroxide (for peroxidase), and with nitroblue tetrazolium and glucose (for glucose oxidase), and counterstained with either 1% methyl green or Giemsa, followed by May-Grunwald stain.

Controls included (a) replacing primary antibody with normal rabbit IgG, (b) using antisera preincubated with TGF-β1-Sepharose resin to remove TGF-β-specific antibodies (Heine et al., 1987), and (c) using primary antibody that had been preincubated with a 20-fold molar excess of the appropriate peptide for 2 h at room temperature before this mixture was applied to the section.

Immunoassays

RIAs and ELISAs were performed as described by Flanders et al. (1988). Antigens used in the ELISA assay included peptides corresponding to amino acids 1-10, 11-20, and 21-30 of TGF-β1 (Peninsula Laboratories, Inc.); porcine TGF-β2 (R & D Systems, Minneapolis, MN); and TGF-β1 purified from human platelets (Assoian et al., 1983). Western blotting of TGF-β1 and 2 was carried out as described by Florini et al. (1986) and immune complexes were detected by an avidin–biotin conjugate immunoperoxidase kit (Vector Laboratories Inc.).

Results

Anti-CC(1-30) and Anti-LC(1-30) Give Different Patterns of Immunohistochemical Localization of TGF-β

Two polyclonal antibodies (anti-CC[1-30] and anti-LC[1-30]) were raised in rabbits against different synthetic preparations (CC[1-30] and LC[1-30]) of the same peptide sequence, corresponding to the amino-terminal 30 amino acids of TGF-β1. Results presented here and by Ellingsworth et al. (1986) show that both antisera recognize TGF-β1 in a number of immunoassays. These two antisera were used to localize TGF-β in a variety of tissues. The staining pattern seen with anti-CC(1-30) IgG was principally extracellular and associated with extracellular matrix, while the staining seen in many cell types with anti-LC(1-30) IgG was intracellular. An example of the two staining patterns in a bovine fibropapilloma, as well as the specificity of the staining, is shown in Fig. 1 (A-D). Intracellular staining of the papilloma epithelium by anti-LC(1-30) (Fig. 1 A) and extracellular

staining of its fibromatous component by anti-CC(1-30) (Fig. 1 C) are clearly evident. Each of these two staining patterns is specific (Fig. 1, B and D) since staining was greatly reduced if the antibody preparations had been preincubated with TGF-β1-Sepharose to remove TGF-β1-specific antibodies (Heine et al., 1987). Staining was also greatly reduced if the primary antibody was normal rabbit IgG (data not shown).

This differential localization of TGF-β was consistently seen in a variety of murine and human cells and tissues, as shown in Fig. 2. Thus, Ha-ras-transformed NIH-3T3 cells, which produce large amounts of TGF-β1 (Anzano et al., 1985; Jakowlew et al., 1987), are stained by anti-LC(1-30) but not by anti-CC(1-30) (Fig. 2, A and B). Tumors raised in nude mice from these cells (as well as from PC3 human prostatic carcinoma cells and from A549 human lung carcinoma cells), all showed intracellular staining by anti-LC(1-30), as shown in Fig. 2 C for the PC3 tumor. However, anti-CC(1-30) stains only the tumor stroma (Fig. 2 D). Immunohistochemical analysis of various organs in the 15-d mouse embryo further demonstrated this differential staining; Fig. 2 (E-H) shows peroxidase-stained sections of a mouse embryo foot and vertebra, respectively. Subdermal mesenchyme of the foot was stained by anti-CC(1-30), while only chondrocytes in cartilage of the developing bones were stained by anti-LC(1-30). In vertebrae where ossification is just beginning, anti-CC(1-30) stained calcifying matrix around chondrocytes, while anti-LC(1-30) again stained proliferating chondrocytes.

This differential staining has also been consistently found in a number of adult and neonatal mouse organs (Thompson et al., 1989), as well as in sections of human colon carcinomas (see below). Furthermore, in human and rat arthritic joints anti-LC(1-30) stains mononuclear cells and synovial cells, while anti-CC(1-30) stains extracellularly around the synovial cells (Lafyatis et al., 1988).

