Exogenous TGF- β 1 Promotes Stromal Development in the Heifer Mammary Gland

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ABSTRACT

The objectives of this study were to determine the local effects of transforming growth factor- β 1 (TGF- β 1) on mammary epithelial and stromal cell proliferation and expression of the TGF- β 1 responsive genes *c-myc* and fibronectin. A single slow-release plastic pellet containing 5 μ g of TGF- β 1 and 20 mg of BSA was implanted in the parenchyma of the right rear quarter of the mammary gland of 9-mo-old prepubertal heifers. A control pellet containing 20 mg of BSA was implanted in the left rear quarter of each heifer. All heifers were treated with bromodeoxyuridine (BrdU) at 4, 12.5, and 22 h after the pellets were implanted to label proliferating cells. Two hours after the last BrdU injection, the animals were euthanatized, and their mammary glands were recovered. Proliferation of mammary stromal cells was significantly higher in TGF- β 1-treated quarters than in BSA-treated, control quarters (3.5 vs. 1.8% BrdU-positive cells). This result coincided with a lack of significant effect of TGF- β 1 on proliferation of mammary epithelial cells and apoptosis. By quantitative reverse transcriptase-polymerase chain reaction, we found that *c-myc* gene expression was unchanged after TGF- β 1 treatment, but fibronectin gene expression was increased 3-fold in TGF- β 1-treated quarters compared with BSA-treated, control quarters. Thus, we concluded that TGF- β 1 selectively acts on the stromal compartment of the bovine mammary gland by increasing cell proliferation and gene expression of the extracellular matrix protein fibronectin.

(Key words: slow release pellet, transforming growth factor- β , mammary gland development)

Abbreviation key: BrdU = bromodeoxyuridine, ECM = extracellular matrix, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, TGF- β 1 = transforming growth factor- β 1, TdT = terminal deoxynucleotidyltransferase, TUNEL = terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling.

INTRODUCTION

Mammary gland development in mammals occurs through invasion of mammary epithelium, surrounded by the supportive stroma, into the mammary fat pad. A crucial phase of this growth occurs during the prepubertal stage of development. During this stage, growth of the mammary epithelium is allometric, meaning it grows at a faster rate than the rest of the body (Tucker, 1987). Stromal and epithelial development are closely linked and are both influenced by formation of the extracellular matrix (ECM) (Yang et al., 1980, Bernfield et al., 1984) endocrine hormones and local growth factors (Plaut, 1993).

One growth factor that regulates mammary gland development is transforming growth factor- β 1 (TGF- β 1). Transforming growth factor- β 1 is a member of the TGF- β family, which also includes two other mammalian isoforms, TGF- β 2 and TGF- β 3. Transforming growth factor- β 1 is the most studied member of the TGF- β family and is known to be involved in cell proliferation, differentiation, apoptosis, and ECM production (Roberts et al., 1990). In vitro studies using mouse mammary cell lines have shown that TGF- β 1 inhibits proliferation of epithelial cells and stimulates the proliferation of certain mesenchyme-derived cells, including fibroblasts (Roberts et al., 1988). In vivo studies have revealed that implantation of a slow-release pellet containing TGF- β 1 reversibly inhibited DNA synthesis in mouse mammary end buds during the prepubertal phase (Silberstein and Daniel, 1987). However, TGF- β 1 did not influence morphology or DNA synthesis during puberty or alveolar development (Daniel et al., 1989). Both TGF- β 2 and TGF- β 3 mimic the effect of TGF- β 1 on the mammary gland (Robinson et al., 1991).

The mechanisms through which TGF- β 1 affects cell growth are not fully understood. Net growth of a cell population is determined by the balance between rates of cell proliferation and apoptosis. Transforming growth factor- β 1 affects the cell cycle through expression of cyclins, cyclin-dependent kinases, cyclin-dependent kinase inhibitors, and *c-myc*, all of which are involved in cell cycle progression (Alexandrow and Moses, 1995). Furthermore, TGF- β 1 regulates apoptosis (Schuster

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and Kriegelstein, 2002). Therefore TGF- β 1 may regulate mammary growth through either or both of these mechanisms.

