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### Research Article

The biological activity of TGF-beta, an important modulator of cell proliferation and extracellular matrix formation, is governed by dissociation of mature TGF-beta from an inactive, latent TGF-beta complex in a process that is critical to its role in vivo. So far, it has not been possible to monitor activation in vivo since conventional immunohistochemical detection does not accurately discriminate latent versus active TGF-beta, nor have events associated with activation been defined well enough to serve as in situ markers of this process. We describe here a modified immunodetection method using differential antibody staining that allows the specific detection of active versus latent TGF-beta. Under these conditions, we report that an antibody raised to latency-associated peptide detects latent TGF-beta, and we demonstrate that LC(1-30) antibodies specifically recognize active TGF-beta 1 in tumor xenografts overproducing active TGF-beta 1, without cross-reactivity in tumors expressing similar levels of latent TGF-beta 1. We previously reported that TGF-beta immunoreactivity increases in murine mammary gland after whole-body <sup>60</sup>Co-gamma radiation exposure. Using differential antibody staining we now show that radiation exposure specifically generates active TGF-beta 1. While latent TGF-beta 1 was widely distributed in unirradiated tissue, active TGF-beta 1 distribution was restricted. Active TGF-beta 1 increased significantly within 1 h of irradiation concomitant with decreased latent TGF-beta immunoreactivity. This rapid shift in immunoreactivity provides the first evidence for [...]

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## Transforming Growth Factor- $\beta$ Activation in Irradiated Murine Mammary Gland

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### Abstract

The biological activity of TGF- $\beta$ , an important modulator of cell proliferation and extracellular matrix formation, is governed by dissociation of mature TGF- $\beta$  from an inactive, latent TGF- $\beta$  complex in a process that is critical to its role in vivo. So far, it has not been possible to monitor activation in vivo since conventional immunohistochemical detection does not accurately discriminate latent versus active TGF- $\beta$ , nor have events associated with activation been defined well enough to serve as in situ markers of this process. We describe here a modified immunodetection method using differential antibody staining that allows the specific detection of active versus latent TGF- $\beta$ . Under these conditions, we report that an antibody raised to latency-associated peptide detects latent TGF- $\beta$ , and we demonstrate that LC(1-30) antibodies specifically recognize active TGF- $\beta$ 1 in tumor xenografts overproducing active TGF- $\beta$ 1, without cross-reactivity in tumors expressing similar levels of latent TGF- $\beta$ 1. We previously reported that TGF- $\beta$  immunoreactivity increases in murine mammary gland after whole-body <sup>60</sup>Co- $\gamma$  radiation exposure. Using differential antibody staining we now show that radiation exposure specifically generates active TGF- $\beta$ 1. While latent TGF- $\beta$ 1 was widely distributed in unirradiated tissue, active TGF- $\beta$ 1 distribution was restricted. Active TGF- $\beta$ 1 increased significantly within 1 h of irradiation concomitant with decreased latent TGF- $\beta$  immunoreactivity. This rapid shift in immunoreactivity provides the first evidence for activation of TGF- $\beta$  in situ. This reciprocal pattern of expression persisted for 3 d and was accompanied by decreased recovery of latent TGF- $\beta$ 1 from irradiated tissue. Radiation-induced activation of TGF- $\beta$  may have profound implications for understanding tissue effects caused by radiation therapy. (*J. Clin. Invest.* 1994. 93:892-899.) Key words: ionizing radiation • transforming growth factor- $\beta$  • mammary gland • immunohistochemistry • extracellular matrix

### Introduction

TGF- $\beta$  is a major modulator of cellular proliferation and extracellular matrix formation. TGF- $\beta$ 1, the best studied protein of

the three differentially expressed and regulated TGF- $\beta$  isoforms, is derived from a 390-amino acid precursor cleaved to produce a 112-amino acid carboxy-terminal peptide (1). The homodimer of this peptide is noncovalently associated with a dimer of the processed amino-terminal pro-segment, called the latency-associated peptide (LAP).<sup>1</sup> This secreted latent TGF- $\beta$  complex is unable to bind to TGF- $\beta$  receptors until the biologically active 24-kD mature TGF- $\beta$  is dissociated from LAP (2, 3). Thus, the biological activity of TGF- $\beta$  is highly controlled by its release from the latent complex, which is considered a critical regulatory event for the function of TGF- $\beta$  in vivo. Although activation has been postulated to occur in a wide variety of physiological and pathological situations in vivo, it has so far not been possible to immunohistochemically distinguish latent and active TGF- $\beta$ .

This technical limitation has resulted in an inability to define events associated with or leading to biological activation of TGF- $\beta$  in situ. Elevated TGF- $\beta$  immunoreactivity has been observed in tissues during normal physiological (4) and pathological (5-7) processes that result in inflammation, tissue repair, or extracellular matrix deposition. We recently showed using immunofluorescence that TGF- $\beta$  is elevated in the murine mammary gland after whole-body exposure to <sup>60</sup>Co- $\gamma$  radiation (8). TGF- $\beta$ 1 immunoreactivity was rapidly induced in the epithelium and the previously negative adipose stroma, and was followed by novel expression of collagen type III, a known target of TGF- $\beta$ . However, whether the altered TGF- $\beta$  immunostaining reflects increased TGF- $\beta$  deposition or altered epitope accessibility, as might occur if TGF- $\beta$  was dissociated from the latent complex, could not be determined with available methods. Whereas altered immunoreactivity is often assumed to represent a corresponding change in TGF- $\beta$  activity, the current methodology detects TGF- $\beta$  irrespective of knowledge of its state of activation. For example, antibodies to LAP are expected to not only recognize latent TGF- $\beta$  but the same pro-segment after dissociation of the latent complex. Likewise, recognition of mature TGF- $\beta$ 1, the active polypeptide that is part of the latent complex, may be impartial to the activation state. However, if the antigenic site is masked, for example by association with LAP, or depends on its conformation, which may change with activation, antibody recognition of TGF- $\beta$ 1 might be wholly dependent upon activation. Furthermore, since accurate immunolocalization in general depends on target protein preservation after tissue fixation, concern about whether tissue processing might activate TGF- $\beta$  has been raised (9). Thus, interpretation of TGF- $\beta$  antibody reactivity is confounded by a number of restrictions, including questions

