Cause of Failure of Lactation in Mouse Mammary Tumor Virus/Human Transforming Growth Factor α Transgenic Mice (43702)

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Abstract. **Transgenic female mice bearing human transforming growth factor-α (TGFα) cDNA under the control of the mouse mammary tumor virus enhancer/promoter became pregnant but failed lactation. TGFα mRNA was detected in the mammary glands of these mice by the reverse transcriptase-polymerase chain reaction. By the use of collagenase-dissociated mammary epithelial cells, the binding of prolactin to its receptor was determined before and after parturition. At the end of pregnancy, the binding in TGFα transgenic (TGFα[+]) mice was small and its amount was comparable to that in the TGFα negative (TGFα[−]) mice. On the day of parturition, prolactin binding in TGFα(+) mice increased approximately 1.9-fold (insignificant), while that in TGFα(−) mice elevated over 5.3-fold ($P < 0.01$). The binding sites per cell were also higher in TGFα(−) mice. Radioimmunoassay of prolactin suggested that in TGFα(+) mice the low level of prolactin binding after parturition was not due to masking effect of serum prolactin. Among six TGFα(+) mice assayed, one mother with the highest prolactin binding activity (3.7-fold increase) initiated lactation, but the others did not. As there was little difference between groups in the growth and synthesis in the mammary glands, it was concluded that the failure of lactation in TGFα(+) mice is principally due to the lack of elevation of mammary prolactin receptor after parturition. At present, the role of TGFα in this process is obscure; however, TGFα was revealed not to interfere with the binding of prolactin to the receptor.**

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Transforming growth factor-α (TGFα) is a 50 amino acid polypeptide and is a potent mitogen. The autocrine mechanism of TGFα is demonstrated in various cancers (1–3) and cancer cell lines (4–7); however, its biological significance on the growth of cancer cells still remains to be clarified. TGFα expression has also been identified in normal cells, including human keratinocytes (8) and human and rodent mammary epithelial cells (9, 10), suggesting that this growth factor has functional roles in normal mammary gland. Snedeker *et al.* (11) have identified TGFα and EGF in the mouse mammary glands at different developmental stages: TGFα, like EGF, was localized to the proliferated epithelial cell layers of the terminal end-buds. Matsui *et al.* (12) and Halter *et al.* (13) reported the consequence of overexpression of TGFα on the growth of normal and neoplastic mammary glands in mice bearing TGFα cDNA transgene under the control of the mouse mammary tumor virus (MMTV) enhancer/promoter and found that these MMTV/TGFα transgenic mice were not able to lactate

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(12). Prolactin is a lactogenic hormone and exerts its action firstly by binding to its receptor (14–16). It is generally known that the number of prolactin receptor in the mammary glands increases after parturition. The failure of initiation of lactation may be caused by no increase in either the number of prolactin receptors in the mammary glands or serum prolactin level. Employing TGFα transgenic mice as the model system, we intended to examine directly either possibility above. Both the number of receptors in the mammary glands and the serum prolactin level were compared, between before and after parturition and between TGFα(+) and TGFα(−) mice. We report here that the failure of lactation of these transgenic mice is principally associated with an insufficient rise of prolactin receptor in the mammary glands at parturition.

Materials and Methods

Animals. Litter mates of MMTV/TGFα transgenic mice (12) were provided by Vanderbilt University School of Medicine, Nashville, TN, as a basal stock. Transgenic mice were generated by mating a transgenic male with females of SHN strain (17, 18). At 50–60 days of age, both female and male offsprings were checked for the presence of TGFα gene by the method described below and divided into TGFα(+) and TGFα(−) groups.

For confirmation of failure of lactation, both TGFα(+) and TGFα(−) females were mated with TGFα(−) males at 60–70 days of age. Pregnant mice were kept individually and placed again with males only near parturition to induce concurrent pregnancy.