Transforming growth factor- β 1 is also known to influence mammary gland development by altering the composition of the ECM. Localized release of TGF- β 1 from a pellet implanted in the stromal compartment induced intense collagen I gene expression (Silberstein et al., 1990). Transforming growth factor- β 1 is known to positively regulate expression of ECM proteins such as fibronectin, collagen IV, and laminin as determined with human mammary epithelial cells (Stampfer et al., 1993).

The effects of TGF- β 1 on bovine mammary gland development have been studied less extensively. Transforming growth factor- β 1 has been shown to inhibit proliferation of primary mammary epithelial cells prepared from heifers in a dose-dependent manner (Purup et al., 2000). Growth inhibition has also been observed in a bovine mammary epithelial cell line (Woodward et al., 1995).

All isoforms of TGF- β mRNA are expressed at different stages of bovine mammary gland development (Plath et al., 1997). Transforming growth factor- β 1 mRNA levels are high in virgin heifers, but decrease during pregnancy and lactogenesis. During involution, when mammary gland remodeling occurs, TGF- β 1 mRNA levels are again elevated (Plath et al., 1997). Therefore, it is likely that TGF- β 1 plays a role in regulating mammatogenesis in dairy cows. However, no studies have been conducted on the effects of TGF- β 1 on mammary gland development in cattle *in vivo*.

We hypothesized that exogenous TGF- β 1 would inhibit mammary epithelial cell proliferation and stimulate stromal cell proliferation and synthesis and secretion of ECM proteins. To test this hypothesis, we implanted plastic polymer pellets, which slowly release TGF- β 1 into the parenchyma of prepubertal heifers, and analyzed mammary cell proliferation and apoptosis. We also measured the effect of TGF- β 1 on gene expression of c-myc and fibronectin, which are involved in regulating cell growth by cell cycle progression and ECM composition, respectively.

MATERIALS AND METHODS

Preparation of TGF- β 1 Ethylene Vinyl Acetate Copolymer (Elvax) Pellets

Elvax pellets (Du Pont Polymers, Wilmington, DE) were washed for 4 d in 95% ethanol with continuous stirring. After washing, 1 g of Elvax was dissolved in 5 mL of 5% dichloromethane (Fisher Scientific, Pittsburgh, PA). The viscous Elvax solution was transferred to 1-inch diameter cup-shaped molds and different mixtures

of protein were dispersed in 1 mL of Elvax. Mixtures were prepared by dissolving 20 mg of BSA (Gibco BRL, Rockville, MD) in PBS, alone or with the addition of either 0.5 or 2 μ Ci 125 I-TGF- β 1 (100 μ Ci/ μ g of protein, specific activity; Amersham Biosciences, Piscataway, NJ) or 5 μ g of unlabeled TGF- β 1 (R&D Systems, Inc., Minneapolis, MN) dissolved in 17 mM acetic acid/1% BSA. The solution was then lyophilized and mixed into the Elvax. Pellets were allowed to dry, and, after 12 h, the pellets were shaped in a circle (0.6-cm diameter) by trimming the edges. Pellets were rolled into cylinders (0.4-cm diameter) and tied in this shape with 15 cm of sterile nylon suture material (3-O Webster, Inc., Sterling, MA) using a sterile needle to perforate the pellets to attach the suture material.

In Vitro Release Kinetics of TGF- β 1 from Elvax Pellets

To determine *in vitro* release kinetics, 5 pellets containing 0.5 μ Ci of 125 I-TGF- β plus 20 mg of BSA were suspended in beakers containing 10 mL of PBS, and 250 μ L aliquots of the PBS were removed 0.5, 1, 2, 6, 8, 14, 24, and 48 h after placing the pellets in the beakers. Total counts in each aliquot were measured using a gamma counter (Wallac, Gaithersburg, MD). Release kinetics were expressed as the percentage released from the pellet compared with total original counts in the pellet.

