Regulated expression and growth inhibitory effects of transforming growth factor-β isoforms in mouse mammary gland development

STEPHEN D. ROBINSON¹*, GARY B. SILBERSTEIN², ANITA B. ROBERTS², KATHLEEN C. FLANDERS² and CHARLES W. DANIEL¹

¹Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064, USA
²Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD 20892, USA

*To whom correspondence should be addressed

Summary

Transforming Growth Factor-β1 (TGF-β1) was previously shown to inhibit reversibly the growth of mouse mammary ducts when administered in vivo by miniature slow-release plastic implants. We now report a comparative analysis of three TGF-β isoforms with respect to gene expression and localization of protein products within the mouse mammary gland. Our studies revealed overlapping expression patterns of TGF-β1, TGF-β2 and TGF-β3 within the epithelium of the actively-growing mammary end buds during branching morphogenesis, as well as within the epithelium of growth-quiescent ducts. However, TGF-β3 was the only isoform detected in myoepithelial progenitor cells (cap cells) of the growing end buds and myoepithelial cells of the mature ducts. During pregnancy, TGF-β2 and TGF-β3 transcripts increased to high levels, in contrast to TGF-β1 transcripts which were moderately abundant; TGF-β2 was significantly transcribed only during pregnancy. Molecular hybridization in situ revealed overlapping patterns of expression for the three TGF-β isoforms during alveolar morphogenesis, but showed that, in contrast to the patterns of TGF-β1 and TGF-β2 expression, TGF-β3 is expressed more heavily in ducts than in alveoli during pregnancy. Developing alveolar tissue and its associated ducts displayed striking TGF-β3 immunoreactivity which was greatly reduced during lactation. All three isoforms showed dramatically reduced expression in lactating tissue.

The biological effects of active, exogenous TGF-β2 and TGF-β3 were tested with slow-release plastic implants. These isoforms, like TGF-β1, inhibited mammary ductal elongation in situ by causing the disappearance of the proliferating stem cell layer (cap cells) and rapid involution of ductal end buds. None of the isoforms were active in inhibiting alveolar morphogenesis. We conclude that under the limited conditions of these tests, the three mammalian isoforms are functionally equivalent. However, striking differences in patterns of gene expression and in the distribution of immunoreactive peptides suggest that TGF-β isoforms may have distinct roles in mammary growth regulation, morphogenesis and functional differentiation.

Key words: TGF-β, isoforms, mouse, northern hybridization, in situ hybridization, mammary gland, growth regulation, morphogenesis.

Introduction

At about 3–4 weeks after birth, with the onset of ovarian function, terminal end buds of the developing mouse mammary gland drive the growth and dichotomous branching of mammary epithelium, eventually filling the adipose-rich stroma with a tree-like ductal network. The development of the gland, although occurring in the subadult animal, is embryonic in nature by several criteria. First, the gland undergoes its complete morphogenesis at this time, which, in many respects, is very similar to that of other ectodermally derived organs such as the lung and salivary gland. Second, postnatal development of the gland is dependent upon reciprocal interactions between epithelium and stroma. This is illustrated by the transplantation of embryonic salivary or mammary mesenchyme into the adult mammary gland; this treatment elicits ductal branching morphogenesis and growth in a pattern which is clearly distinct from mammary epithelium growing in subadult mammary fat pad (Daniel et al. 1968; DeOme et al. 1959; Hoshino, 1964; Sakakura et al. 1976, 1982). Third, the structural and molecular changes in the basal lamina and extracellular matrix accompanying duct formation are embryonic in nature (Bernfield and Banerjee, 1982; Bernfield et al. 1984). In addition, the mammary gland retains embryonic potential well into postnatal life, which is demonstrated by the ability of the previously growth-static ducts to reinitiate ductal growth and morphogenesis when im-
planted into parenchyma-free mammary fat pad. The regulation of mammary development is poorly understood, but a variety of regulatory factors and processes have been implicated, including epithelial-mesenchymal interactions, as mentioned above (Bemfield et al. 1973), hormones (Topper and Freeman, 1980) and local regulation by growth factors (Daniel and Silberstein, 1987).

After the mammary ductal tree has been established, ductal spacing must be maintained so that alveolar development and secretory differentiation can occur at the onset of pregnancy. The maintenance of an open pattern of branching requires the active suppression of lateral buds (Faulkin and DeOme, 1960; Daniel et al. 1989). In addition, the ability of the mammary gland to undergo repeated rounds of alveolar development (a process of rapid growth, morphogenesis and differentiation) and subsequent involution following the cessation of lactation, implies the ongoing maintenance of an alveolar stem cell population. The development of lobulo-alveolar structures during pregnancy also depends on circulating hormones, interactions with the mammary stroma, and presumably also requires local regulatory factors (Daniel and Silberstein, 1987). The hormones associated with mammary development during pregnancy have been studied extensively (Topper and Freeman, 1980) and there is compelling evidence for the essential role of extracellular matrix in functional differentiation (Bissell and Ram, 1989). The role of growth factors remains to be elucidated.