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1. Abbreviation used in this paper: LAP, latency-associated peptide.

regarding antibody specificity in processed tissue, a lack of information on the preservation of endogenous latent and active TGF- $\beta$ , and the inability to independently demonstrate the status of TGF- $\beta$  in situ.

The specificity of antibody detection using various tissue fixation methods could be resolved by using a tissue in which the activation status of TGF- $\beta$  is known. Thus, we used two xenograft tumors constructed to express active and latent TGF- $\beta$  to develop modified tissue fixation and immunodetection procedures permitting differential antibody staining of active vs. latent TGF- $\beta$ 1. This method was used to evaluate the activation state of TGF- $\beta$  after irradiation of the murine mammary gland in vivo. Immunostaining revealed that radiation induces a rapid shift from predominantly latent to active TGF- $\beta$ , which indicates that irradiation leads to activation of TGF- $\beta$ 1 in situ.

## Methods

**Antibodies.** Polyclonal rabbit antiserum raised against a synthetic peptide corresponding to the amino-terminal 30 amino acids of mature TGF- $\beta$ , designated LC(1-30) (10), was provided by Dr. M. Sporn (National Institutes of Health, Bethesda, MD).

Anti-LAP antibody was produced in goats immunized with recombinant human LAP. The recombinant LAP used as immunogen was purified from medium conditioned by a Chinese hamster ovary cell line transfected with an expression plasmid for the human TGF- $\beta$ 1 pre-pro-protein. LAP was dissociated and purified from mature TGF- $\beta$ 1 by acidification and reverse phase chromatography. Total IgG was purified by protein G affinity chromatography. Both the dimeric LAP and its monomers were detectable using this antibody on Western blot analysis. Approximately 2 ng/lane of LAP was detected in Western blot assays using an antibody concentration of 1  $\mu$ g/ml. Direct ELISA demonstrated that this antibody is specific for the LAP derived from the TGF- $\beta$ 1 precursor and does not react with LAP from TGF- $\beta$ 2 nor with mature TGF- $\beta$ 1, TGF- $\beta$ 2, or TGF- $\beta$ 3.

**Irradiation.** Adult (> 12 wk of age) virgin female BALB/c mice were irradiated with  $^{60}\text{Co}$ - $\gamma$  radiation using a dose rate of 0.35 Gy/min to a total dose of 5 Gy. After 1 h or 1, 3, or 7 d, animals from each exposure group were killed by cervical dislocation in accordance with animal welfare procedures and the inguinal mammary glands were removed for immunohistochemistry.

**Tumor tissue.** Two cell clones of the 293 renal sarcoma cell line that were transfected with TGF- $\beta$ 1 expression plasmids were used (11). Clone B9 overproduces latent TGF- $\beta$ 1 whereas clone C19, transfected with an expression plasmid for a modified TGF- $\beta$ 1 precursor, secretes predominantly active TGF- $\beta$ 1. Cells were cultured to subconfluence and  $2 \times 10^6$  cells were injected into each flank of adult female nude mice as described (11). Palpable tumors were obtained within 3–5 wk and were excised from mice killed by cervical dislocation in accordance with approved animal welfare guidelines. Tissue was processed as described below for immunohistochemistry.

**Immunohistochemistry.** Tissues were embedded in OCT compound (Miles Inc., Elkhart, IN) and frozen in a dry ice/ethanol bath. The blocks were stored at  $-70^\circ\text{C}$  until sectioning. 4- $\mu\text{m}$  thick sections were obtained from sectioning at  $-30$  to  $-35^\circ\text{C}$ . For use with anti-LC(1-30), the sections were immediately fixed onto gelatin-coated coverslips using  $-20^\circ\text{C}$  methanol/acetone (1:1), air dried, and stored at  $-20^\circ\text{C}$ . Anti-LAP antibodies were used with sections fixed for 20 min with 2% paraformaldehyde and rinsed three times with PBS containing 0.1 M glycine. The best fixative was determined by comparing each antibody using sections fixed in various ways and selecting the condition under which reactivity was strongest.

Before antibody treatment, the tissue sections were treated for 60 min in supernatant from a solution of 0.5% casein in phosphate-buf-

fered saline ( $\text{Na}_2\text{PO}_4$ , 0.9% NaCl), pH 7.4 (blocking buffer). The sections were then incubated with 25  $\mu\text{l}$  of the primary antibody diluted to working concentration in blocking buffer and incubated overnight at  $4^\circ\text{C}$ . Antibody controls were incubated without primary antibody or with similar concentrations of normal serum or IgG from the same species as the primary antibody. Sections were washed and incubated with a 1:100 dilution of the appropriate fluorescein isothiocyanate-conjugated secondary antibody for 1 h at room temperature. After several washes, the sections were mounted in Vectashield (Vector Laboratories, Palo Alto, CA). Sections were viewed using a microscope (Olympus Corp. Precision Instr. Div., Lake Success, NY) equipped with epifluorescence and photographed using Kodak film TriX 400. Identical exposures were taken for a given antigen in each experiment and printed using identical parameters in order to facilitate comparisons. Each antibody was examined in at least three independent staining experiments from two independent sets of irradiated animals. Antibody controls were negative in comparison to specific antibody staining.