Implantation of Elvax Pellets

Heifers were sedated by administering xylazine (1 mg/mL) *i.v.* (0.05 mL/100 kg of BW). Level of sedation was evaluated by loss of the palpebral reflex. Udders were clipped, washed with a sanitizing solution, and rinsed with 70% ethanol. Lidocaine (1.5 mL per quarter) was injected as a field block around the incision site. The rear quarters were palpated to localize the parenchyma. The parenchyma was distinguished from the fat pad by its greater density and firmness. A scalpel was used to make an incision (~0.3 cm) through the skin and underlying connective tissue capsule at the midpoint of each rear quarter. Next, a 0.5-cm diameter, 10-cm long cannula filled with a trocar (Popper & Sons, New Hyde Park, NY) was inserted into the parenchyma. The trocar was removed and used as a plunger to insert the pellet, with sterile suture material attached, into the parenchyma. The end of the nylon suture was placed under the skin, and the wound was then closed with wound clips. The suture material was attached to the pellet to facilitate the recovery of the pellet after euthanasia.

In Vivo Diffusion Distance of TGF- β 1 from Elvax Pellets

To determine the distance that TGF- β 1 diffused in vivo and, hence, to determine the amount of tissue to cut from around the pellet during the experiment described in detail below, a pellet containing 2 μ Ci of 125 I-TGF- β 1 was implanted in the left rear quarter of a 9-mo-old heifer. After 24 h, the heifer was killed, and the mammary gland was recovered. Five tissue sections (each 0.2-cm wide) were taken from each side of the pellet, parallel to the pellet. The sections were weighed, and radioactivity was measured in a gamma counter to determine the total radioactivity each tissue section had been exposed to. Release kinetics were expressed as cpm/mg tissue for each section. In addition, the radioactivity that remained in the pellets after 24 h of implantation was determined in a gamma counter to calculate the percentage of 125 I-TGF- β 1 released from the pellet.

Experimental Design

Four prepubertal Holstein heifers, 9 mo old and weighing 225 ± 20 kg, were used. A pellet containing 5 μ g of TGF- β 1 plus 20 mg of BSA was implanted in the parenchyma of the right rear quarter of the mammary gland of each heifer. A control pellet containing 20 mg of BSA was implanted in the parenchyma of the left rear quarter of the mammary gland of the same heifers. Bromodeoxyuridine (**BrdU**; Roche, Indianapolis, IN; 2.25 mg/kg of BW) was administered at a concentration of 20 mg/mL in 0.9% saline via a jugular catheter at 4, 12.5, and 22 h after implantation of the pellets. Two hours after the last BrdU injection, the heifers were euthanatized by injecting euthanasia solution i.v. (Sleep Away; Ford Dodge Animal Health, Overland Park, KS; 1 mL/5 kg of BW). The mammary glands were recovered and transported to the laboratory (less than 5 min in transit). The mammary glands were placed skin side up on a sterile surgical drape. An incision was made through the skin at the point of implantation entry with a sterile razor blade, and the nylon suture material was followed to find the BSA pellet and the TGF- β 1 pellet in the peripheral parenchymal zone. A 0.216-cm³ (0.6- $\times$ 0.6- $\times$ 0.6-cm) block of tissue section around the pellet, with the pellet being in the middle, was excised. The block of tissue was divided into two equal halves by cutting a cross section. One-half was fixed in 10% buffered formalin (Fisher Diagnostics, Middletown, VA) for 4 h. Fixed samples were transferred to PBS and sectioned as described subsequently. The other one-half was frozen in liquid nitrogen to be used for RNA extraction.

All procedures involving animals were conducted humanely under the approval of the University of Vermont Institutional Animal Care and Use Committee.

Immunohistochemistry and Tissue Staining

BrdU and fibronectin localization. Immunohistochemistry was performed to detect BrdU and fibronectin. Formalin-fixed tissue was embedded in paraffin, sectioned at 5 μ m, and mounted onto slides. To detect cells that incorporated BrdU, a mouse monoclonal anti-BrdU antibody (cat# 1202693; Roche) was used. To detect fibronectin protein, a mouse monoclonal fibronectin Ab-11 clone FBN11 antibody (cat# MS-1351-R7; Neomarkers, Fremont, CA) was used. For both stainings, a Histostain broad-spectrum detection kit (Zymed Laboratories Inc., San Francisco, CA) was used. In brief, slides were deparaffinized in xylene and rehydrated through a graded series of alcohols. Endogenous peroxidases were quenched by incubating slides in 3% H₂O₂ in methanol for 15 min. The sections were rinsed in distilled water and boiled in 10 mM sodium citrate (pH 6.0) twice for 5 min with a 5-min cooling time in between. After the second boil, the slides were cooled for 30 min and rinsed in PBS. After a 10-min block with Ready-to-Use blocking solution (Zymed kit), the BrdU antibody was added at 1:100, diluted in 1% BSA/PBS. The manufacturer's working concentration was used for the fibronectin primary antibody.