Anti-LC(1-30) and Anti-CC(1-30) Recognize Different Epitopes of the TGF-β1 Molecule

To determine the basis of the different staining patterns, we have measured the reactivities of anti-LC(1-30) and anti-CC(1-30) in several immunoassays. Each antibody was assayed for its ability to immunoprecipitate ^{125}I-TGF-β1. The ED$_{50}$ for anti-LC(1-30) was 1:250 with a maximum immunoprecipitation of 75% of the ^{125}I-TGF-β1 added, while the corresponding values for anti-CC(1-30) were 1:100 and 35%, respectively. While these differences in immunoprecipitation of ^{125}I-TGF-β1 from solution may reflect varying concentrations of TGF-β-specific antibodies in the two antisera, differences in the accessibility of the epitope recognized by each of these antibodies in native TGF-β1 may also contribute.

To investigate this possibility, the antibodies were tested for their reactivities with various peptides in an ELISA assay (Table I). When tested against peptide LC(1-30), the antisera generated against this peptide, anti-LC(1-30), gave an ED$_{50}$ of 1:4,000, while anti-CC(1-30), which was generated to the other synthetic preparation of peptide, gave an ED$_{50}$ of only 1:200 against the peptide LC(1-30). Ellingsworth et al. (1986) have reported an ED$_{50}$ of 1:10,000 for anti-CC(1-30) when tested against the peptide to which it was raised (CC[1-30]), but none of the peptide from this synthesis re-

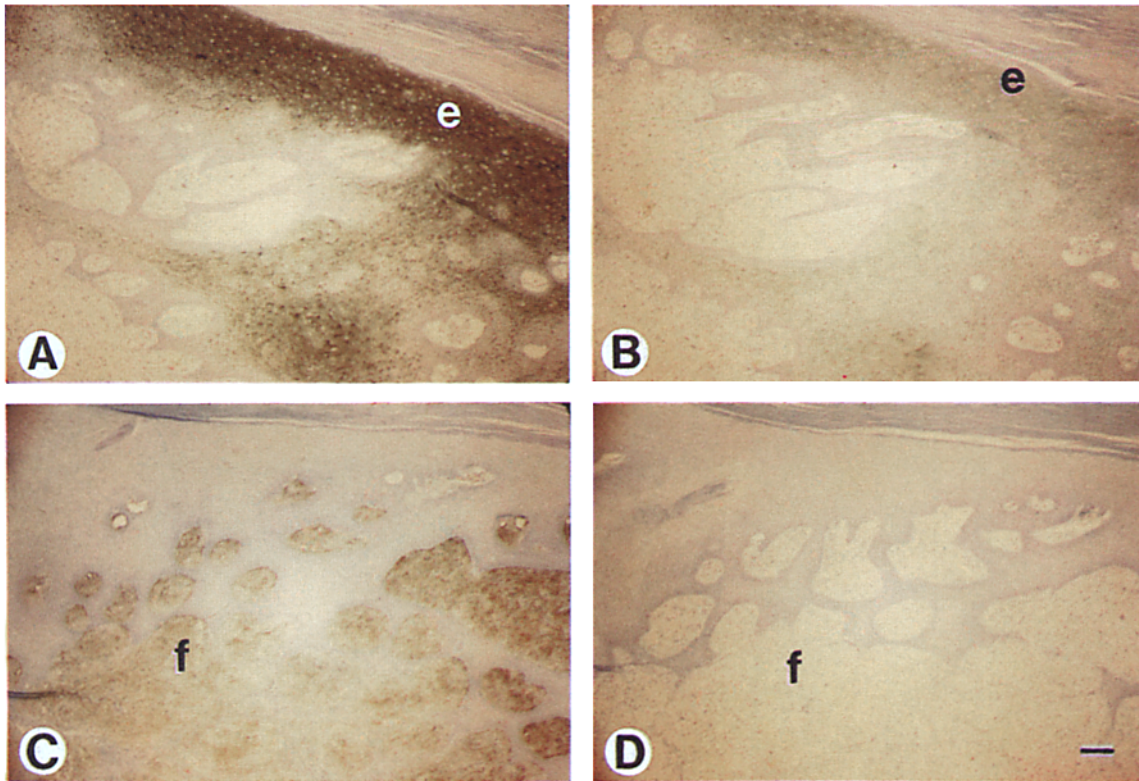*Figure 1.* Demonstration of staining specificity. Bovine fibropapillomas were stained by (*A*) anti–LC(1–30) preincubated with Sepharose-control resin; (*B*) anti–LC(1–30) preincubated with TGF-β1 coupled to Sepharose; (*C*) anti–CC(1–30) preincubated with Sepharose-control resin; (*D*) anti–CC(1–30) preincubated with TGF-β1 coupled with Sepharose. Antisera were prepared as described in Heine et al. (1987). Anti-LC(1–30) stains epithelium (*e*), while anti–CC(1–30) stains the fibromatous component (*f*) of the fibropapilloma. In each case, staining was greatly reduced when antibody was preincubated with TGF-β1–Sepharose. Sections were stained with peroxidase and counterstained with Giemsa and May–Grunwald stains. Bar, 100 µm.