**Assay for TGF- $\beta$  activity.** Mammary gland was excised from animals at various times postirradiation, chopped, and incubated at  $37^\circ\text{C}$  for 1 h in DMEM/F12 media containing 2% BSA. 2.5  $\mu\text{l}$  of 0.2 M phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) and 5  $\mu\text{l}$  of aprotinin ( $\sim 10$  trypsin inhibitor units/ml; Sigma Chemical Co.) were added to each milliliter of conditioned medium samples, which were frozen on dry ice and stored at  $-70^\circ\text{C}$ . Conditioned media were tested for the presence of latent TGF- $\beta$  using inhibition of mink lung epithelial cell DNA synthesis assayed by [ $^3\text{H}$ ]thymidine incorporation (12). Samples were heated at  $80^\circ\text{C}$  for 10 min to activate latent TGF- $\beta$  (13). 80  $\mu\text{l}$  of a 1:1 dilution of each experimental sample was added 2 h after plating to wells containing  $5 \times 10^4$  cells in 0.2 ml DMEM containing 0.2% serum. 20 h later, the cell cultures were labeled for 2 h by the addition of 0.5 mCi [ $^3\text{H}$ ]thymidine (4 mCi/ml; Amersham Corp., Arlington Heights, IL) and  $^3\text{H}$  incorporation into DNA was determined by scintillation counting. Neutralizing antibody to TGF- $\beta$  (R & D Systems, Minneapolis, MN) was used to demonstrate specificity of TGF- $\beta$  inhibition of DNA synthesis in experimental samples.

## Results

**Differential use of antibodies distinguishes latent and active TGF- $\beta$ .** We compared the specificity of several antibodies using two xenograft tumors derived from transfected 293 renal sarcoma cells that overexpress TGF- $\beta$  (11). The clonal tumor cell lines B9 and C19 were derived from the same parental cell line and secrete similar levels of TGF- $\beta$ 1; however, B9 secretes latent TGF- $\beta$ 1, whereas C19 makes predominantly active TGF- $\beta$ 1. This difference in TGF- $\beta$  activation between the tumor clones results from directed mutation of two cysteines to serines in the pre-pro-segment TGF- $\beta$  peptide (11), which prevents disulfide bond formation in LAP resulting in constitutively active TGF- $\beta$  (14). The activation of TGF- $\beta$ 1 secreted from C19 cells is presumably a consequence of decreased interaction of the mutated LAP with TGF- $\beta$ 1, thus resulting in a high level of dissociation of LAP from TGF- $\beta$ 1. This is in contrast with the physiological activation mechanisms of TGF- $\beta$ 1, which are thought to result from proteolytic degradation of LAP and subsequent release of active TGF- $\beta$ 1.

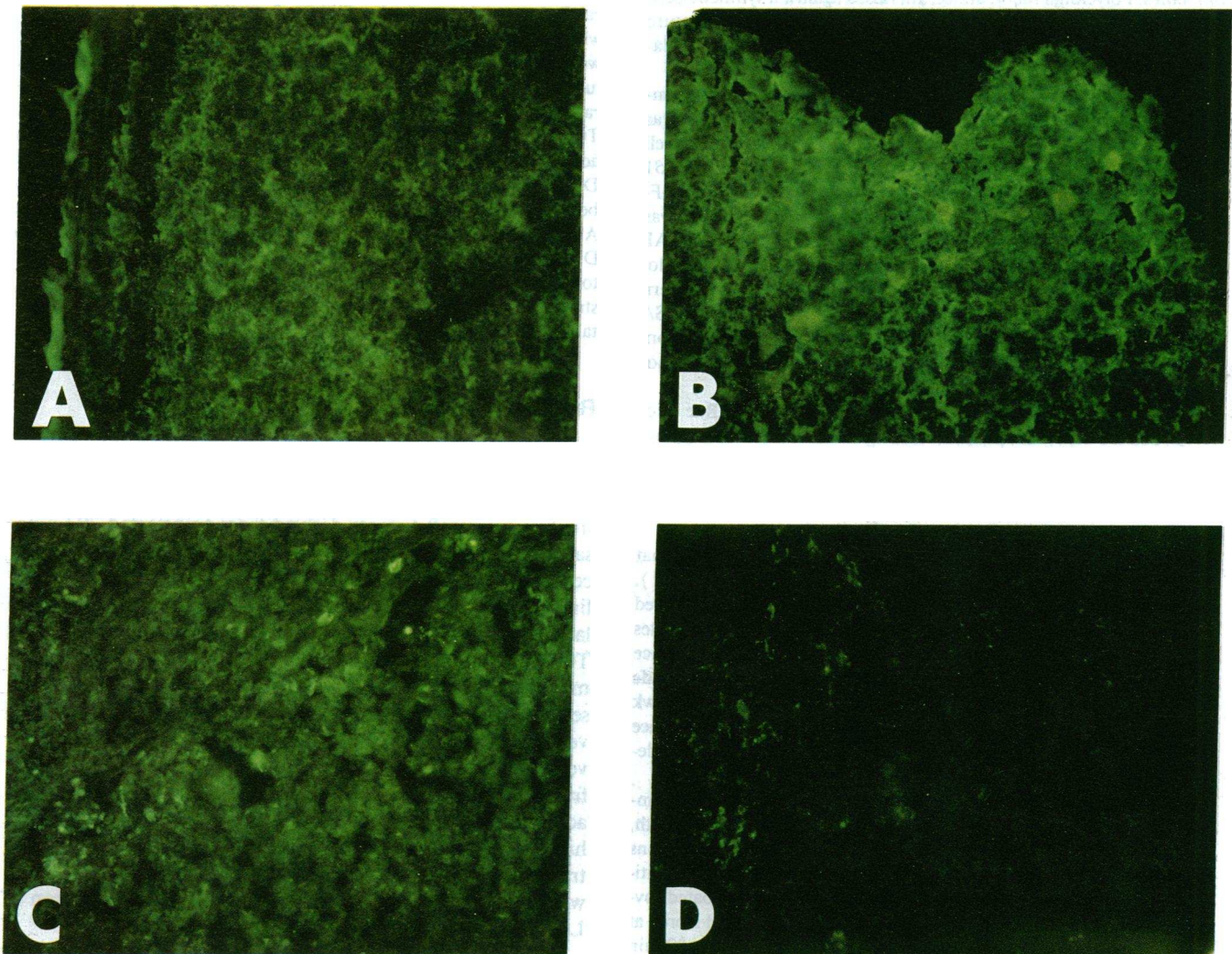
Previous immunohistochemical detection methods for TGF- $\beta$  have used paraffin-embedded tissue fixed in either Bouin's fluid and neutral buffered formalin (10), Bouin's fluid alone (15), or 10% neutral buffered formalin alone (6). To potentiate the preservation of latent and active TGF- $\beta$ , we elected to use cryosections from frozen unfixed tissue for im-