Slides were incubated in a humid chamber for 16 h at 4°C and washed with PBS before application of a biotin-conjugated anti-mouse secondary antibody for 30 min at room temperature. Detection of the biotin-avidin complex was carried out by incubating the slides for 10 min at room temperature in streptavidin-peroxidase conjugate followed by a 5-min incubation in diaminobenzidine. Sections were counterstained with hematoxylin for 3 min, dehydrated, mounted with Histomount, and cover-slipped. For all immunohistochemistry procedures, a mouse non-specific IgG was used as a negative control (Zymed Laboratories Inc.).

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling staining. In situ detection of DNA fragmentation was performed using terminal deoxynucleotidyltransferase (**TdT**)-mediated dUTP nick-end labeling (**TUNEL**) staining (Gavrieli et al., 1992). In brief, sections were deparaffinized in xylene and rehydrated through a graded series of alcohol. Proteinase K (Sigma, St. Louis, MO) digestion was carried out at 25 μ g/mL for 15 min at room temperature preceding inactivation of endogenous peroxidases with 3% H₂O₂ in methanol for 15 min at room temperature. Tissue sections were labeled with 30 U of TdT (Roche) and 1 nmol of 16-dUTP-biotin (Roche) in 100 μ L of TdT

buffer for 1 h at 37°C. After stopping the DNA nick-end labeling reaction, the sections were blocked for 30 min at room temperature with 2% BSA in tris-buffered saline. Labeled sections were treated with streptavidin-peroxidase (Zymed Laboratories, Inc.) for 20 min at room temperature and then incubated with amino ethyl carbazole substrate (Zymed Laboratories) under the same conditions. Tissue sections were counterstained with hematoxylin for 1.5 min, rinsed in distilled water, and mounted with GVA (glycerol vinyl alcohol) aqueous mounting solution.

All analyses included positive control sections that were treated with 10 μ g/mL DNase I (Sigma) for 15 min at room temperature. Negative control sections were incubated with 1 nmol of 16-dUTP-biotin in 100 μ L of TdT buffer, without TdT.

Quantitation of apoptosis and cell proliferation. Slides stained for BrdU and TUNEL were examined with an Olympus BX41 light microscope (Olympus America Inc., Melville, NY) to quantify BrdU-labeled cells and apoptotic cells. Photomicrographs were taken at 400 $\times$ magnification from 12 fields per slide, containing epithelial and stromal cells, using Optronics Magnafire-SP software. For each BrdU-stained slide, a total of 3000 epithelial and 3000 stromal cells were counted. Each cell was scored as negative or positive for BrdU staining. For each TUNEL-stained slide, the TUNEL-positive cells were counted out of a total of 8 fields (approximately 2000 cells). Cells were counted using Image Pro Express (Media Cybernetics, L.P, Silver Spring, MD). Data are reported as the percentage of positive cells per total number of epithelial or stromal cells counted.

Quantitative RT-PCR

Ribonucleic acid was isolated from the tissue with Trizol according to the manufacturers protocol (Life Technologies, Rockville, MD). The RNA was treated with RNase-free DNase (Ambion Inc., Austin, TX) at a concentration of 2 U/ μ L for 35 min at 37°C to remove genomic DNA.

Using the isolated RNA as a template, cDNA was prepared using the Promega Reverse Transcription kit (Promega, Madison, WI). In brief, the RNA (2.5 μ g) was incubated with random hexamer primers for 5 min at 70°C. Then, 1 $\times$ reverse transcriptase buffer, RNase inhibitor, 10 mM dNTP mix, and Molony murine leukemia virus-reverse transcriptase were added, and the samples were incubated for 10 min at room temperature, followed by 60 min at 42°C, and 15 min at 70°C. For each sample, a negative control was made by taking some of the RNA and performing a RT-PCR without the Molony murine leukemia virus-reverse transcriptase.