The TGF-βs are a family of highly conserved proteins with 65–80% amino acid homology within the C-terminal portion of the mature proteins (Roberts and Sporn, 1990). The family now comprises at least five vertebrate members termed TGF-β1 (Derynck et al. 1985), TGF-β2 (Madisen et al. 1988; Miller et al. 1989a), TGF-β3 (ten Dijke et al. 1988; Derynck et al. 1988; Jakowlew et al. 1988a; Miller et al. 1989b), TGF-β4 (Jakowlew et al. 1988b) and TGF-β5 (Kondaiah et al. 1990). Each of these proteins is highly conserved through evolution, and belongs to a larger superfamily of growth factors (Sporn et al. 1985; Madisen et al. 1988; Miller et al. 1989), TGF-β1 and -β3 have been cloned from mammalian cDNA libraries, whereas expression of TGF-β4 and TGF-β5 is apparently limited to avian and amphibian systems, respectively.

In biological assays TGF-β1, -β2 and -β3 act similarly to stimulate or inhibit cell proliferation, depending on the cell type, but there are differences in potencies among the three isoforms (Graycar et al. 1989; ten Dijke et al. 1990). As well, specific and unique activities have been assigned to both TGF-β1 and TGF-β2: TGF-β2 has potent mesoderm-inducing activity in a Xenopus bioassay (Rosa et al. 1988), while TGF-β1 can act in this capacity only in synergy with fibroblast growth factor (Kimmelman and Kirscher, 1987); on the other hand, TGF-β1 is a more potent inhibitor of endothelial cells in culture (Jennings et al. 1988). In situ hybridization analyses of all three mammalian TGF-β isoforms during murine embryogenesis show very different spatial and temporal expression patterns for the isoform-specific RNAs (Pelton et al. 1989; Fitzpatrick et al. 1990; Pelton et al. 1990a, 1990b; Millan et al. 1991; Schmid et al. 1991).

The results of our study provide evidence that a developing organ in vivo has the ability to respond to all three mammalian isoforms of TGF-β when administered exogenously, and that it does so with an identical gross morphological response. At this time in development, when the mammary gland is susceptible to exogenous TGF-βs, the actively growing ductal tips (end buds) show overlapping mRNA and protein expression patterns for the three TGF-βs. However, there is also compelling evidence that the individual factors are expressed differentially throughout mammary gland development and distributed within the gland in unique patterns, suggesting distinct intrinsic roles for the individual factors based on their temporal and spatial localization.

Materials and methods

Animals

Virgin C57/BL/crl mice, 5 weeks of age and weighing ca 16 g, were used for all implant studies. C57/BL/crl mice were also used for tissue collection for all stages of mammary gland development, with the exception of parenchyma-free mammary fat pad (clear fat pad) which was from Balb/C mice. One μm sections were from C3H mice.

Implants

EVAC (Elvax 40P) was a gift of DuPont Chemical Co. (Universal City, CA). TGF-β1 was from human platelets (A. Roberts, Laboratory of Chemoprevention, National Institutes of Health, Bethesda, MD). TGF-β2 was from porcine platelets and was a gift of R&D Systems. TGF-β3 was from the recombinant chicken cDNA expressed in NIH3T3 cells under the control of a metallothionein promoter and purified to homogeneity (A. Roberts). Implant preparation is described in detail elsewhere (Silberstein and Daniel, 1982). Briefly, a lyophilized mixture of 20 mg BSA and varying amounts of TGF-β1, -β2 or -β3 were dispersed in 0.125 ml of EVAc that had been dissolved in dichloromethane [20% wt/vol]. This mixture was quick-frozen and dried, and the polymer matrix with entrapped chemical was then cut to size (typical implant weight: 0.5–1 mg) and surgically implanted. Recipient mice were anesthetized with Nembutal (60 μg/g body wt) and the number 3 mammary glands were exposed by reflecting the abdominal skin. A small pocket was made in the mammary fat pad using Dumont forceps, which were then used to insert the implant. The abdominal skin was closed with wound clips and the animals were allowed to recover in an atmosphere of 95% O₂, 5% CO₂. 6–10 animals were used for implanting each of the TGF-β isoforms.

Histology and DNA autoradiography

Glands were fixed overnight in Tellyesniczkys' fixative. For whole-mount preparations, glands were defatted in three changes of acetone, hydrated through graded alcohols, stained with hematoxylin, dehydrated through graded alcohols to xylene and photographed. After whole-mount examination, pieces of tissue were embedded in paraffin and sectioned at 5 μm for DNA autoradiography. Animals were injected intraperitoneally with 100 μCi of [3H]thymidine. After 40 min, the mammary glands were removed, fixed,
processed and paraffin-sectioned by standard methods. Slides were dipped in Kodak NTB-2 emulsion diluted 1:1 with water, exposed for 11 days, developed and stained with hematoxylin/eosin.