munodetection of TGF- $\beta$ . We then tested various fixatives in conjunction with immunofluorescence detection of antibodies raised to distinct regions of the latent TGF- $\beta$  complex. We chose two antibodies for use in this study. LC(1-30) antibodies raised against a 30-amino acid peptide sequence residing at the amino terminus of mature TGF- $\beta$  (10) were used with cryosections fixed with methanol/acetone. Antibodies raised against purified recombinant LAP were used with paraformaldehyde-fixed tissue.

Anti-LAP showed similar immunoreactivity for both types of tumor sections (Fig. 1, *A* and *B*), indicating that they express similar levels of LAP and, by inference, of mature TGF- $\beta$ . In comparison, substantial LC(1-30) immunostaining was observed in C19-derived tumor tissue producing active TGF- $\beta$ 1, whereas negligible immunostaining was detected in B9 tumor sections (Fig. 1, *C* and *D*). The lack of immunostaining in B9 tumor despite abundant latent TGF- $\beta$  implies that the embedding and staining procedure did not activate endogenous latent TGF- $\beta$ . Furthermore, these data indicate that the LC(1-30)

epitope of mature TGF- $\beta$ 1 is masked when TGF- $\beta$  is associated with LAP in the latent complex. Thus, since LC(1-30) was raised against an epitope of mature TGF- $\beta$ 1 (10), but did not react with B9 tumor tissue, even though the total level of latent TGF- $\beta$ 1 was similar, LC(1-30) appears to specifically recognize active TGF- $\beta$  while anti-LAP detects total TGF- $\beta$  under these conditions.

*Active TGF- $\beta$  increases in mammary gland after irradiation.* The ability to selectively detect active TGF- $\beta$ 1 by immunohistochemistry and to preserve endogenous latent TGF- $\beta$ 1 allows examination of the relative distribution of both forms of TGF- $\beta$ 1 in tissues during physiological and pathological processes. We used this method to evaluate the distribution of TGF- $\beta$ 1 in the murine mammary gland after irradiation. We have previously shown TGF- $\beta$ 1 immunoreactivity is faint in normal mammary gland but prominent in irradiated mammary gland from 1 h to 7 d postradiation (8). This rapid rise in immunoreactivity could result from either increased total protein, i.e., due to new synthesis and/or deposition of TGF- $\beta$ 1, or