Primers and probes for c-myc and fibronectin were made using the *Bos taurus* c-myc proto-oncogene and *Bos taurus* fibronectin DNA sequences (Gen Bank accession nos. AF519455 and K00800, respectively). Primers and probes were designed using the software Primer Express (Applied Biosystems, Foster City, CA) purchased from Sigma Genosys (The Woodlands, TX). Primers and probe for the glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) housekeeping gene were a gift from D. Kerr (University of Vermont, Burlington). Sequences for primers and probes are given in Table 1. Using a Perkin-Elmer ABI 7700 Prism Sequence Detection System (Applied Biosystems), the relative abundance of cDNA corresponding to c-myc, fibronectin, and GAPDH were each quantified relative to the levels in standard curves. The standard curves were made from cDNA from the mammary gland of a 9-mo-old heifer. Levels of c-myc and fibronectin were then normalized to levels of GAPDH in the same samples to allow comparison between samples. Probes for all three genes were used at a concentration of 200 nM. Primers were used at a concentration of 900 nM in a total volume of 50 μ L.

Statistical Analysis

All data are presented as means $\pm$ SE. Statistical significance was evaluated using SAS (Statistical Analysis Software, Cary, NC). Data obtained from BrdU and TUNEL experiments were analyzed using a two-sided, paired *t*-test, and data from quantitative RT-PCR were analyzed using a one-sided, paired *t*-test to compare means for TGF- β 1-treated and control quarters within animals. Data with $P \leq 0.05$ were considered statistically significant. If $0.05 > P \geq 0.10$, data were considered to exhibit a trend.

RESULTS

In Vitro Release Kinetics of 125 I-TGF- β 1 from Elvax Pellets

Release kinetics of 125 I-TGF- β 1 from Elvax were determined in vitro in a preliminary experiment. Release of 125 I-TGF- β 1 from Elvax pellets in vitro reached 32% by 24 h and 38% by 48 h (Figure 1). The percentage of 125 I-TGF- β 1 that was released per hour steadily declined from 15% during the first hour to <2% after 12 h.

In Vivo Diffusion Distance of 125 I-TGF- β 1

To determine the distance TGF- β 1 diffuses from the Elvax pellet into the surrounding mammary tissue, we counted radiolabeled ligand in mammary tissue sections 24 h after implantation of a pellet containing 2 μ Ci

Table 1. Primer and probe sequences for quantitative RT-PCR.¹

Gene	Forward primer	Probe	Reverse primer
c-myc	CGTGTCTACCCATCAGCACAA	TACGCAGCGCCCCCTCCA	CTTGGCGGCGGGATAGT
Fibronectin	GGAGAACAGTGGCAGAAGGAAT	TCTTGGTGCCATTTGCTCCTGCAC	AGGTCTGCGCAGTTGTCA
GAPDH ²	GCATCGTGGAGGGACTTATGA	CCACTGTCCACGCCATCACTGCCACC	CAGAAGACTGTGGATGGCCC

¹Probes were labeled with 6-carboxyfluorescein (6-FAM) on the 5' end and with black hole quencher (BHQ-1) on the 3' end.

²GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

¹²⁵I-TGF- β 1. As expected, Figure 2 shows that counts of ¹²⁵I-TGF- β 1 were highest in tissue recovered closest to the pellet. As determined by total counts remaining in the pellet after 24 h, up to 47% of ¹²⁵I-TGF- β 1 was released from the pellet. However, only 25% of the ¹²⁵I-TGF- β 1 released was localized within 0.2 cm on either side of the pellet after measuring radioactivity in each tissue section. A second zone of less intense radioactivity extended the area by another 0.2 cm on each side of the implant so that the total zone of diffusion was 0.8 cm (i.e., 0.4 cm on each side of the implant). Radioactivity was not detected at a distance >0.8 cm around the pellet. In the following experiment, a zone of 0.6 cm around the pellet was used to ensure that tissue was exposed to TGF- β 1.

Based on the percentage of ¹²⁵I-TGF- β 1 recovered in the 0.2-cm tissue section surrounding the Elvax pellet, at least 25% of the 5 μ g of TGF- β 1 used for each pellet was delivered into the mammary tissue of the 4 prepubertal heifers.