**Immuno histochemical staining**

Tissue fixation was accomplished by perfusion (Kiernan, 1981). Briefly, animals were heparinized (subcutaneous injection; 1 unit g⁻¹ body wt) 30 min before perfusion, anesthetized with Nembutal (60 µg·g⁻¹ body wt), and after puncturing the left ventricle with a blunted 18-gauge hypodermic needle and nicking the right atrium, perfused with freshly prepared 1 % paraformaldehyde/PBS until the solution flowing out of the right atrium was clear (about 10 ml), followed by perfusion with 70 ml freshly prepared 4 % paraformaldehyde/PBS. After perfusion, tissue was left in situ for 30 min, excised, post-fixed in Bouin's solution for an additional 5 h, transferred to 50 % ethanol and rinsed until free of excess Bouin's. Tissue was then dehydrated through graded alcohols to xylene, embedded in paraffin (not exceeding a temperature of 60°C) and sectioned at 5 µm for immunostaining.

TGF-βs were localized in sections essentially as described by Heine et al. (1987), using an avidin–biotin–peroxidase kit (Vector Laboratories Inc., Burlingame, CA – Elite kit). Deparaffinized sections were first treated to several blocking steps, (30 min each: 0.2 % glycine to block aldehyde groups; 0.3 % hydrogen peroxide in methanol to block endogenous peroxidases; 10 % goat serum), then incubated with IgG fractions of antibody (1–15 µg·ml⁻¹) overnight at room temperature. After incubation, sections were washed with 1 % goat serum followed by the detection kit protocol. Sections were counterstained with hematoxylin (10 s) before mounting coverslips. For TGF-β2 immunostaining, deparaffinized sections were treated in 0.1 % trypsin/PBS (Sigma T-8128), 5 min at 37°C prior to the blocking steps. On average, tissue from 3–6 individual animals was used for each developmental stage examined.

**Antibodies to TGF-β**

TGF-βs are synthesized as pre-pro-TGF-βs, composed of a pro-region or 'latency' domain adjacent to a 112–114 amino acid domain that becomes the mature or active moiety. The pre-pro-molecule is processed to a glycosylated, dimeric latent form by proteolytic cleavage following the N terminus signal sequence and at a peptide bond between the latency domain and the mature domain (pro-TGF-β). Latent TGF-β is secreted from cells and can subsequently be activated by dissociation from the remainder of the precursor.

Rabbit polyclonal antibodies raised to (1) a synthetic peptide corresponding to amino acids 50–75 of the mature domain of TGF-β1 (anti-50–75–1), (2) a synthetic peptide corresponding to amino acids 266–278 of the latency domain of TGF-β1 (anti-266–278–1), (3) a synthetic peptide corresponding to amino acids 50–75 of the mature domain of TGF-β2 (anti-50–75–2), or (4) a synthetic peptide representing amino acids 81–100 within the latency domain of TGF-β3 (anti-81–100–3), were used in these studies.

An IgG fraction from normal rabbit serum was used as the major control for most studies. In addition, specificity was assessed by preincubating the anti-TGF-β IgG with a solution of the peptide against which it was raised. This procedure resulted in the complete loss of staining. On western blots there is some cross-reactivity of anti-50–75–1 and anti-50–75–2 with TGF-β3, but this cross-reactivity is not seen in ELISA assays. There is no other isofrom cross-reactivity on western blots (Flanders et al. 1988, 1990).

**RNA preparation and northern hybridization**

Tissues were frozen in liquid nitrogen immediately after removal and total RNA was prepared by the guanidine isothiocyanate (4 M), cesium chloride (5.7 M) method (Ausubel et al. 1989). Total RNA from 12–18 animals for each developmental stage was isolated, with the exception of tissue from pregnant or lactating stages where 2–3 animals were used. DNA was electrophoresed in 1.0 % agarose containing 2.3 M formaldehyde in Mops buffer (0.2 M morpholinopropane sulfonic acid, 50 mM sodium acetate, 5.0 mM EDTA, pH 7.0). RNA was transferred to Nytran membrane (Schleicher and Schuell, Keene, NH) by the established procedure of Maniatis et al. (1982). Northern hybridizations were carried out under high stringency conditions using 32P-random-primed labeled (1–10x10⁸ cts·min⁻¹·µg⁻¹) murine TGF-β cDNAs (β1 fragment corresponding to bases 1241–1519 of the cDNA; β2 and β3 – entire coding region of cDNA). Washes after the hybridizations were in 0.1xSSPE/0.1 % sodium dodecyl sulphate at 65°C. Under these conditions, cross-hybridization of related but nonspecific transcripts to the probes does not occur. All TGF-β clones were gifts of Paturu Kondiah (Laboratory of Chemoprevention, Bethesda, MD).

**In situ hybridizations**

Tissue from 3–6 individual animals for each developmental stage was fixed 3 h in 4 % paraformaldehyde/PBS, dehydrated in a graded series of ethanol to xylene and embedded in paraffin. 7 µm sections were cut and floated on slides coated with 3-aminopropyltriethoxysilane (Sigma). Sections were then baked onto slides overnight at 37°C. Sections were dewaxed through xylene, rehydrated through a graded series of ethanol and re-fixed in 4 % paraformaldehyde/PBS. Sections were pretreated with 1 % BSA, followed by 0.2 % HCl, then digested with proteinase K (20 µg·ml⁻¹; Sigma) at room temperature for 5 min, refixed in 4 % paraformaldehyde/PBS, rinsed in 2 % glycine/PBS, acetylated (100 mM triethanolamine, 25 mM acetic anhydride) and dehydrated through ethanol.