For the prolactin receptor studies, both TGFα(+) and TGFα(−) females were mated with TGFα(−) males, and the day of the presence of vaginal plug was designated as Day 1 of pregnancy. Pregnant mice were killed under light ether anesthesia at 09:00–10:00 hr on Day 18 or 19 of pregnancy or at 11:00–12:00 hr on the day of parturition (Day 0 of lactation) after litter removal for 2 hr and blood was collected from the trunk. At autopsy, all 10 mammary glands were removed and subjected immediately to prolactin receptor assay.

For other experiments, pregnant and lactating mice were produced as described above except that mice on Day 0 of lactation were killed without litter removal. A part of the third thoracic mammary glands were used for histological examination and the remainder were stored at −80°C for the determination of TGFα mRNA. Bilateral inguinal mammary glands were also stored at −80°C for determination of lactose and nucleic acids.

Throughout the experiments, animals were kept in plastic cages (16 × 28 × 13 cm) with wood shavings, maintained in a windowless animal room, which was air-conditioned (21°–22°C and 50%–70% relative humidity), artificially illuminated (14 hr of light from 05:00 to 19:00 hr) and ventilated 16 times/hr, and were provided with a commercial diet (Nihon Nosan Kogyo KK, Yokohama, Japan) and tap water *ad libitum*.

DNA Preparation. Genomic DNA was prepared from whole blood by the method of Higuchi (19). Heparinized blood was obtained from mice at 40–50 days of age by orbital venous puncture under the light ether anesthesia, and 0.1 ml of blood was mixed with 0.1 ml lysis buffer containing 0.32 *M* sucrose, 10 m*M* Tris-HCl (pH 7.5), 5 m*M* $MgCl_2$ and 1% Triton X-100 in a 1.5-ml Eppendorf microcentrifuge tube, and centrifuged at 13,000*g* for 20 sec. The pellet was resuspended in 0.1 ml lysis buffer using vortex mixer, and centrifuged at 13,000*g* for 20 sec followed by resuspension of the pellet in 0.1 ml polymerase chain reaction (PCR) buffer containing Proteinase K (0.1 mg/ml), and incubation at 50°C for 50 min and at 90°C for 10 min to inactivate the proteinase.

Enzyme Amplification. PCR was performed in the PCR buffer solution containing 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% (w/v) gelatin, 10 m*M* Tris-HCl (pH 8.3), 25 μl of genomic DNA preparation described above, 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), each dNTP at 200 μ*M*, 1.0 μ*M* of each primer (upstream hTGFα primer; 5′-GATCCAGTGTGACCTAGAGAAGAAAT-3′ and downstream β-globin primer; 5′-GATCTTTTCTATGGAATAAGGAATGGA-3′) (Matsui, personal communication) (total vol = 100 μl). The target sequences in samples were amplified for 40 cycles (1 cycle = 94°C—1 min, 54°C—2 min, 72°C—3 min) in a program temperature control system (Astec, Fukuoka, Japan). The amplified products were analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Prolactin Receptor Assay Using Dissociated Mammary Epithelial Cells. Ovine prolactin (NIADDK-P-S17, 30 IU/mg) was kindly supplied by NIADDK, Bethesda, MD. Biological active [I^{125}]-labeled prolactin ([I^{125}]prolactin) was prepared by a lactoperoxidase-H_2O_2 method (20). Specific radioactivity of [I^{125}]prolactin was about 2.6 MBq/μg.

A precise protocol for digestion of mammary tissue, which predominantly consisted of alveolar cells as well as duct cells, with collagenase and *in vitro* prolactin binding assay has previously been described in detail (21). The mammary tissue was incubated for 90 min at 37°C in 40 ml (/animal) Medium 199 (Hank's salt; GIBCO, Grand Island, NY) (pH 7.4) containing 0.05% collagenase (Type Ia, 320 U/mg; Sigma Co., St. Louis, MO), 0.01% DNase (510 Kunitz units/mg; Sigma) and 2% bovine albumin (BSA) in a shaking water bath. After the incubation, the solution was passed through one layer of nylon mesh. Residue of tissue on nylon mesh was washed extensively with

Medium 199%–0.25% BSA (assay medium) and with gentle agitation. These filtrated cells were washed three times with 50 ml of the assay medium by centrifugation at 80*g* (tube bottom) for 2 min. For estimation of cell number in the suspension, the cells were suspended in 0.04% crystal violet-0.1 *M* citric acid and vortexed vigorously. Nuclei stained with crystal violet were counted in a hemocytometer. Finally, remaining cell was adjusted by dilution to 1.0×10^7 cells/ml in the assay medium.