TGF- β 1 Induces Stromal Cell Proliferation In Vivo

Deoxyribonucleic acid synthesis was measured by counting the proportion of cells positive for BrdU incor-

poration. Proliferating cells were distinguished by the presence of brown granules atop the nucleus, indicating the incorporation of BrdU into DNA (Figure 3, A and B). Proliferation of mammary stromal cells was increased 1.8-fold in quarters treated with 5 μ g of TGF- β 1 for 24 h compared with BSA-treated control quarters (3.5% vs 1.9%; $P < 0.0002$; Figure 4A). Transforming growth factor- β 1 did not have a significant effect on mammary epithelial cell proliferation as determined by comparing the mean analyzed with a paired t -test. However, there was a trend toward an increase in DNA synthesis in mammary epithelial cells ($P < 0.10$; Figure 4A).

The Apoptotic Rate of Mammary Epithelial and Stromal Cells Is Low

The pattern of apoptosis was measured by TUNEL staining of sections from mammary gland quarters exposed to BSA or TGF- β 1 (5 μ g) for 24 h. Figure 3 (C and D) shows TUNEL-positive cells, indicated by red nuclei that represent the incorporation of d-UTP in the nicked DNA ends. The overall apoptotic rate was very low during this stage of mammary gland development. There was no significant difference observed in the percentage of apoptotic mammary epithelial or stromal

Figure 1. Release kinetics of ¹²⁵I-transforming growth factor- β 1 (TGF- β 1) from Elvax pellets (Du Pont Polymers, Wilmington, DE). Release kinetics of 5 pellets containing 20 mg of BSA and 0.5 μ Ci of ¹²⁵I-TGF- β 1. Samples of PBS were taken at 0.5, 1, 2, 6, 8, 14, 24, and 48 h after placement of the pellets in beakers with PBS and measured in a gamma counter. The average counts from each time point are expressed as the cumulative percentage released compared with the total counts per pellet. Thirty-eight percent of the ¹²⁵I-TGF- β 1 was released by 48 h.

Figure 2. Diffusion of ¹²⁵I-transforming growth factor- β 1 (TGF- β 1) from an Elvax pellet (Du Pont Polymers, Wilmington, DE) into mammary tissue. A pellet containing 20 mg of BSA and 2 μ Ci of ¹²⁵I-TGF- β 1 was implanted in the left rear quarter of a prepubertal heifer. Ten zones of 0.2-cm width around the implants were cut, weighed, and counted in a gamma counter. The counts of each zone were divided by the weight of the tissue. * = pellet location.

Figure 3. Immunohistochemical detection of bromodeoxyuridine- (BrdU-) and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling- (TUNEL-) positive cells in the mammary gland of prepubertal heifers. Quarters were implanted with pellets containing 20 mg of BSA (control) or 5 μ g of transforming growth factor- β 1 (TGF- β 1) plus 20 mg of BSA for 24 h. Immunohistochemistry, using a BrdU antibody, was performed on both BSA- (A) and TGF- β 1-treated (B) quarters. Arrows indicate BrdU-positive cells. Also, TUNEL staining was performed on either BSA- (C) or TGF- β 1-treated (D) sections. Arrows indicate TUNEL-positive cells. (Bar = 25 μ m).

cells after TGF- β 1 treatment in comparison with BSA control (Figure 4B).

TGF- β 1 Increases Fibronectin Gene Expression, But Does Not Affect c-myc Gene Expression

To determine whether TGF- β 1 had an effect on c-myc or fibronectin gene expression, cDNA from parenchymal tissue from TGF- β 1- and BSA-treated quarters was analyzed by quantitative RT-PCR. The effect of TGF- β 1 on c-myc gene expression is shown in Figure 5A. Treatment of quarters with 5 μ g of TGF- β 1 for 24 h resulted in no significant change in c-myc gene expression compared with BSA-treated, control quarters. Figure 5B shows a 3-fold increase in relative expression of the fibronectin gene after treatment with 5 μ g of TGF- β 1 for 24 h compared with the BSA-treated control ($P < 0.06$).