To avoid nonspecific binding of RNA probes, slides were pre-hybridized 16 h at 57°C with 4 µg·slide⁻¹ 'irrelevant', cold mRNA transcripts (Silberstein et al. 1990) in hybridization buffer (see below).

Slides were hybridized at 57°C for 18 h under silanized coverslips in a solution containing 50 % formamide, 300 mM NaCl, 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 1 mM DTT and 25 µg·ml⁻¹ yeast tRNA. Slides were then baked onto slides overnight at 37°C. Slides were dewaxed through xylene, rehydrated through a graded series of ethanol and re-fixed in 4 % paraformaldehyde/PBS. Sections were pretreated with 1 % BSA, followed by 0.2 % HCl, then digested with proteinase K (20 µg·ml⁻¹; Sigma) at room temperature for 5 min, refixed in 4 % paraformaldehyde/PBS, rinsed in 2 % glycine/PBS, acetylated (100 mM triethanolamine, 25 mM acetic anhydride) and dehydrated through ethanol.

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Slides were hybridized at 57°C for 18 h under silanized coverslips in a solution containing 30 % formamide, 300 mM NaCl, 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 1 mM DTT and 25 µg·ml⁻¹ yeast tRNA. Slides were then baked onto slides overnight at 37°C. Slides were washed in 2xSSC, 50 mM DTT at 50°C, and then rinsed one hour in 5xSSC, 10 mM DTT at 50°C. The slides were then washed in 2xSSC, 50 % formamide, 0.1 % SDS, 100 mM DTT for one hour at 65°C followed by RNase A (20 µg·ml⁻¹; Calbiochem)/RNase T1 (1 U·ml⁻¹; Sigma) treatment at 37°C for 20 min in a solution containing 5.0 M NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA. Slides were rinsed in RNase buffer/20 mM β-mercaptoethanol for 30 min followed by washes in 2xSSC and 1xSSC each for 30 min at 65°C. Slides were then dehydrated through graded alcohols containing 0.3 % ammonium acetate, dipped in photographic emulsion (Kodak, NTB2) diluted 1:1 with 2 % glycerol/water and exposed at 4°C for 5–7 weeks in the presence of desiccant. The slides were developed (Kodak D19), counterstained with hematoxylin (10 s) and analyzed with a Leitz Aristoplan microscope. The slides were photographed using Kodak Panatomic-X film.
Riboprobes were $^{35}$S-UTP labeled to a specific activity of $10^6$ cts min$^{-1}$ per $\mu$g using the appropriate SP6, T7 or T3 transcription system. The probes were digested to an average size of 100–150 nucleotides by controlled alkaline hydrolysis (Cox et al. 1984) and used at a final concentration of $4\times10^4$ cts min$^{-1}$ per $\mu$l of hybridization buffer.

In order to minimize the possibility of cross-hybridization between the TGF-β, transcript-specific probes were constructed. The TGF-β1-specific antisense probe was a 279 nucleotide fragment from the mature protein coding region of the cDNA (bases 1241–1519 of the cDNA). The TGF-β2 transcript-specific probe was a 502 nucleotide PstI–SacI fragment (bases 236–737 from the latency protein encoding region of the cDNA (Miller et al. 1989) subcloned into pGEM3ZF (Promega) in an antisense orientation to the T7 promoter. The TGF-β3-specific probe was a 281 nucleotide SmaI–SacI fragment (bases 223–503 from the latency protein encoding region of the cDNA (Miller et al. 1989b)) subcloned into pGEM3ZF in an antisense orientation to the T7 promoter. The control probe was TGF-β2 sense from the subclone mentioned above. These probes recognize only their respective isoforms on northern blots and show different hybridization patterns on mouse embryos, indicating that each probe recognizes a specific TGF-β mRNA(s) and does not cross-hybridize with the other TGF-β transcripts.

Results

TGF-β gene expression

The expression of TGF-β1, -β2, and -β3 transcripts in various stages of development was evaluated by northern hybridization to total RNA isolated from mammary glands (Fig. 1). Hybridization results for TGF-β1 and TGF-β3 revealed single transcripts for each isoform (2.4 kb and 3.8 kb, respectively). Substantial expression of TGF-β1 and TGF-β3 was noted in all stages of mammary gland development with the exception of the lactating gland, which showed substantially reduced levels of transcripts (Figs 1A and 1C). However, the patterns of expression for these two TGF-β mRNA species differed; TGF-β3 transcript levels were significantly increased in glands from mid-pregnant animals (Fig. 1C). Also noteworthy was the presence of TGF-β1 and TGF-β3 transcripts in RNA from 'cleared' mammary fat pads from which the glandular components had been removed by the method of DeOme et al. (1959). The mammary gland fat pad contains a wide variety of cell types and the cellular sources of cleared fat pad transcript synthesis have not been determined. However, these data do indicate the ability of cells within the mammary gland fat pad to synthesize TGF-β1 and TGF-β3 transcripts. In accordance with this, TGF-β3 immunostaining is frequently seen in association with blood vessels within the fat pad (not shown).