mained for testing with anti–LC(1–30). These data suggest that the peptides generated by the two syntheses are in different conformations and that the two antibodies recognize different epitopes of the peptides. In order to test this possibility, the two antisera were tested against shorter peptides corresponding to residues 1–10, 11–20, and 21–30 of TGF-β1. Both antisera showed reactivity with the amino-terminal peptide 1–10, neither showed reactivity with the middle peptide 11–20, while only anti–LC(1–30) showed reactivity with peptide 21–30. It should be noted that anti–CC(1–30) had greater reactivity with TGF-β1 than it did with any of the short peptides, suggesting that the epitope of TGF-β1 recognized by anti–CC(1–30) is a discontinuous one that does not exist in the shorter peptides. Furthermore, anti–LC(1–30) also had significant reactivity to the peptide corresponding to the amino-terminal 30 amino acids of TGF-β2; this probably results from the ability of anti–LC(1–30) to react with the peptide corresponding to residues 21–30 of TGF-β1, since this region is highly conserved between TGF-β1 and 2, as well as in the deduced sequences of the newly described TGF-β3 and 4 (Jakowlew et al., 1988*a,b*; ten Dijke et al., 1988). Thus, it must be considered that under fixation conditions leading to exposure of this epitope, anti–LC(1–30) might detect TGF-β2, 3, or 4, as well as TGF-β1, in immunohistochemical localizations. This cross-reactivity is not seen in Western blots however, where both antibodies detect 10 ng of TGF-β1, but do not detect 100 ng of TGF-β2.

Anti–CC(1–30) May Recognize TGF-β Associated with Extracellular Matrix

Since the two antibodies seem to recognize different epitopes of TGF-β1, we conducted additional immunohistochemical studies to investigate the differences in the two forms of TGF-β detected by these antibodies. Mesenchymal areas stained with anti–CC(1–30) are rich in extracellular matrix proteins such as collagen and fibronectin. In fibromatous areas of the bovine fibropapilloma, the staining pattern of anti–CC(1–30) is generally restricted to areas which also stain with a trichrome stain for collagen. However, while trichrome staining for collagen is uniform throughout the fibromatous area (data not shown), staining with anti–CC(1–30) is localized to fibromatous areas near the epithelium (Fig. 1 *C*). An analogous situation was found in human colon carcinomas (Fig. 3). Thus, antifibronectin antibody (Fig. 3 *B*) gave uniform extracellular staining throughout tumor stroma, while staining by anti–CC(1–30) was localized (Fig. 3 *A*) to stromal areas near epithelial cells, which are stained by anti–LC(1–30) (see Fig. 4).

Intracellular Localization of TGF-β with Anti–LC(1–30) May Represent Sites of TGF-β Synthesis