The 0.4 ml cell suspension, 50 μl unlabeled prolactin (0 or 5 μg) and 50 μl [I^{125}]prolactin (about 1×10^5 cpm) were incubated for 3 hr at room temperature with constant shaking. After the incubation, the mixture was diluted in 3 ml of the assay medium. The cells were precipitated by centrifugation as described above. Radioactivity was measured in an auto gamma counter with counting efficiency of 60%. Nonspecific binding of [I^{125}]prolactin to the cells was determined in the presence of 5 μg of unlabeled prolactin, and specific binding was obtained by subtracting nonspecific binding from total binding. For Scatchard analysis (22), the concentration of unlabeled prolactin varied between 4 and 64 ng/ml. Each prolactin binding assay was performed in triplicate.

In order to examine the effects of TGFα on the binding of [I^{125}]prolactin, (i) the prolactin binding assay was carried out in the presence of increasing doses of unlabeled TGFα from 1 ng/ml to 3 μg/ml (human recombinant, GIBCO BRL, Gaithersburg, MD), and (ii) the cell homogenate was treated with 3 *M* $MgCl_2$ or H_2O_2 according to the procedures of Edery *et al.* (23) and used for the prolactin binding assay.

Mammary Gland Contents of Lactose and Nucleic Acids. Each frozen gland was thawed and homogenized in 1.5 ml of distilled water. Lactose and nucleic acids were extracted with trichloroacetic acid by the method of Schneider (24). The first extract was lyophilized and dissolved in 2.0 ml of distilled water. Lactose was determined by the measurement of D-glucose after hydrolysis of lactose with β-galactosidase. D-glucose released from lactose was measured by the glucose oxidase and peroxidase method (25). DNA and RNA were determined by the diphenylamine (26) and the orcinol (27) reactions, respectively.

Mammary Gland Histology. For histological examination, portions of inguinal mammary glands were removed from some mice of both groups at the end of pregnancy, fixed in Bouin's solution, embedded in paraffin, sectioned at 6 μm and stained with hematoxylin-eosin.

Serum Prolactin Level. Blood was left at room temperature for 8 hr, kept in the refrigerator overnight, and centrifuged at 1000*g* for 20 min at 4°C; serum was stored at −20°C. Prolactin level was determined by homologous radioimmunoassay.

Weights and Histology of Endocrine Organs. At autopsy, anterior pituitary, adrenals and ovaries were removed and weighed. Adrenals and ovaries were further examined histologically.

RNA Isolation and the Detection of Human TGFα mRNA. RNA was isolated from 100 mg of frozen mammary tissue by the acid guanidium-phenol-chloroform method (28). RNA concentration was determined at 260 nm by spectrophotometer. TGFα mRNA expressed in the mammary gland was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) method. The cDNA for human TGFα was synthesized in 20 μl buffer containing 5 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, each dNTP at 1 m*M*, 1 U/μl of RNase inhibitor, 2.5 U/μl of cloned moloney murine leukemia virus (M-MLV) reverse transcriptase (Perkin Elmer Cetus), 0.75 μ*M* of downstream primer for human TGFα and 1 μg of total RNA. The reaction was performed at 42°C for 30 min and terminated by heating for 5 min at 99°C. The sample was then stored at 5°C. The cDNA obtained by reverse transcriptase reaction was amplified by PCR for 40 cycles in the buffer containing synthesized cDNA, 2 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2.5 U of Taq DNA polymerase, and 0.75 μ*M* upstream primer for human TGFα (total vol = 100 μl). The human TGFα primer sequences were corresponded at nucleotides 110–131 (5′-AGCACGTCCCCGCTGAGTGCAG-3′) for a sense strand and at nucleotides 650–630 (5′-GCTGCACAGGTGATTACAGGC-3′) for an antisense strand, respectively (29).