Hybridization of a TGF-β2-specific probe to total RNA demonstrated the presence of four TGF-β2 transcripts (6.0 kb, 5.0 kb, 4.0 kb, and 3.5 kb), (Fig. 1B). However, in most stages of mammary development, TGF-β2 mRNA signals were very low; in fact, TGF-β2 transcripts were present in conspicuous amounts only in lobulo-alveolar mammary tissue. RNA loading was assessed by probing with a $^{32}$P-end-labeled synthetic oligonucleotide directed against murine 18S ribosomal RNA (Fig. 1D).

Hybridization in situ

In situ hybridization was performed on sections of mammary gland tissue using $^{35}$S-labeled gene-specific probes and is shown in Figs 2 and 3. Mouse TGF-β2 sense probe was used as a negative control. This probe showed no specific hybridization signals (Fig. 2H).

Fig. 1. Northern analysis of TGF-β isoform expression during mammary gland development. (A) TGF-β1, (B) TGF-β2, (C) TGF-β3, (D) 18S ribosomal RNA as a control for loading. Numbers refer to the following: (1) cleared (parenchyma-free) mammary fat pad, (2) 5-week mammary gland, (3) 3-month mammary gland, (4) mammary gland from mid-pregnant animals, (5) lactating mammary gland. 15 μg of total RNA was loaded per lane. The membrane was probed consecutively with TGF-β1, TGF-β2, TGF-β3 and 18S rRNA probes. Transcripts are marked by arrows: TGF-β1 – 2.4 kb; TGF-β2 – 6.0 kb, 5.0 kb, 4.0 kb, and 3.5 kb; TGF-β3 – 3.8 kb.

Fig. 2. End bud expression of TGF-β mRNAs. Bright-field (A,B,C,D) and corresponding dark-ground (E,F,G,H) photomicrographs of adjacent sections through a bifurcating end bud. (A,E) TGF-β1 antisense probe. TGF-β1 expression is seen over the end bud epithelium with heaviest labeling at the tips (arrows). (B,F) TGF-β2 antisense probe. TGF-β2 expression is weak over both epithelial and stromal components. (C,G) TGF-β3 antisense probe. Expression is seen over epithelium, with heaviest labeling in the flank region of the bifurcating end bud (arrow). (D,H) TGF-β2 sense control probe; arrows indicate corresponding reference points in the bright-field and dark-ground micrographs. No specific labeling is seen. Bar=70 μm.
TGF-β2 signals were weak at all stages of mammary development with the exception of tissue from pregnant animals, a result that is consistent with northern analysis (Fig. 1). TGF-β3 was the only isoform detected in lactating tissue, and this localization was primarily within the stroma (not shown).

**TGF-β expression during branching morphogenesis**

The terminal end bud of the developing mammary gland drives ductal morphogenesis in the gland by producing a supply of differentiated ductal and myoepithelial cells for elongation of the subtending ducts. All three isoforms of TGF-β showed overlapping epithelial expression within the actively growing mammary end buds (Fig. 2) and the growth-quiescent subtending ducts (not shown). TGF-β2 signals were weak but detectable over background (Fig. 2F). Expression of TGF-β1 and TGF-β3 was seen in the epithelial cells of the growing end bud (Figs 2E and 2G, respectively). Both isoforms showed non-overlapping areas of heavier labeling, TGF-β1 at the tips of the developing end buds, and TGF-β3 in the flank region of the end bud. The flank region of the end bud is the site of active matrix development and basal lamina hypertrophy that serves to ensheath the subtending duct within a connective tissue tunic.

**TGF-β expression during pregnancy**

The pregnant mammary gland consists of actively growing alveolar structures which bud off from pre-existing ducts. The alveoli will, eventually, occupy all of the available fat pad stroma and will, following parturition, produce milk. TGF-β1 was expressed at low levels, within both alveolar structures and ductal...
Fig. 4. End bud expression of TGF-β proteins. Photomicrographs illustrating the overlapping expression patterns of TGF-β isoforms in actively growing end buds. Body cells (large arrowheads) are immunopositive for each isoform, as are occasional fibroblasts and adipocytes of the stroma. Only TGF-β3 shows heavy staining in cap cells (small arrowheads). (A) TGF-β1 (anti-266–278–1), (B) TGF-β2 (anti-50–75–2), (C) TGF-β3 (anti-81–100–3), (D) a representative of end bud staining with blocked antibody (anti-81–100–3). Bar: (A, D)=36 μm; (B)=45 μm; (C)=50 μm.