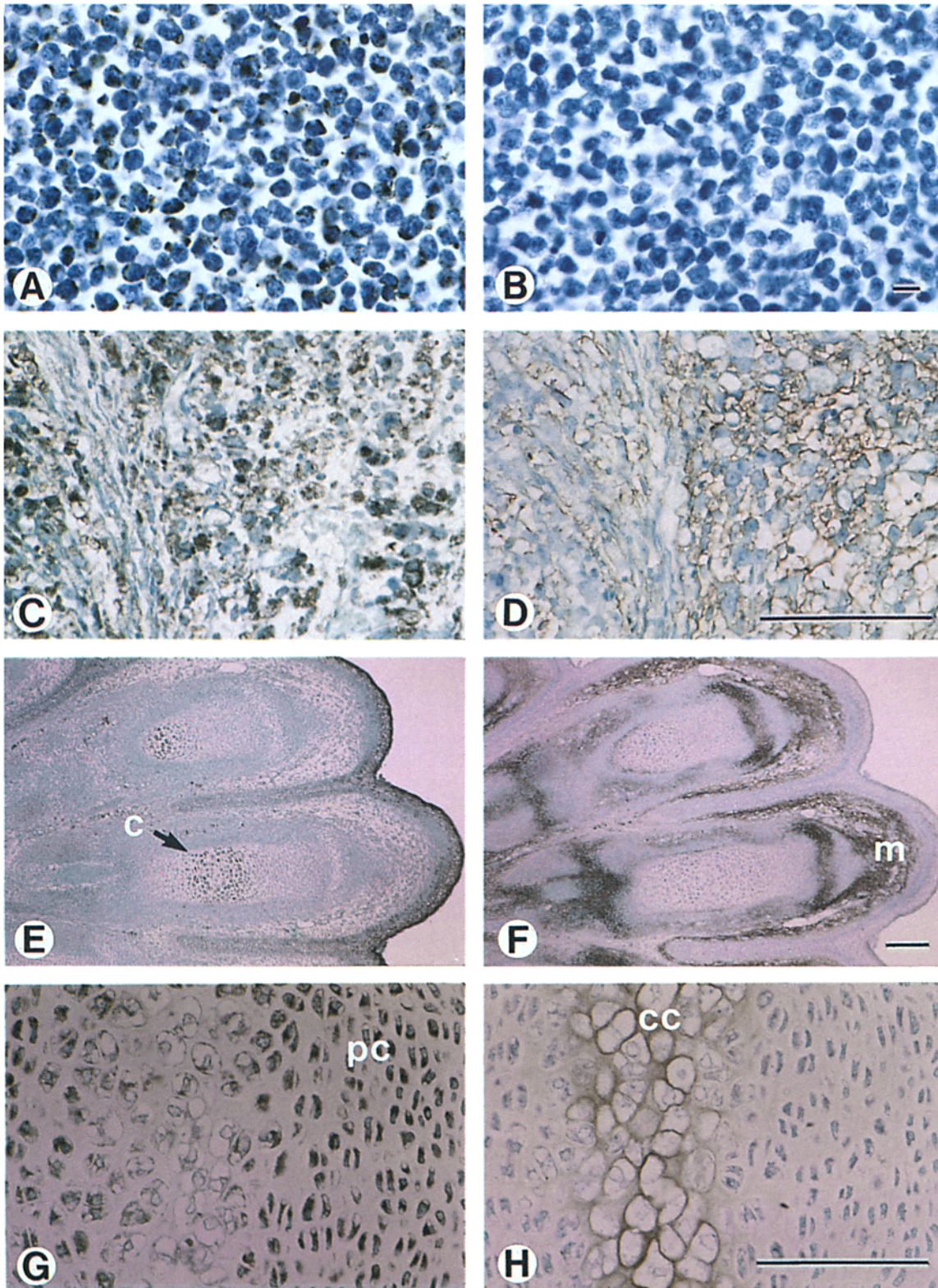Three other antibodies raised to TGF-β1 peptides showed in-

Figure 2. Differential staining patterns with anti–LC(1–30) and anti–CC(1–30). (*A* and *B*) Sections of pellets of Ha-*ras* NIH-3T3 cells; anti–LC(1–30) (*A*) stains cell cytoplasm and anti–CC(1–30) (*B*) gives no staining. (*C* and *D*) Tumors raised in nude mice after injection of PC-3 cells; anti–LC(1–30) (*C*) stains intracellularly, while anti–CC(1–30) (*D*) stains stroma. (*E* and *F*) Developing foot of a 15-d mouse embryo; chondrocytes (*c*) stained by anti–LC(1–30) (*E*) and mesenchyme (*m*) stained by anti–CC(1–30) (*F*). (*G* and *H*) 15-d mouse embryo vertebra; proliferating chondrocytes (*pc*) stained by anti–LC(1–30) (*G*), or calcifying matrix (*cc*) stained by anti–CC(1–30) (*H*). Peroxidase, with Geimsa and May–Grunwald counterstain. Bars: (*A* and *B*) 10 μm; (*C–H*) 100 μm.