β-actin primers were synthesized and used as a control RT-PCR analysis (30). Primer sequences were corresponded to the mouse sequence at nucleotides 35–41 (5′-GTGGGCCGCTCTAGGCACCA-3′) for a sense strand and at nucleotides 116–108 (5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′) for an antisense strand, respectively.

Statistics. The statistical significance of difference between groups in each parameter was evaluated by Duncan's multiple range test.

Results

Confirmation of Failure of Lactation. As shown in Table I, none of the TGFα(+) mice was able to suckle its young successfully, and pups died in a day after parturition. Meanwhile, all of the TGFα(−) mice lactated normally.

Prolactin Receptor. After digestion with collagenase, mammary epithelial cells were collected by low-speed centrifugation. In any cell preparations, the collagenase-dissociated cells remained clumps (for aggregates) as determined under a phase contrast microscope. The fat cells and most single cells had been removed by repeating low-speed centrifugation. By histological examination, the cell aggregates main-

Table I. Confirmation of Failure of Lactation (Mean ± SEM)

Group	No. of mice	No. of delivery[a]	Lactation	Failure of lactation	Mother wt at parturition	No. of pups
TGFα(+)	4	9	0	9	34.2 ± 1.4	5.5 ± 2.4
TGFα(−)	2	6	6	0	33.9 ± 0.7	9.0 ± 1.0

[a] Each mouse had two to three parturitions.

tained their original alveolar structure. No differences in the morphological structure were found between TGFα(+) and TGFα(−) mice. The number of cells per animals in TGFα(−) mice was 2.02 ± 0.19 ($\times 10^{-8}$) [n = 5] cells per animal at the end of pregnancy or 1.62 ± 0.15 ($\times 10^{-8}$) (6) cells on Day 0 of lactation (mean ± SEM and the number of estimates in parentheses). In TGFα(+) mice, the cell yield changed from 2.78 ± 0.35 ($\times 10^{-8}$) (4) cells to 2.18 ± 0.16 ($\times 10^{-8}$) (6) cells during the same period. The cell yield was always higher in TGFα(+) mice than in TGFα(−) mice and the difference on Day 0 of lactation was statistically significant ($P < 0.05$).

The *in vitro* prolactin binding assay was performed using the dissociated cells. As shown in Figure 1, the amount of specific binding of prolactin was the same when TGFα(+) and TGFα(−) mice were compared at the end of pregnancy. In TGFα(−) mice, the amount of specific binding of prolactin on Day 0 of lactation increased about 5.3-fold as compared with that at late pregnancy ($P < 0.01$). In TGFα(+) mice, specific binding of prolactin remained low on Day 0 of lactation and differed insignificantly from the value at late pregnancy, the increase being about 1.9-fold. The amount

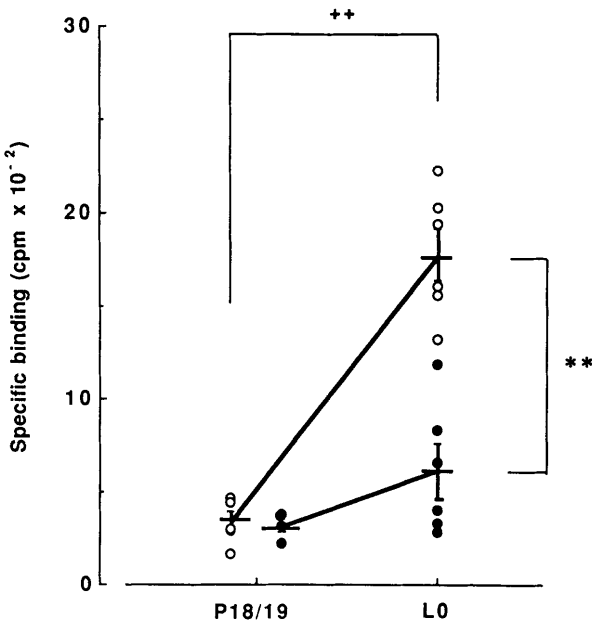

Figure 1. Specific binding of prolactin in the mammary glands of each group. Data are presented as a basis of 4.0 × 10^6 cells. P18/19: Day 18–19 of pregnancy, L0: Day 0 of lactation, ○ TGFα(−), ● TGFα(+). **,++ $P < 0.01$.

of prolactin binding on Day 0 of lactation was significantly smaller in TGFα(+) mice than in TGFα(−) mice ($P < 0.01$). Among six TGFα(+) mice assayed, one mother with the highest prolactin binding activity appeared to initiate lactation, since whitish milk-like substances existed in her mammary gland as well as in the stomach of the young. By the above criteria, the remaining five mothers showed no evidence of lactation.