Fig. 6. TGF-β3 immunostaining in pregnant and lactating tissue. (A) Alveoli and duct from mid-pregnant animal (anti-81–100–3). Numerous alveolar cells (large arrowheads) are heavily immunopositive. (B) Alveoli from lactating animal (anti-81–100–3). Alveolar cells are now nearly completely devoid of TGF-β3 (large arrowheads). Spotty staining is present within the small stromal spaces between alveoli (small arrowhead). Bar=42 μm.
**Fig. 5.** Comparison of TGF-β1 and TGF-β3 expression in growth-quiescent ducts. (A) TGF-β1 (anti-50–75–1). The fibrous stroma immediately adjacent to the duct is heavily immunopositive for TGF-β1 (arrow). The ductal epithelial cells (large arrowhead) are devoid of staining, as are the myoepithelial cells (small arrowhead). (B) TGF-β1 (anti-266–278–1). Numerous epithelial cells are immunopositive for pro-TGF-β1 (large arrowheads). There is no evidence of myoepithelial staining (small arrowhead). (C) Duct immunostained with blocked anti-266–278–1. Note the absence of staining. (D) TGF-β3 (anti-81–100–3). Numerous ductal epithelial cells (large arrowheads) are immunopositive for TGF-β3. Myoepithelial cells (small arrowheads) are also heavily stained. (E) Duct immunostained with blocked anti-81–100–3. Note the complete absence of any specific staining. (F) One micron section of a duct showing heavy intra-myoeipithelial staining (small arrowheads) for TGF-β3 (anti-81–100–3). Bar: (A)=45 μm; (B, D, E)=28 μm; (C)=20 μm; (F)=13 μm.

components of the pregnant mammary gland (not shown). TGF-β2 probes showed hybridization both over alveolar structures of the gland from pregnant animals, as well as over ductal elements (Fig. 3C). TGF-β3 showed heavy labeling over alveolar structures and very intense labeling over ducts from the pregnant animal (Fig. 3D).

**Immunolocalization**

**Immunolocalization of TGF-β isoforms in growth-active mammary end buds**

TGF-β2 immunoreactivity was very low in all developmental stages examined and is shown only for the end bud (Fig. 4).

Fig. 4 illustrates the distribution of TGF-β isoforms in the end buds of glands that were undergoing branching morphogenesis, at similar stages to the end bud shown in Fig. 2. All three mammalian TGF-βs, TGF-β1 (Fig. 4A), TGF-β2 (Fig. 4B), and TGF-β3 (Fig. 4C), showed intraepithelial immunoreactivity within the cells of the end bud which corresponded well with transcript localization (Fig. 2). Only TGF-β3 showed appreciable staining in myoepithelial progenitor (cap) cells (see next section). No extracellular staining was seen in front of the growing end buds for any TGF-β isoform. Stromal cells, both fibroblasts and adipocytes, demonstrated some staining with all three antibodies. Fig. 4D shows an end bud stained with antibody previously incubated with the peptide against which it was raised (blocked antibody, see Materials and methods; shown for anti-81–100–3). No specific staining is present. This is representative of the immunostaining obtained with any of the blocked antibodies.

**TGF-β1 and TGF-β3 peptides in growth-quiescent ducts**

Fig. 5 illustrates the distribution of TGF-β1 and TGF-β3 in growth-quiescent ducts. Immunostaining with blocked TGF-β1 antibody (anti-266–278–1) is shown in Fig. 5C and blocked TGF-β3 antibody is shown in Fig. 5E. Staining for epitopes within the mature form of TGF-β1 showed intense extracellular reactivity concentrated in stroma adjacent to ducts (Fig. 5A). This extracellular staining localizes TGF-β1 that is associated with fibrous elements of the extracellular matrix and is not obviously concentrated within any particular group of stromal cells. The cells that make up the duct, including the myoepithelial cells, do not stain with this antibody.

Figs 5B and 5D compare the staining patterns of TGF-β1 and TGF-β3, respectively, using antibodies against peptides in the latency regions of the molecules. Both antibodies showed substantial intracellular epithelial staining of body cells, as well as stromal cell staining. However, only TGF-β3 was appreciably associated with the myoepithelial cells of the duct (Fig. 5D). Myoepithelial staining was confirmed with 1μm mammary sections and is shown in Fig. 5F.

**Immunohistochemical localization during pregnancy and lactation**

In general, the intensity of TGF-β3 epithelial immunostaining reflected the relative levels of TGF-β3 mRNA transcripts observed in northern blots and protein localization was consistent with transcript localization seen by *in situ* hybridization. Quite striking was the heavy immunostaining by anti-TGF-β3 of the alveolar epithelial cells of the mammary gland from mid-pregnant animals (Fig. 6A). Occasional stromal cells were also immunopositive. The ductal staining pattern was identical to that of the growth-quiescent mammary gland (Fig. 5D). TGF-β1 was localized only extraepithelially in tissue from mid-pregnant animals with either anti-50–75–1 or anti-266–278–1. This staining was primarily associated with ducts, but occasional staining was observed around alveolar structures (not shown). In the lactating gland, TGF-β3 was sometimes seen as a partial stain around alveoli. Very rarely, spotty or weak intracellular staining was observed in lactating alveolar (epithelial) cells (Fig. 6B).

**Effects of exogenous TGF-β**

The direct effects of TGF-β2 and TGF-β3 on ductal growth in virgin mice were investigated using ethylene vinyl acetate copolymer (EVAc) implants, which provide the slow release of bioactive molecules to small zones of the mammary gland (Silberstein and Daniel, 1982). Implants containing either TGF-β2 or TGF-β3 were placed ahead of the advancing front of mammary end buds, which are the foci for regulatory influences on ductal growth in the glands of subadult, virgin mice (Nandi, 1958).