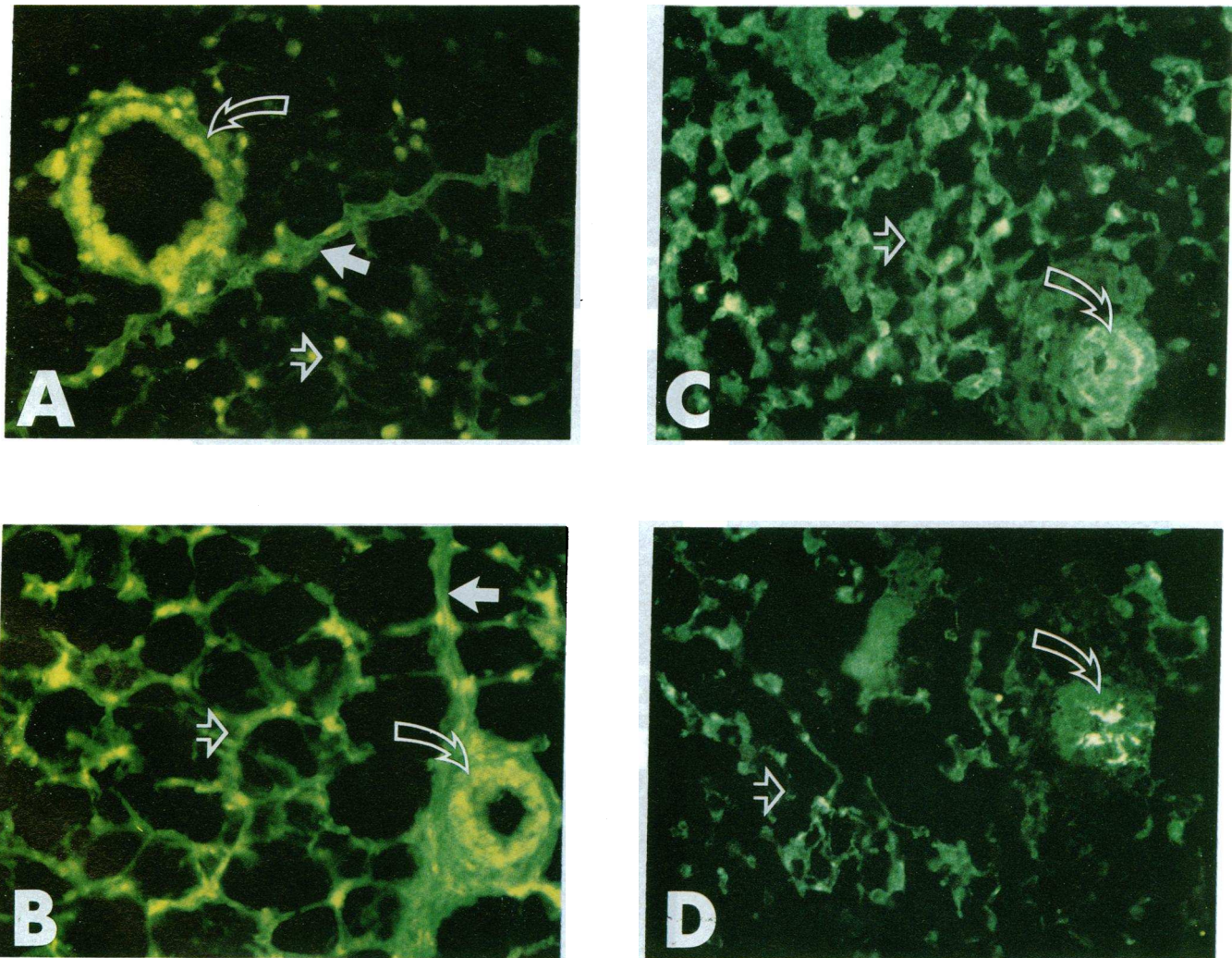


**Figure 1.** Immunofluorescent localization of anti-LAP (*A* and *B*) and LC(1-30) (*C* and *D*) immunoreactivity in human xenograft tumors. Tumors derived from 293 clone C19 cells (*A* and *C*), which overexpress active TGF- $\beta$ , and clone B9 cells (*B* and *D*), which overexpress latent TGF- $\beta$ , are both immunostained using anti-LAP but only C19 is positive with LC(1-30). Thus, LC(1-30) reactivity appears to be contingent upon activation. It should be noted that the intensity of the immunofluorescence should only be compared for a given antibody since differences between distinct antibodies do not necessarily reflect their relative abundance.  $\times 20$ .