Table I. Antisera Titers by ELISA*

| Antigen | ED$_{50}$ (antiserum dilution) | |
	Anti–LC(1–30)	Anti–CC(1–30)
Peptide 1–10	1:1,350	1:150
11–20	0	0
21–30	1:300	0
1–30 (LC)	1:4,000	1:200
1–30 (TGF-β2)	1:600	1:100
TGF-β1	1:1,000	1:450
TGF-β2	0	0

* Antigens were plated in microtiter plates and tested with serial dilutions of IgG fractions of each antiserum, beginning with equal amounts of IgG. Values reported are the dilutions which gave a half-maximal response.

tracellular staining patterns similar to those obtained with anti–LC(1–30). The first antibody was raised to a peptide corresponding to amino acids 50–75 of mature human TGF-β1, while the second antibody was generated to amino acids 266–278 of the human TGF-β1 precursor. Like anti–LC(1–30), both of these antibodies showed intracellular TGF-β localization in A549 cells grown in culture, as well as in tumors raised in nude mice from PC3 cells. Fig. 4 shows an example of this colocalization of staining in the epithelium of human colon carcinomas. The staining obtained with anti–P(50–75), raised to a second epitope of mature TGF-β1, provides further evidence that the intracellular staining is specific for TGF-β1. Specificity was further shown by the reduction in staining which occurred when this antibody was removed by preincubation with TGF-β1–Sepharose (data not shown). A third antibody raised against amino acids 46–56 of the TGF-β1 precursor showed the same localization to epithelial cells of the bovine fibropapillomas as did anti–LC(1–30) (data not shown).

The staining of cells with the antibodies raised to the precursor peptides indicates that these cells may be sites of TGF-β synthesis. Production of mature TGF-β1 occurs by synthesis of the entire TGF-β1 precursor molecule, dimerization and formation of the appropriate disulfide bonds, proteolytic cleavage of the mature TGF-β1 molecule from the precursor sequence, and association with a binding protein to generate latent TGF-β (Miyazono et al., 1988; Wakefield et al., 1988). A punctate pattern of intracellular staining was especially apparent in A549 cells (Fig. 5) with both anti–LC(1–30) and anti–pre(266–278). Furthermore, two recombinant Chinese hamster ovary (CHO) cell lines expressing either low or high amounts of TGF-β showed significantly more staining with both anti–LC(1–30) and anti–pre(266–278) in the high expressing cells than in the low expressing cells, again suggesting that anti–LC(1–30) staining correlated with TGF-β biosynthesis (data not shown).

Discussion

We have found marked differences in the histochemical localization of TGF-β, using two antibodies raised to two different synthetic preparations of the same peptide sequence corresponding to the first 30 amino acids of TGF-β1. The amino-terminal 30 amino acids of TGF-β1 contain three cysteine residues; since both peptides were synthesized with unprotected cysteines, it is likely that different chemical treat-
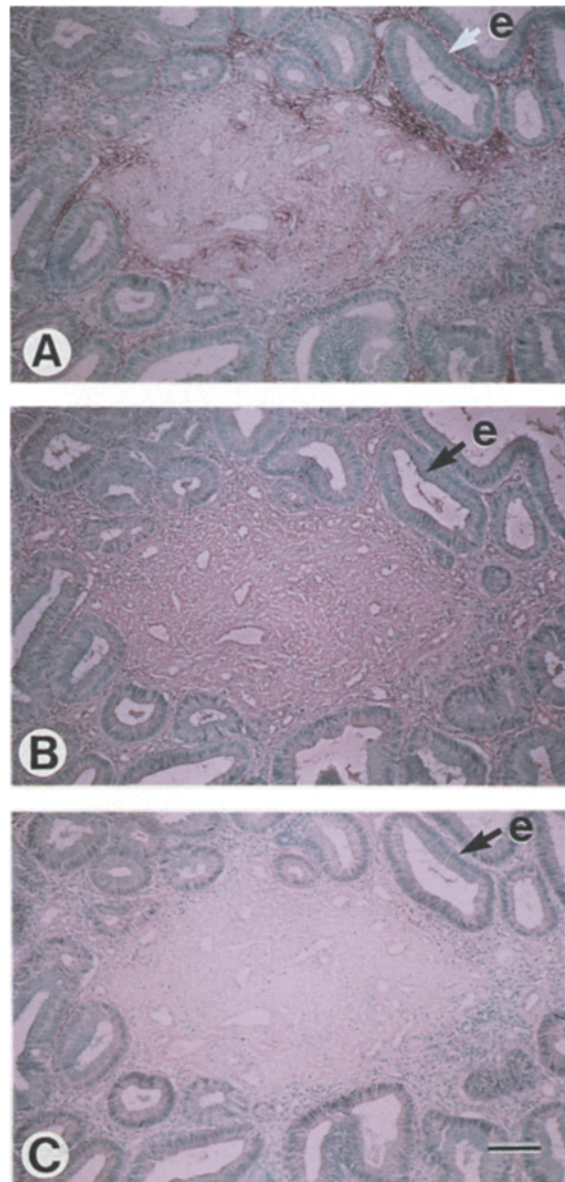