Fig. 7B illustrates the effect of implanted TGF-β3 (100 ng) on ductal elongation. Two days of treatment with implanted TGF-β3 resulted in the complete inhibition of growth and involution of end buds. A control implant containing bovine serum albumin (BSA) had no discernible effect on the contralateral gland in the same animal (Fig. 7A), indicating that TGF-β3 acts directly on the gland and not through a systemic intermediary. Both treatment and control implants were placed in approximately the same
Fig. 7. Photomicrographs illustrating the effect of exogenous TGF-β3 on ductal growth. Five-week-old virgin mice were implanted with EVAc pellets containing either BSA (control) or 100 ng TGF-β3 for 2 days. (A) Control. End buds (large arrowhead) at the ductal growth front have grown past a BSA implant (*). Blunt-tipped, growth-quiescent, terminal ducts (small arrowheads) are seen on either side of the end bud array as well as in the middle of the gland. (B) Effect of TGF-β3 on ductal growth. Implanted TGF-β3 (*) resulted in the disappearance of end buds. This gland is contralateral to the pictured control. (C) DNA autoradiograph of an end bud treated with BSA for 2 days. Both cap cells (small arrowhead) and luminal epithelial cells (large arrowheads) show extensive labeling, characteristic of normal end buds. (D) DNA autoradiograph of an end bud treated with TGF-β3 (100 ng) for 2 days. The cap cell layer is absent and the tip of the end bud is ensheathed in dense connective tissue (arrow). DNA synthetic cells are absent within the treated duct. Bar: (A, B)=2 mm; (C, D)=54 μm.

Discussion

Several studies have suggested a role in murine development for TGF-β1 (Lehnert and Akhurst, 1988; Akhurst et al. 1990) and TGF-β2 (Pelton et al. 1989), and, more recently, for TGF-β3 (Fitzpatrick et al. 1990; Pelton et al. 1990a, 1990b; Millan et al. 1991; Schmid et al. 1991), particularly with respect to differentiation and morphogenesis. We extend these studies by investigating the temporal and spatial distribution of the three mammalian TGF-β isoforms in the developing mammary gland.

In general, we have shown that RNA transcripts and intraepithelial protein products for the three mammalian TGF-β isoforms co-localize. This localization is interpreted as identifying sites of synthesis of the isoforms within the gland and is indicative of autocrine and paracrine mechanisms, as reported for TGF-β1 in other systems (Heine et al. 1987; Lehnert and Akhurst, 1988; Akhurst et al. 1990). This is the first demonstration of the co-localization of TGF-β2 and TGF-β3 transcripts with their respective protein products. However, we have no information of whether the identified TGF-β proteins are in a latent or active form. Given that most of the localization reported here is intracellular and that it coincides with transcript localization, and given the many mechanisms operating to clear active TGF-β from tissues (Roberts and Sporn, 1990), the protein that we localize is most likely latent. However, latent TGF-β must be considered a storehouse of the peptide, which can be activated under the appropriate conditions.

TGF-β expression in different developmental stages

Table 1 summarizes the localization of TGF-β isoforms during mammary gland development based on a composite of northern analysis and in situ hybridizations. All three isoforms are present during most stages of mammary development, but in many instances the expression patterns of the three TGF-β isoforms are regulated differently. For example, TGF-β2 and TGF-β3 transcript levels increase during pregnancy, whereas TGF-β1 transcript levels do not; TGF-β3 transcripts are expressed more intensely in the ducts of the pregnant...
animal than in the alveoli. In addition, TGF-β2 transcripts are detected at appreciable levels only during pregnancy. These data are, in general, supported by immunohistochemical analysis. Table 1 also shows that the mammary gland fat pad has the capacity to express the various TGF-β isoforms. This is supported by northern analysis which demonstrates the expression of, in particular, TGF-β1 and TGF-β3 in fat pads that have had their parenchyma components removed. Immunohistochemical results show that TGF-β proteins are present within the fibroblasts and adipocytes of the stroma; the significance of these observations is unknown (see Fig. 4). In addition, expression of TGF-βs within other structures of the fat pad could contribute to a cleared fat pad signal. For example, immunohistochemical analysis shows TGF-β3 protein in the smooth muscle cells associated with blood vessels of the fat pad (not shown).

Overlapping patterns of expression during the ductal phase of development

Previous reports have demonstrated that TGF-β isoforms have overlapping patterns of transcript expression in morphogenetically active epithelia such as whisker follicle, salivary gland and tooth bud (all, like the mammary gland, ectodermal derivatives) (Millan et al. 1991; Schmid et al. 1991). The results of this paper demonstrate that all three mammalian TGF-β RNAs and proteins are present in the epithelial components of the mammary gland which are undergoing active growth and are involved in branching morphogenesis (end buds). There are two interpretations of this pattern of staining: First, the TGF-β proteins may act in an autocrine fashion to stimulate growth in the end bud region as well as to inhibit the premature differentiation of end bud parenchymal cells, similar to the role proposed for TGF-β1 during lung branching morphogenesis (Heine et al. 1990). However, it should be pointed out that within the developing lung, TGF-β1 is quite abundant within the extracellular matrix around the developing lung buds (Heine et al. 1990) and TGF-β1 transcripts have been seen within the mesenchymal cells surrounding the lung buds (Millan et al. 1991; Schmid et al. 1991). This hypothesis is inconsistent with our implant studies, which show growth inhibition and the disappearance of myoepithelial stem cells (cap cells) when glands are treated with exogenous, active TGF-β (see below). We have not seen extracellular accumula-

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Results represent a composite of northern analysis and *in situ* hybridization. Fat pad includes, but is not limited to, adipocytes and fibroblasts.