In our preliminary experiments, the binding of [I^{125}]prolactin to the cell receptor was not interfered with the presence of TGFα. In TGFα(+) mice, the amount of specific binding of [I^{125}]prolactin did not increase after the treatment with 3 M $MgCl_2$ (data not shown).

The *in vitro* prolactin binding assay was carried out in the presence of various doses of prolactin, and the effects of prolactin on the binding of [I^{125}]prolactin to the receptor was examined. Bound/free prolactin ratio was plotted as a function of bound prolactin according to the procedures of Scatchard (Fig. 2). In the present experiments, two individuals among six TGFα(+) mice and all six TGFα(−) mice could be successfully analyzed. In other individuals, including the late pregnant control, the amount of specific prolactin binding was too small to be analyzed by Scatchard analysis. In TGFα(−) mice, the plots line was linear ($r < -0.95$). The K_d for prolactin binding and binding capacity were estimated to be 0.31 ± 0.04 nM and 544.6 ± 40.4 sites per cell, respectively. In TGFα(+) mice, the plots line was parallel with each other and with that of TGFα(−) mice, indicating that prolactin binding affinity differed little between TGFα(+) and TGFα(−) mice. The two plots lines shifted to the left, indicating that the mammary cells contained more prolactin receptors in TGFα(−) mice than in TGFα(+) mice. Scatchard analysis estimated that the K_d for prolactin binding and binding capacity were 0.27 nM and 258.0 sites per cell (the mean of the two determinations) in TGFα(+) mice, respectively. Among eight mice assayed, the amount of specific binding of prolactin correlated well with the number of prolactin receptors per cell ($r > 0.92$).

Mammary Gland Contents of Lactose and Nucleic Acids. The results are shown in Table II. There was little difference between groups in mammary lactose content at the end of pregnancy. A significant increase was observed in the lactose content in

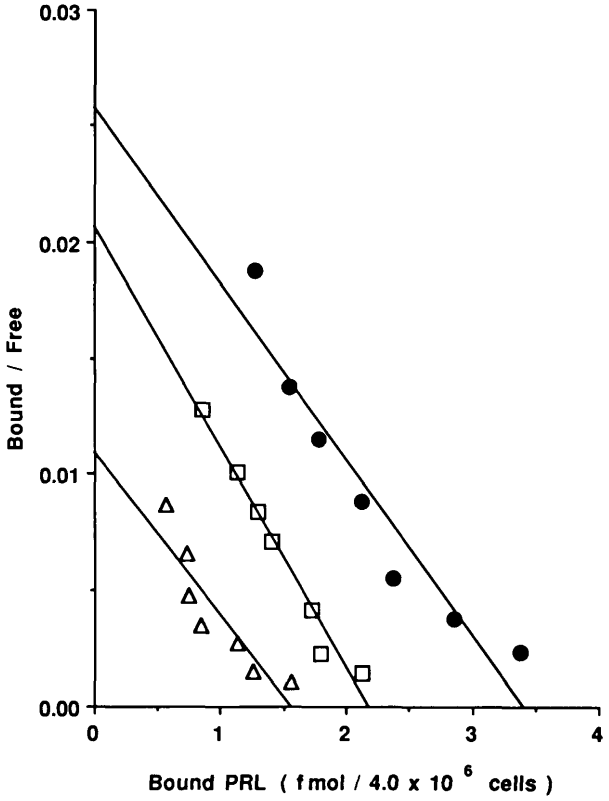

Figure 2. Scatchard analysis of prolactin binding in the mammary glands of each group. The dissociated mammary epithelial cells were 4 × 10^6 cells/tube. In TGFα(−), the means of six estimates are plotted (●). In TGFα(+), two estimates which are possible to be plotted are presented separately (□, △).