Figure 3. Comparison of staining by anti–CC(1–30) and by anti-fibronectin antibody. Human colon carcinoma stained by (A) anti–CC(1–30), (B) anti–human fibronectin, and (C) normal rabbit serum IgG. While staining by antifibronectin is uniform throughout the tumor stroma, staining by anti–CC(1–30) is localized in the stroma near the tumor epithelium (e). Sections were stained with glucose oxidase (pink reaction product) and counterstained with methyl green. Bar, 100 μm.

ments of the peptide while on the resin or while being removed from the resin have resulted in alternate folding patterns of the peptide. Since antigenic determinants can be as few as 5–10 amino acids (Tanaka et al., 1985; Dyrberg and Oldstone, 1986; Van Regenmortel, 1987), it is conceivable that antibodies raised against the two peptides were generated against unique epitopes of the TGF-β1 molecule. Data presented here and by Ellingsworth et al. (1986) show that both antibodies against P(1–30) recognize TGF-β1 in Western blots, RIA, and ELISA systems. However, our results (Table I) suggest that each antibody recognizes a different epitope
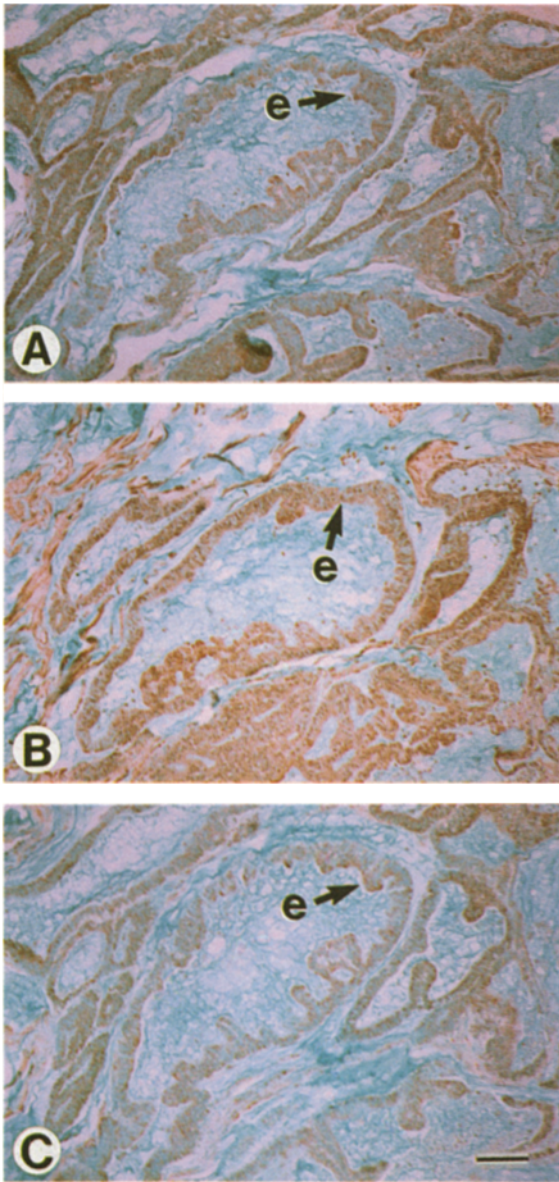
Figure 4. Comparison of intracellular staining by anti-LC(1-30) and other peptide antibodies. Human colon carcinomas were stained by (*A*) anti-LC(1-30), (*B*) anti-P(50-75) raised to a peptide corresponding to amino acids 50-75 of human mature TGF-β1, and (*C*) anti-pre(266-278) raised to a peptide corresponding to amino acids 266-278 of the human TGF-β1 precursor. All antibodies stain tumor epithelium (*e*). Peroxidase, with methyl green counterstain. Bar, 100 μm.