Effect of TGF- β 1 on Fibronectin Production

To investigate whether there was an increase in accumulation of ECM proteins caused by TGF- β 1, we examined the distribution of fibronectin by immunohistochemistry. Fibronectin was abundantly expressed in the mammary tissue sections from both BSA- (Figure 6A) and TGF- β 1- treated (Figure 6B) quarters. We observed more fibrous sheets of intense fibronectin staining in sections of TGF- β 1-treated tissue compared with sections from BSA-treated quarters.

DISCUSSION

Plastic polymer pellets containing TGF- β 1 have been used to show that TGF- β 1 plays a role in mammary gland development by inhibiting DNA synthesis of epithelial structures in mice. In the stromal compartment, implanted TGF- β 1 was associated with increased colla-

Figure 4. Transforming growth factor- β 1 (TGF- β 1) increases stromal cell proliferation and has no effect on apoptosis. A) The percentage of bromodeoxyuridine- (BrdU-) positive epithelial and stromal cells after treatment with 20 mg of BSA or 5 μ g of TGF- β 1 plus 20 mg of BSA for 24 h. Transforming growth factor- β 1 increased stromal cell proliferation compared with BSA control as determined by paired *t*-test (**P* < 0.0002). B) The percentage of terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling- (TUNEL-) positive cells in epithelium and stroma after treatment with BSA or TGF- β 1.

gen I gene expression, which forced premature differentiation of the end buds in the mouse mammary gland (Silberstein and Daniel, 1987, Silberstein et al., 1990). However, the role of TGF- β 1 in bovine mammary gland development had not been studied *in vivo*. In this study, we measured the effect of TGF- β 1 on proliferation of mammary epithelial and stromal cells and on expression of the TGF- β 1 responsive genes, c-myc and fibronectin, in prepubertal heifers by using slow-release pellets containing TGF- β 1.

Delivery of TGF- β 1 from Elvax pellets into the gland was consistent with the release pattern shown in previous studies with mouse mammary glands (Daniel et al., 1989). Those investigators observed that 40% of the TGF- β 1 was released from the pellet *in vitro* after 48 h. This result is consistent with our findings where 38% of the TGF- β 1 was released in 48 h (Figure 1). From our studies *in vivo* (Figure 2), it was concluded that a dose of approximately 1.2 μ g of TGF- β 1 was delivered to mammary tissue surrounding the pellet. The minimum dose at which TGF- β 1 has been shown to have a significant effect in the mouse is 100 ng per pellet (Daniel et al., 1989). Therefore, our dose of 1.2 μ g should be enough

Figure 5. Transforming growth factor- β 1 (TGF- β 1) increases fibronectin, but not c-myc gene expression. Ribonucleic acid isolated from parenchymal tissue from quarters that received 20 mg of BSA or 5 μ g of TGF- β 1 plus 20 mg of BSA was reverse transcribed into cDNA, and quantitative RT-PCR was performed. A) Difference in c-myc gene expression after TGF- β 1 treatment for 24 h compared with BSA treatment. B) Difference in fibronectin gene expression compared with BSA after the same treatment (†*P* < 0.06).

to show bioactivity. To determine whether TGF- β 1 exerted a positive or negative effect on mammary cell growth, we undertook this overexpression approach. Thus, we consider the dose of 1.2 μ g of TGF- β 1 to be supra-physiological.

Silberstein and Daniel (1987) showed the effect of TGF- β 1 on epithelial cells by demonstrating that end buds, in response to the growth factor, ceased proliferation and regressed into quiescent mammary ducts. In our study, TGF- β 1 did not inhibit proliferation of the epithelial cells (Figure 4A). Interestingly, while not significantly different, DNA synthesis was stimulated in mammary epithelial cells treated with TGF- β 1 compared with BSA (*P* < 0.10). Thus, our study is the first to suggest that TGF- β 1 increases mammary epithelial cell proliferation in the bovine *in vivo*. This stimulation of DNA synthesis may be of biological importance, as Purup et al. (2000) reported a stimulation of DNA synthesis by TGF- β 1 in primary bovine mammary epithelial cells *in vitro* at a concentration of <50 pg/mL. Furthermore, a recent study using *in vitro* tissue culture experiments showed a difference in response of differ-

Figure 6. Fibronectin protein deposition in response to transforming growth factor- β 1 (TGF- β 1). Immunohistochemistry to detect fibronectin protein was performed on slides from quarters treated with A) 20 mg of BSA or B) 5 μ g of TGF- β 1 for 24 h. Fibrous sheets are stained brown for both treatments. (Bar = 25 μ m).

ent parenchymal zones of the bovine mammary gland to TGF- β 1. The investigators found that cells of the peripheral parenchyma are more sensitive to TGF- β 1 than cells of the medial parenchyma (Ellis et al., 2000). Because we harvested tissue from peripheral parenchymal zones, it may be that TGF- β 1 differentially regulates proliferation of epithelial cells in these regions.