TGF-β isoforms in mouse mammary development

When implanted into the developing mammary gland, TGF-β1, -β2 and -β3 gave morphogenetically similar results: the complete inhibition of ductal growth with concomitant accumulation of fibrous matrix in front of the inhibited end bud (shown for TGF-β3 in Fig. 7). While many questions remain to be asked about the response of the gland to TGF-β2 and TGF-β3, such as dosage and time course effects, the observed response to all three growth factors is identical. These results imply that, within the limited scope of our bioassay, each of the isoforms can function interchangenably when introduced into the same location within the gland (the stroma immediately in front of growing end buds). These data also support at least partially overlapping roles for all three TGF-βs during the growth of normal end buds. In conjunction with the localization data, these results suggest that all three isoforms act in a cooperative manner.
TGF-βs may act similarly in governing ductal morphogenesis by influencing extracellular matrix deposition.

**TGF-β in growth-quietant ducts**

Fig. 5 demonstrates that, in ductal epithelial luminal cells, the intracellular expression pattern for TGF-β overlaps with the pattern for TGF-β1. This result is also supported by in situ hybridization (not shown). However, TGF-β3 seems to be the only isoform expressed appreciably in myoepithelial cells. This expression of TGF-β3 in contractile cells may be compared with high expression of TGF-β3 in skeletal and cardiac muscle, and also with studies that have identified elements in the TGF-β3 promoter that are responsible for high basal level expression in myocytes and increased expression in differentiating myocytes (Lafyatis et al. in press).

Myoepithelial cells form the outermost monolayer of mammary ducts, with processes extending laterally along ducts. The functional significance of the myoepithelium in secretory mammary tissue, where it causes milk ejection, is clear, but in the virgin animal this contractile tissue has no obvious function. It has been suggested, however, that ductal myoepithelial cells are capable of reforming cap cells during the formation of lateral branches or alveoli (Daniel and Silberstein, 1987). It is possible that, within this cell population, TGF-β3 acts in an autocrine fashion to inhibit terminal differentiation of this cell population to allow for the eventual formation of lateral buds. TGF-β1 is known to inhibit differentiation of myoblasts into myotubes and is highly expressed in both cell types (Fiorini et al. 1986; Massagué et al. 1986; Olson et al. 1986). Interestingly, TGF-β3 is also highly expressed in the stem cell population that gives rise to myoepithelial cells (Fig. 4).

Myoepithelial cells are also directly responsible for the synthesis of the basal lamina that ensheaths the ductal components of the gland, and it is possible, especially in light of the known role of TGF-β in extracellular matrix synthesis and turnover (as mentioned above), that the TGF-β3 produced by these cells may be involved in the elaboration of basal lamina components.

**TGF-βs during pregnancy and lactation**

Although the levels of TGF-β2 and TGF-β3 transcripts are high during pregnancy, the levels for all three TGF-βs are dramatically reduced during lactation (Fig. 1). TGF-β3, in particular, is expressed at high levels in alveoli and ducts from pregnant animals, but is expressed only in the stroma of the lactating gland (Figs 3 and 6). Neither TGF-β1 (Daniel et al. 1989) nor TGF-β2 or TGF-β3 (unpublished) have any effect on the morphogenesis of alveoli; however, TGF-β1 has been shown to inhibit the induction by lactogenic hormones of β-casein in HC11 mouse mammary epithelial cells (Mieth et al. 1991). These findings suggest that the TGF-βs may be involved in regulating the onset of lactation during pregnancy, by inhibiting the synthesis of milk proteins. We are currently investigating this possibility in vitro. It should be noted that TGF-β2 protein has been found in milk (Stoeck et al. 1989; D. Danielpour, personal communication); it is possible that some of the expression of TGF-β2 during pregnancy is due to the active secretion of the protein into milk.

In conclusion, we report overlapping expression patterns for TGF-β1, -β2 and -β3, both at the transcript and protein level, and the functional interchangeability of the three factors when added exogenously to the growing murine mammary gland. However, we also demonstrate unique temporal and spatial patterns of expression for the three isoforms during mammary gland development as well as region-specific differences even where the patterns overlap. These findings are consistent with the distinct regulatory features of the promoters for each of the genes (Kim et al. 1990; Noma et al. in press; Lafyatis et al. 1990) and suggest that the factors serve distinct roles in vivo solely on the basis of their differential expression patterns. This is one of two models for TGF-β action proposed by Pelton et al. (1990b). This model does not rule out the possibility that the precursor domains of each TGF-β may confer biological specificity in tissue targeting, receptor binding and/or activation of the latent forms (Roberts and Sporn, 1990), or that within particular systems the factors could have very distinct biological functions. Indeed, distinct TGF-β receptor subsets that preferentially recognize one TGF-β isoform over the others have been noted in various cell lines (Cheifetz et al. 1991).

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**References**


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