of the 1-30 region of TGF-β1, since in an ELISA assay anti-LC(1-30) showed relatively strong reactivity to peptides corresponding to amino acids 1-10 and 21-30 of TGF-β1, while anti-CC(1-30) exhibited greater reactivity to TGF-β1 than to any of the 10-amino acid peptides. This suggests that the epitope anti-CC(1-30) recognizes most strongly is made up of noncontinuous amino acids (Barlow et al., 1986) that are only brought together correctly in TGF-β1 or P(1-30).

The intracellular localization of immunoreactive TGF-β1 by anti-LC(1-30) was also seen with anti-P(50-75), as well as with two different antibodies raised to the precursor se-

quences not found in mature TGF-β, anti-pre(46-56) and anti-pre(266-278). Transformed cells known to produce relatively large amounts of TGF-β1 are stained particularly well by these antibodies, as are recombinant CHO cells expressing high levels of TGF-β1. Since the precursor remainder is lost from TGF-β upon activation, it is unlikely that the staining pattern seen with anti-pre(266-278) would result from endocytosis of TGF-β after receptor binding; rather, the data suggest that cells which are stained by these antibodies are sites of TGF-β1 synthesis.

The typical mutually exclusive staining of intracellular TGF-β1 by anti-LC(1-30) and extracellular TGF-β1 by anti-CC(1-30) suggests a possible conformational change in TGF-β1 upon secretion. Since little is known about the processing, secretion, and activation of TGF-β, the exact nature of this conformational change is not known. The intracellular form of TGF-β recognized by anti-LC(1-30) may be the unprocessed precursor form or TGF-β that has been proteolytically cleaved from its precursor, but still associated with the precursor remainder, as is known to occur in latent TGF-β (Miyazono et al., 1988; Wakefield et al., 1988). Secretion may change the spatial relationship of TGF-β with other proteins in the latent TGF-β complex and mask the epitope recognized by anti-LC(1-30) while exposing the epitope recognized by anti-CC(1-30).

Anti-CC(1-30), anticollagen, and antifibronectin antibodies all stain extracellular matrix. However, in contrast to the uniformly distributed staining of matrix found with anticollagen and antifibronectin antibodies, the extracellular staining by anti-CC(1-30) is localized near cells which show intracellular staining by anti-LC(1-30). This is especially striking in human colon carcinomas, in which antifibronectin antibody gives uniform staining throughout the stroma, while anti-CC(1-30) shows staining of the stroma only near epithelial cells that stain positive for TGF-β with anti-LC(1-30) (Fig. 4). This presence of TGF-β in the stroma may be partially responsible for the desmoplastic response associated with invasive carcinomas. Similar variations in the patterns of staining by anti-CC(1-30), anti-LC(1-30), antifibronectin, and anticollagen antibodies have also recently been found in the developing mouse lung (Heine, U., personal communication). TGF-β has been shown to copurify with fibronectin under neutral conditions (Fava and McClure, 1987); whether the association of TGF-β with fibronectin in vivo is the result of specific binding of either active or latent TGF-β1 to fibronectin, or it is the result of a nonspecific association of TGF-β1 with fibronectin and possibly with other matrix proteins, remains to be determined.