The increase in stromal DNA synthesis, as affected by TGF- β 1 (Figure 4A), is similar to that reported *in vitro* where mouse and rat mesenchyme-derived fibroblasts were treated with TGF- β 1. Both studies showed an increase in fibroblast proliferation after TGF- β 1 treatment (Roberts et al., 1988, Zhao and Young, 1996). In conclusion, the results obtained from BrdU staining demonstrated that TGF- β 1 acts on the stromal compartment of the mammary gland by increasing DNA synthesis of stromal cells.

An independent marker of the rate of cell proliferation is c-myc. Transcriptional regulation by the c-myc protein is required for cell cycle progression, which was shown to be downregulated by TGF- β 1 *in vitro* (Alexandrow and Moses, 1995). This suggests that downregulation of c-myc by TGF- β 1 in epithelial cells is an important mechanism for cell growth arrest. The fact that c-myc was not downregulated in our study is consistent with the lack of inhibition of DNA synthesis in epithelial cells (Figure 5A). Given our results, we would expect, if anything, c-myc to be upregulated. However, we did not observe a change in c-myc mRNA expression, which can be explained by the fact that only 1 to 3% of cells are dividing. Therefore, 97% of the cells are not dividing, so there is no measurable increase in c-myc gene expression.

In contrast to the lack of an effect on c-myc expression by TGF- β 1, fibronectin gene expression was upregu-

lated by TGF- β 1 (Figure 5B), even though RNA for this experiment was isolated from both epithelial and stromal cells. Transforming growth factor- β 1 is known to stimulate the expression of fibronectin and its incorporation into the ECM (Ignatz and Massague, 1986). Combined with the fact that TGF- β 1 significantly stimulated stromal cell proliferation, as shown with the BrdU staining data, we believe that this increase is biologically important.

Because we observed an increase in fibronectin gene expression upon treatment with TGF- β 1, we examined fibronectin protein deposition by immunohistochemistry. More fibrous sheets of intense fibronectin staining appeared to be present in sections from TGF- β 1-treated quarters compared with BSA-treated, control quarters (Figure 6). In contrast, TGF- β 1 did not affect collagen IV protein deposition (data not shown), indicating a specific effect of TGF- β 1 on fibronectin deposition.

Epithelium and stroma communicate with each other through the ECM. Because TGF- β 1 induced stromal cell proliferation and ECM protein synthesis, our data suggest a possible role for TGF- β 1 in modulating mammary epithelial-stromal interactions. However, it is not clear whether a positive effect on the ECM by TGF- β 1 will promote epithelial cell growth. Our suggestion is supported by studies that have shown strong localization of TGF- β 1 in the stromal tissue during mouse mammary ductal morphogenesis, as determined by immunohistochemistry. Transforming growth factor- β 1 hereby inhibited ductal budding indirectly through ECM deposition and directly by using the ECM as a reservoir to affect the epithelial cells (Silberstein et al., 1990, 1992). In addition, mice overexpressing a dominant negative TGF- β type II receptor in the stroma exhibited increased lateral branching of the mammary ductal tree,

suggesting that TGF- β 1 negatively regulates branching morphogenesis by influencing stromal-epithelial interactions (Joseph et al., 1999).

Our findings here are important, as they show for the first time that TGF- β 1 affects the stromal compartment of the bovine mammary gland in vivo through increased stromal cell proliferation and fibronectin gene expression. We speculate that TGF- β 1 stimulates bovine mammary gland development by providing a matrix for epithelial cells to grow in after stimulation of the stroma. Second, TGF- β 1 could cause an imbalance in epithelial versus stromal cell proliferation and ECM synthesis to control branching morphogenesis. Which of these speculations holds up will have to be investigated in further studies.

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