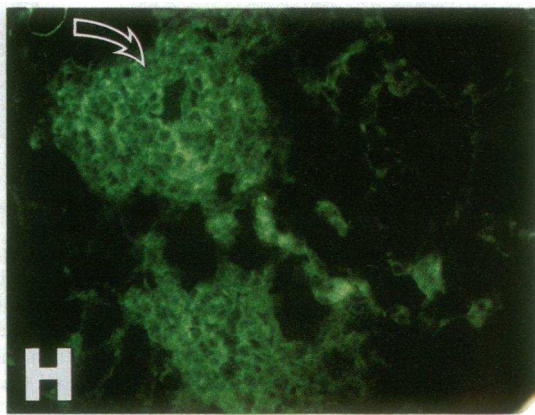
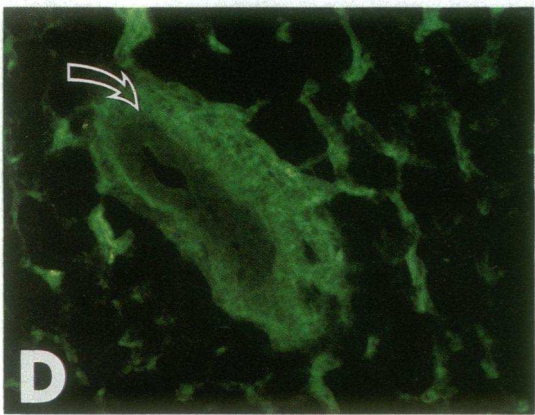
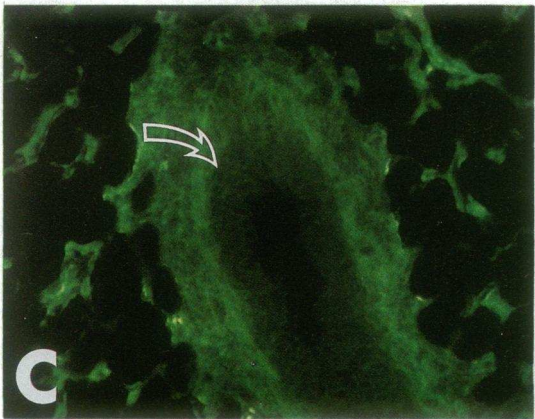
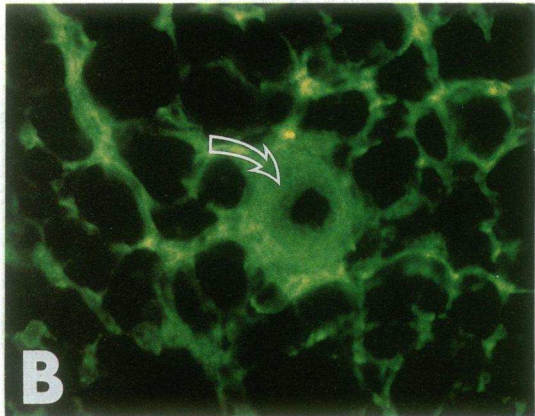
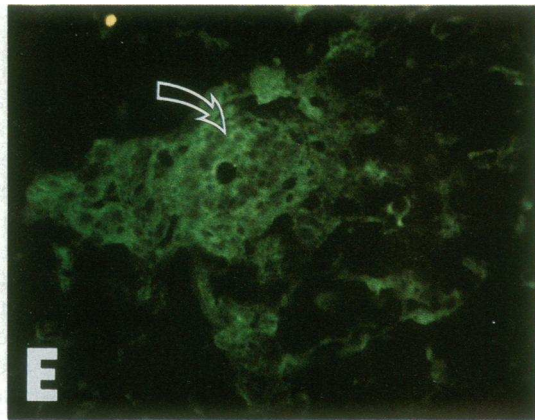
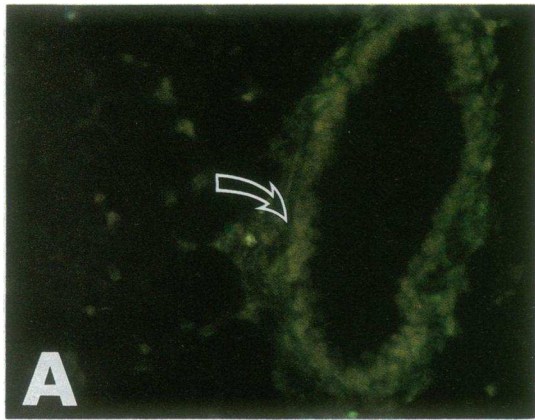
to increased epitope access, which could occur after local activation of latent TGF- $\beta$ 1. Increased deposition without proteolytic activation would presumably result in increased LAP immunoreactivity since TGF- $\beta$ 1 is secreted as a latent complex.

To evaluate these possibilities, we analyzed normal and irradiated mammary gland tissue using the immunofluorescence detection procedures outlined above. In the unirradiated mammary gland, anti-LAP was strongly expressed in the epithelium and was less abundant in the periepithelial stroma, while LC(1-30) faintly immunostained the epithelium, periepithelial stroma, and tissue septa in normal mammary gland (Fig. 2, *A* and *C*). Remarkably, whereas the adipose stroma was strongly positive for anti-LAP, it was essentially negative with LC(1-30). The distinct pattern of staining by these two antibodies indicates that while latent TGF- $\beta$ 1 is abundant, active TGF- $\beta$  is restricted and is particularly limited in the adipose stroma.

This pattern of TGF- $\beta$  immunoreactivity altered significantly 1 h after radiation exposure (Fig. 2, *B* and *D*). Anti-LAP immunoreactivity was strikingly diminished in the adipose stroma, while LC(1-30) immunoreactivity was induced in the previously negative adipose stroma and was dramatically increased in the epithelium and periepithelial stroma. The pattern of reversal in which LC(1-30) immunoreactivity was induced in parallel with loss of LAP immunoreactivity was particularly marked in the adipose stroma. Assuming that LAP is degraded (16) or otherwise altered after release from the TGF- $\beta$ 1 latent complex and that the LC(1-30) antigenic site is masked by its association with LAP, these data support the conclusion that, either directly or indirectly, ionizing radiation exposure leads to TGF- $\beta$ 1 activation. Further evidence of TGF- $\beta$ 1 biological activity is that LC(1-30) immunostaining in the adipose stroma corresponds with novel collagen type III expression 3 d after irradiation (8).



**Figure 2.** Immunofluorescent localization of active TGF- $\beta$  using LC(1-30) (*A* and *B*) and latent TGF- $\beta$ 1 complex using anti-LAP (*C* and *D*) in normal (*A* and *C*) and irradiated (*B* and *D*) mammary gland. The nuclei in *A* are lightly counterstained with propidium iodide and thus appear yellow. In unirradiated mammary gland, active TGF- $\beta$  (*A*) was detected principally in the epithelium and stromal sheath (*curved arrow*) as well as the tissue septa (*filled arrow*), whereas latent TGF- $\beta$  (*C*) was strongest in the epithelium (*curved arrow*) and adipose stroma (*open arrow*). 1 h after 5-Gy radiation, active TGF- $\beta$  was dramatically increased in epithelium, peri-epithelial stroma, and tissue septa (*B*). In addition, new staining was observed in cells in the adipose stroma (*open arrow*). In contrast, latent TGF- $\beta$  decreased, particularly in the adipose stroma, presumably due to proteolytic degradation of LAP (*D*). The rapid shift in LAP vs. LC(1-30) immunoreactivity provides the first marker of TGF- $\beta$ 1 activation in situ.  $\times 40$ .