The predominantly extracellular staining pattern reported here for anti-CC(1-30) has been reported previously in the mouse embryo by Heine et al. (1987), who found widespread and abundant extracellular TGF-β in many mesenchymal tissues, although some occasional intracellular localization of TGF-β with anti-CC(1-30) was found in specialized cells of mesenchymal origin, such as osteoblasts and chondrocytes. With the new anti-LC(1-30) that we have described here, we now have a new tool for further investigation of the spatial and temporal localization of TGF-β1 in the mouse embryo. We have recently found that anti-LC(1-30) stains many epithelial cells of the embryo, with intracellular mesenchymal staining occurring to a lesser degree (Flanders, K., unpublished observations). Interestingly, in situ hybridization of
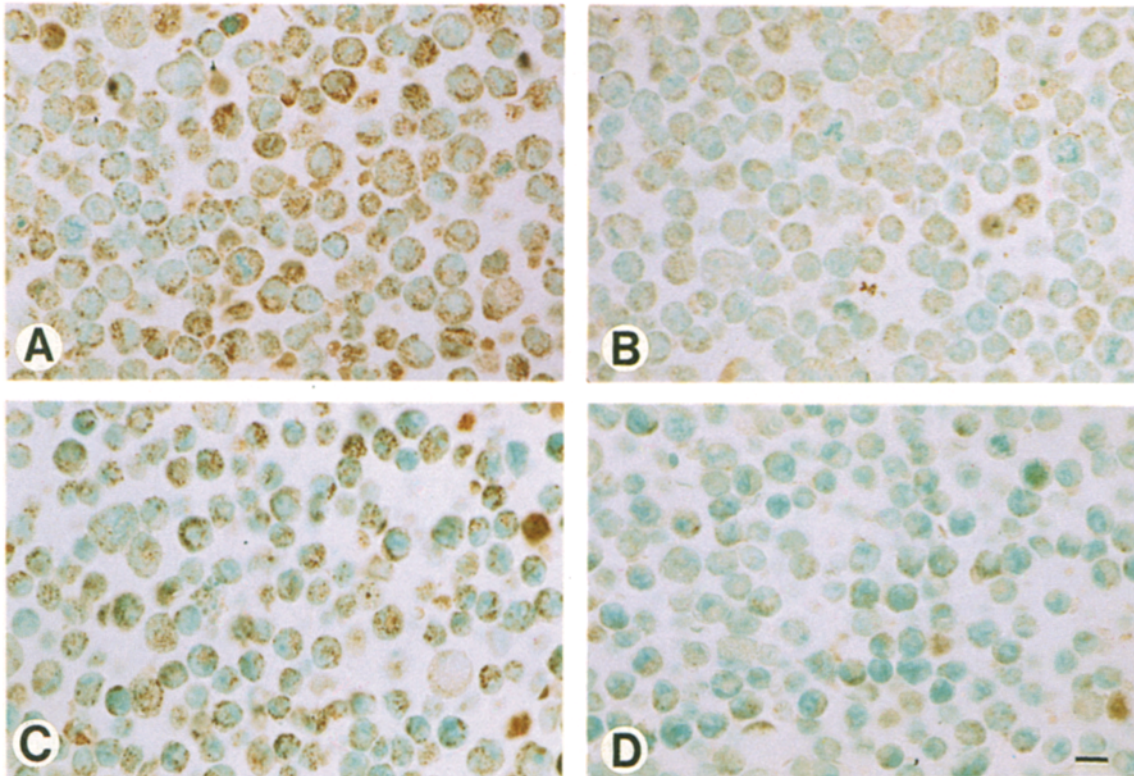
Figure 5. Staining of sections of pellets of A549 cells by anti–LC(1–30) and anti–pre(266–278). Sections were stained by (*A*) anti–LC(1–30), (*B*) anti–LC(1–30) incubated with a 20-fold molar excess of peptide LC(1–30) over IgG, (*C*) anti–pre(266–278), and (*D*) anti–pre(266–278) preincubated with a 20-fold molar excess of peptide pre(266–278) over IgG. Both antibodies show punctate cytoplasmic staining that is abolished when antibody is preincubated with appropriate peptide. Peroxidase, with methyl green counterstain. Bar, 100 μm.

TGF-β1 in the mouse embryo indicates that TGF-β1 mRNA is present in both epithelial and mesenchymal cells (Lehnert and Akhurst, 1988), similar to the localization seen with anti–LC(1–30).

In conclusion, it is clear that the combined use of anti–LC(1–30) and anti–CC(1–30) antibodies will be a valuable tool for investigating the role of TGF-β in reciprocal relationships between epithelium and mesenchyme in many physiological and pathological states, such as embryogenesis, morphogenesis, repair of tissue damage, and carcinogenesis. The additional use of in situ hybridization techniques for TGF-β, which have been described recently (Lehnert and Akhurst, 1988; Sandberg et al., 1988; Wilcox and Derynck, 1988) combined with immunohistochemistry, will provide further important insights into the mechanism of TGF-β action. The present studies should also focus attention on the future importance of physicochemical studies on the structure of the TGF-β molecule itself (including its latent form), since the ultimate understanding of the complex staining patterns reported here will rest on elucidation of the molecular structure of the actual epitopes that are stained by different antibodies.

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