TGFα(−) mice, but not in TGFα(+) mice, on Day 0 of lactation and a significant difference in the mammary lactose content on Day 0 was observed between TGFα(+) and TGFα(−) mice.

Either DNA or RNA content in the mammary glands did not differ at the end of pregnancy and on Day 0 of lactation in both TGFα(+) and TGFα(−) mice. RNA/DNA ratio tended to be higher in TGFα(−) mice than TGFα(+) mice near parturition; however, the difference was not statistically significant.

Mammary Gland Structure. There was no apparent difference between groups in the histological structure of mammary glands at the end of pregnancy; the glands were well developed and were extended with secretion (Fig. 3).

Serum Prolactin Level. There was no significant difference in serum prolactin level between TGFα(+) and TGFα(−) groups either at the end of pregnancy (194 ± 65 ng/ml [3] vs 116 ± 37 ng/ml [3]) or on Day 0 of lactation (53 ± 4 ng/ml [5] vs 125 ± 31 ng/ml [8]) (mean ± SEM and the number of estimates in the parentheses).

Mother Weight, Number of Pups, and Weights and Histology of Endocrine Organs. Little difference was observed between TGFα(+) and TGFα(−) groups in any parameter before and after parturition (data not shown). Furthermore, the structures of adrenals and ovaries near parturition were similar in both groups; adrenals were composed of the cortices with three zones and the medulla. Ovaries consisted of both functional corpora lutea and follicles at the several developmental stages. These observations suggest that the secretory activity of steroid hormones from these glands differs little between TGFα(+) and TGFα(−) groups.

Expression of TGFα mRNA. As presented in Figure 4A, in all samples, human TGFα mRNA transcripts were detected in mammary glands of TGFα(+) mice both at the end of pregnancy and on Day 0 of lactation. Amplification of mRNA transcripts from mammary glands of TGFα(−) mice showed no products of target cDNA region at either stage.

Amplification product (245 bp) obtained by PCR analysis with β-actin primers was detected in all samples (Fig. 4B).

Discussion

The elevation of prolactin receptor in the mammary glands is a prerequisite condition for lactogenesis as well as maintenance of lactation (14–16). In this study, only a slight elevation of the receptor was observed in TGFα(+) mice at parturition, whereas in TGFα(−) mice, the receptor level increased markedly at this time. Associated with this, the lactose content in the mammary glands on Day 0 was significantly

Table II. Mammary Gland Contents of Lactose and Nucleic Acids in Each Group (Mean ± SEM)

Group	No. of estimates	Lactose (mg)	DNA (mg)	RNA (mg)	RNA/DNA
End of pregnancy					
TGFα(+)	4	32.19 ± 4.53	4.19 ± 0.17	9.79 ± 1.30	2.03 ± 0.20
TGFα(−)	4	35.67 ± 2.18	3.87 ± 0.34	11.96 ± 2.01	3.34 ± 0.84
Day 0 of lactation					
TGFα(+)	5	35.62 ± 6.50	4.34 ± 0.56	9.01 ± 0.73	2.24 ± 0.35
TGFα(−)	7	79.79 ± 6.55*	3.85 ± 0.32	9.32 ± 0.67	3.34 ± 0.84

* Significantly higher than the other groups at $P < 0.05$.