Additional changes in the immunostaining pattern occurred at longer intervals after radiation exposure (Fig. 3). Anti-LAP immunoreactivity progressively decreased to barely detectable levels during the first 3 d after radiation exposure and returned to levels comparable to unirradiated tissue only at ~ 7 d. In contrast, LC(1-30) immunoreactivity remained elevated for 7 d postirradiation.

The decreased anti-LAP immunoreactivity could be due to decreased latent TGF- $\beta$ 1 or the result of masking by association with other proteins, such as those of the extracellular matrix. We measured the relative abundance of latent TGF- $\beta$  protein in medium conditioned by tissue fragments from irradiated mice using a standard TGF- $\beta$  assay based on its inhibition of DNA synthesis in mink lung epithelial cells (Fig. 4). Conditioned media from unirradiated tissue inhibited DNA synthesis more than that from irradiated tissue fragments, which indicates that there was no significant increase in latent TGF- $\beta$ 1 release, and in fact, was somewhat less at all time points. This activity could be neutralized using TGF- $\beta$ -specific antibodies (Fig. 4 B). These data argue against increased TGF- $\beta$ 1 protein production as the basis for elevated LC-1-30 immunoreactivity and support the observed decrease in LAP immunoreactivity.

In summary, this study shows that radiation exposure leads to: (a) a rapid shift from predominantly LAP immunoreactivity to LC(1-30) reactivity; (b) a rapid induction of LC(1-30) immunoreactivity in the adipose stroma; (c) parallel loss of LAP immunoreactivity; and (d) decreased latent TGF- $\beta$ 1 in media conditioned by irradiated mammary gland. These data provide in situ evidence consistent with TGF- $\beta$ 1 activation.

## Discussion

The biological activity of TGF- $\beta$  is governed by dissociation of mature TGF- $\beta$  from an inactive, latent TGF- $\beta$  complex in a process that is critical to the role of TGF- $\beta$  in vivo. So far, it has not been possible to monitor activation in vivo, since conventional immunohistochemical detection does not accurately discriminate latent vs. active TGF- $\beta$  nor have events associated with activation been defined well enough to serve as in situ markers of this process. We describe a modified immunodetection method based on the use of differential antibody detection of two regions of latent TGF- $\beta$  that allows the specific detection of active TGF- $\beta$  and identification of events associated with activation. This method was used to evaluate the status of TGF- $\beta$  in tissue after ionizing radiation exposure and shows that irradiation leads to increased immunoreactivity using antibodies specific for active TGF- $\beta$  concomitant with loss of latent TGF- $\beta$ 1 immunoreactivity. Active TGF- $\beta$  was apparent within 1 h after radiation exposure and persisted for 7 d postirradiation, and was inversely correlated with latent TGF- $\beta$ 1 detection over a similar time course. These two complementary and reciprocal changes are best explained by the conversion of the

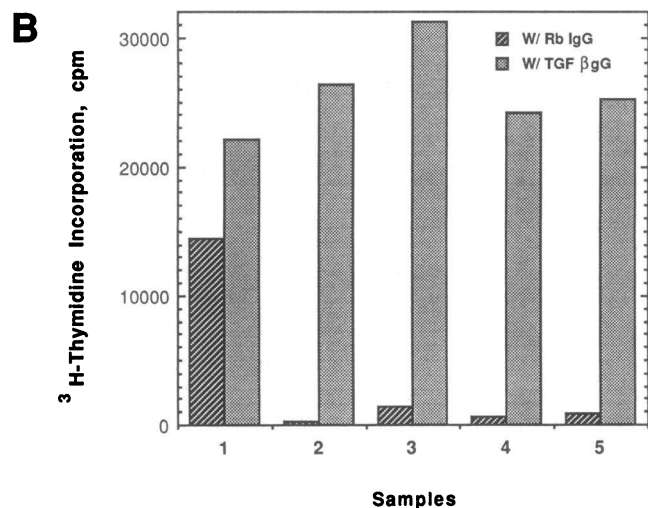
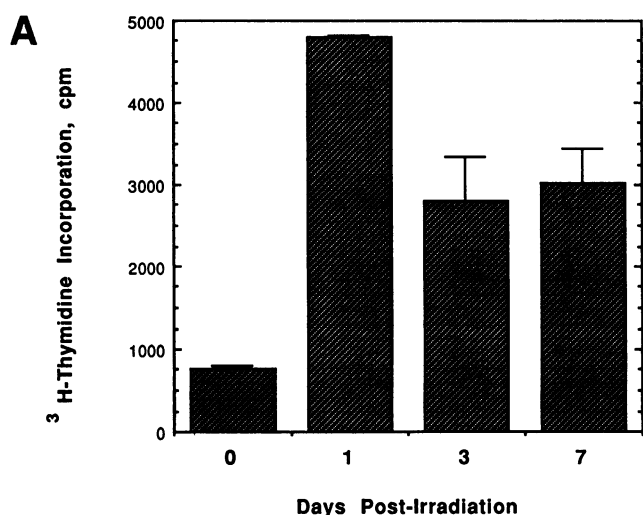
available pool of latent TGF- $\beta$ 1 into active TGF- $\beta$ 1. The rapid shift from predominantly latent to active TGF- $\beta$ , together with our previous report that stromal collagens rapidly remodel in the irradiated mammary gland (8), lead to the conclusion that radiation exposure induces activation of latent TGF- $\beta$ 1 in situ. Radiation-induced TGF- $\beta$ 1 activation is the first documented example of TGF- $\beta$  activation in vivo.

Our studies indicate that a discrete event, such as radiation exposure, leading to activation is accompanied by two significant alterations in TGF- $\beta$  immunoreactivity: increased access to amino-terminal epitopes of mature TGF- $\beta$ 1 and decreased stability of, or access to, LAP. Indeed, while both events may have been predicted based on our knowledge of TGF- $\beta$  activation in vitro, there was little prior basis for interpreting changes in TGF- $\beta$  immunoreactivity in vivo since it is generally assumed that TGF- $\beta$  antibodies are insensitive to its activation status. These postirradiation changes are not unique to the specific antibodies used herein since increased staining is also seen using CC(1-30), which was raised to the same peptide sequence as LC(1-30)(8), and decreased immunoreactivity similar to anti-LAP is seen with an antibody raised to recombinant latent TGF- $\beta$  complex (Barcellos-Hoff, M. H., and M. L.-S. Tsang, unpublished data). Thus, the elevated immunoreactivity of antibodies directed against specific regions of mature TGF- $\beta$ 1 in conjunction with loss of LAP immunoreactivity may serve as indicators of in situ TGF- $\beta$  activation during physiological and pathologic processes.

Little is as yet known about the in vivo mechanisms of latent TGF- $\beta$  activation. Proteases are thought to be involved in selectively degrading LAP, resulting in the release of active TGF- $\beta$  (16, 17). The functional involvement of proteases in latent TGF- $\beta$  activation is best substantiated in the studies on cocultured endothelial and smooth muscle cells. In this culture system, proteolytic conversion of plasminogen into plasmin results in plasmin-mediated activation of TGF- $\beta$ 1 (18, 19). This plasminogen activator-induced cascade may also be responsible for the activation of TGF- $\beta$ 1 in several other systems and during wound healing. Whereas the generality of this enzymatic cascade in TGF- $\beta$  activation remains to be established, plasmin may be the responsible agent in radiation-induced TGF- $\beta$ 1 activation as well since radiation elicits rapid elevation of plasminogen activator expression and activity in cultured cells (20), which is reminiscent of the rapid radiation-induced TGF- $\beta$ 1 activation in the murine mammary gland.

The physiological consequences of radiation-induced TGF- $\beta$  activation are apparent from the rapid remodeling of collagen types I and III observed in the irradiated mammary gland (8). In particular, the abundant collagen type III expression in the previously negative adipose stroma 3 d after radiation exposure colocalized with the rapid activation of TGF- $\beta$ 1 (8). The temporal sequence, unique expression of collagen III in the irradiated tissue, and the extensively documented ability of TGF- $\beta$  to induce secretion of several major extracellular ma-

*Figure 3.* Immunofluorescent staining of active TGF- $\beta$  using LC(1-30) (A-D) and latent TGF- $\beta$  complex using the anti-LAP antibody (E-H) in mammary gland sections at different times after irradiation. (A and E) Unirradiated tissue; (B and F) 1 d; (C and G) 3 d; and (D and H) 7 d after irradiation. Cross sections of an epithelial duct (curved arrow) surrounded by peri-epithelial stroma embedded in adipose stroma are shown. Whereas there is little active TGF- $\beta$  in the adipose stroma of the unirradiated tissue (A), it is strongly induced in the adipose stroma of the irradiated tissue (B-D). Increased active TGF- $\beta$  persisted for 7 d. Latent TGF- $\beta$  complex is abundant in unirradiated tissue (E) but radiation exposure (F-H) resulted in a progressive loss of anti-LAP reactivity in both the epithelium (curved arrow) and periepipithelial stroma. Anti-LAP immunoreactivity was barely detectable by day 3 but returned to control levels by day 7.  $\times 20$ .



**Figure 4.** Mink lung epithelial assay of total latent TGF- $\beta$  in medium conditioned by irradiated tissue fragments as a function of time post-irradiation. Total TGF- $\beta$  activity in heat-activated media (A) was decreased as a function of radiation exposure as evidenced by elevated DNA synthesis compared with the media from unirradiated tissue. Results shown are mean cpm $\pm$ SD of triplicate determinations from two experiments (A). The specificity of DNA synthesis inhibition by conditioned media was demonstrated by the addition of neutralizing TGF- $\beta$  antibody (B). TGF- $\beta$ -neutralizing IgG or nonspecific IgG were added (50  $\mu$ g/ml) to medium alone (sample 1) or conditioned medium from unirradiated mammary gland (sample 2) or from irradiated mammary gland, 1 d (sample 3), 3 d (sample 4), or 7 d (sample 5) after exposure. The bars designate the average of two to four determinations. [<sup>3</sup>H]Thymidine incorporation in the presence of 100 pg/ml of rTGF- $\beta$  (positive control) was 775 $\pm$ 68 cpm (A) and 100 $\pm$ 40 cpm (B).

trix proteins and chemotaxis of fibroblasts (21, 22), strongly suggest that TGF- $\beta$  activation may initiate the extracellular matrix remodeling observed within 24 h of radiation exposure (8). Although collagen changes associated with radiogenic fibrosis evolve over a protracted period (23) and TGF- $\beta$  immunoreactivity accompanies fibrosis in irradiated tissue (5, 24,

25), our data suggest that TGF- $\beta$ 1 activation may be the initial event.

Clearly, TGF- $\beta$  activation by radiation and subsequent TGF- $\beta$ -induced extracellular matrix remodeling provide a new focus for the study of mechanisms of tissue response to radiation. Since normal tissue tolerance is the main limiting factor in radiotherapy of many cancers, TGF- $\beta$  activation may provide a novel target for manipulating the physiological consequences of radiation therapy.

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