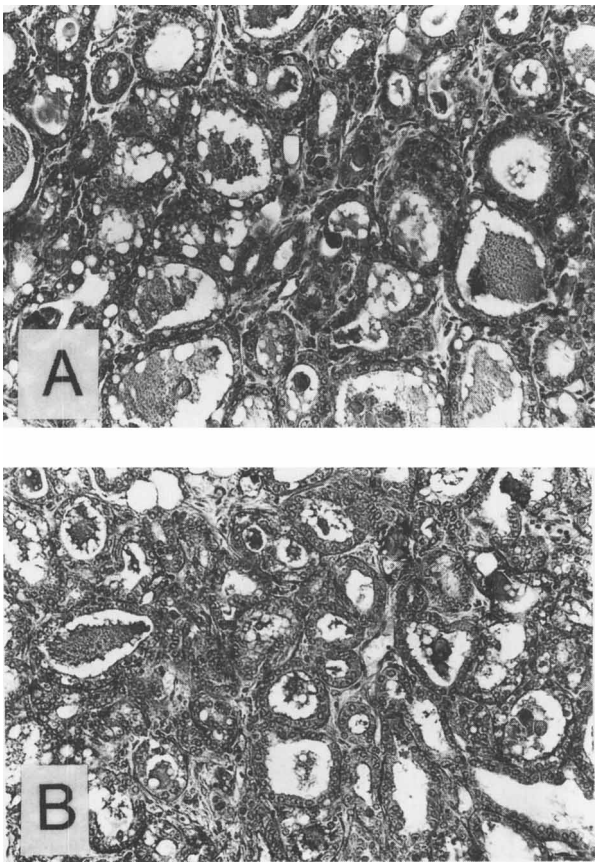

Figure 3. Representative mammary gland structures of TGFα(+) (A) and TGFα(−) (B) mice at the end of pregnancy (×200, H-E stain).

lower in TGFα(+) mice than in TGFα(−) mice, which indicates no rise of milk synthesis in TGFα(+) mice. On the other hand, mammary gland contents of lactose and nucleic acids, RNA/DNA ratio and the histological structure of mammary glands at the end of pregnancy differed little between groups, indicating that

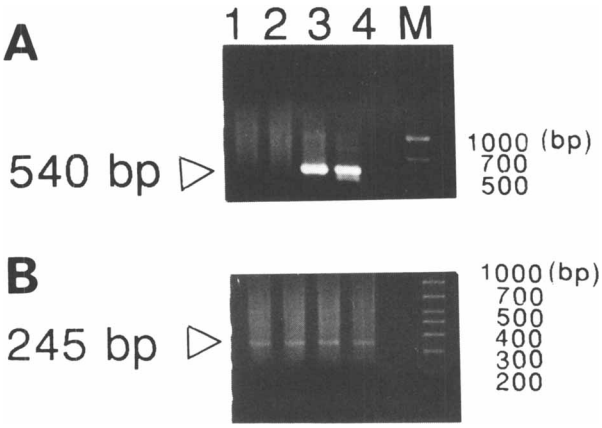

Figure 4. Detection of human TGFα (A) and β-actin (B) transcripts in the mammary gland of each group. Arrows indicate TGFα and β-actin transcripts as predicted 540 bp and 245 bp, respectively. 1: TGFα(−), end of pregnancy; 2: TGFα(−), Day 0 of lactation; 3: TGFα(+), end of pregnancy, 4: TGFα(+), Day 0 of lactation; M: nucleotide size marker.

even the mammary glands of TGFα(+) mice were ready to lactate at the end of pregnancy. Furthermore, we observed that all newborns from TGFα(+) mothers grew quite normally when they were fosternursed by nontransgenic control mothers. These results strongly suggest that the failure of lactation in TGFα(+) mice is principally due to the lack of elevation of prolactin receptor in the mammary glands following parturition. Snedeker *et al.* (11) reported that transcription of mouse TGFα mRNA was detected in virgin and pregnant mice but not in lactating animals. In this study, TGFα(+) mice showed an apparent expression of human TGFα mRNA after parturition.

The role of TGFα in this process remains to be solved; however, the present study revealed that the amount of prolactin binding did not increase after parturition in TGFα(+) mice, and this was not due to the masking of the receptor by TGFα or endogenous prolactin. It is also unlikely that this factor may influence the secretion of some lactogenic hormones, such as estrogen, progesterone, glucocorticoids, and/or prolactin, all of which are associated essentially with lactogenesis directly and indirectly through the induction of prolactin receptor (31), since little modulation of these hormones by TGFα was suggested in the present study. We speculate that the expression of TGFα in the mammary gland may interfere with the process of either prolactin receptor gene transcription or translation.

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