RESEARCH COMMUNICATION

Transforming growth factor-β1 inhibits casein secretion from differentiating mammary-gland explants but not from lactating mammary cells

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Transforming growth factor-β (TGFβ) is important in the maturation and function of the mammary gland and is present in milk. We have examined whether, in addition to inhibiting lactogenesis, TGFβ exerts acute regulatory effects on lactating mammary cells. The isoform TGFβ1 at 5 and 50 ng/ml suppressed the onset of lactation and the subsequent production of β-casein by differentiating mouse mammary explants from pregnant mice. By contrast, it did not inhibit protein synthesis or secretion from acini isolated from lactating-mouse mammary gland or protein secretion from explants from lactating mice. These data indicate that TGFβ inhibits the onset of casein secretion, but is not an acute regulator of casein synthesis or secretion from differentiated lactating mammary cells.

INTRODUCTION

In the past few years, results from several studies have implicated the family of transforming growth factor-β (TGFβ) isoforms in the maturation and function of the mammary gland. Levels of mRNAs for the three mammalian isoforms of TGFβ (denoted β1, β2 and β3) peak during pregnancy, decrease during lactation and become elevated again during mammary-gland involution (Robinson et al., 1993; Streuli et al., 1993). Introduction of implants containing the three TGFβ isoforms into the mammary glands of subadult virgin mice has been shown to inhibit ductal development (Robinson et al., 1991). When TGFβ1 was overexpressed in the mammary gland of transgenic mice, pregnancy-induced lobulalveolar development and milk protein production were impaired (Jhappan et al., 1993).

In addition to the inhibitory effects of TGFβ on mammary-gland growth and development, results from *in vitro* experiments have demonstrated a variety of effects of TGFβ on mammary epithelial-cell function. For example, TGFβ1 has been shown to induce expression of a number of extracellular-matrix (ECM) proteins by human mammary epithelial cells in culture (Stampfer et al., 1993). This ability of TGFβ1 to stimulate ECM formation may be under feedback regulation, as TGFβ1 expression in mouse mammary epithelial cells was strongly reduced by the presence of ECM components (Streuli et al., 1993). It has been also shown that the three isoforms of TGFβ inhibit β-casein production in a mammary epithelial cell line (HC11) and in mammary-tissue explants from mid-pregnant mice (Mieth et al., 1990; Robinson et al., 1993). Robinson et al. (1993) reported that TGFβ did not appear to affect the morphological development of mammary explants from mid-pregnant mice, but inhibited casein synthesis and release at the post-transcriptional level.

It is not known whether TGFβ exerts acute regulatory effects on differentiated lactating mammary cells. Earlier work on the effect of TGFβ on casein secretion from mammary-gland explants (Robinson et al., 1993) did not directly address the issue of acute regulation of casein secretion. Recently, it has been shown that TGFβ1 is present in milk from lactating mice (Letterio et al., 1994), raising the possibility that TGFβ could act as an autocrine regulator of casein synthesis or secretion. Inhibitory feedback regulation of milk protein synthesis and secretion from lactating mammary cells (Blatchford and Peaker, 1982) is thought to involve a milk protein termed 'FIL' (feedback inhibitor of lactation) (Wilde et al., 1987, 1994). Like TGFβ, FIL can exert chronic autocrine effects on mammary-gland differentiation (Wilde et al., 1990). FIL has also been shown to inhibit acutely constitutive casein synthesis and secretion by lactating mouse mammary epithelial acini (Renninson et al., 1993). The data presented here suggest that, while developmental effects of TGFβ1 can modify casein secretion, unlike FIL, TGFβ1 is not an acute regulator of casein production by lactating mammary cells.

MATERIALS AND METHODS

Materials

Unless stated otherwise, tissue-culture media were obtained from Gibco Laboratories, Paisley, Renfrewshire, Scotland, U.K., and other reagents were from the Sigma Chemical Co., Poole, Dorset, U.K.

Preparation of mammary epithelial acini from the lactating mouse

Mouse mammary epithelial acini were prepared in a similar manner to that described by Turner et al. (1992). Mammary tissue was removed from mid-lactation CD1 mice (10 days *post partum*), chopped and dissociated by incubation with collagenase

Abbreviations used: DME, Dulbecco's modified Eagle's; ECM, extracellular matrix; FIL, feedback inhibitor of lactation; HBSS, Hanks balanced salt solution; TGFβ, transforming growth factor-β.

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(150 i.u./ml; Lorne Laboratories, Reading, U.K.) in Hanks balanced salt solution (HBSS) supplemented with 5 μg/ml insulin, 0.01 μg/ml cortisol, 40 mg/ml BSA, 5.5 mM glucose, 20 μM $CaCl_2$, 10μM $MgSO_4$, 0.7 mM glutamine and 2% (v/v) minimum essential amino acids. The suspension was incubated with shaking for up to 90 min at 37 °C until the majority of acini were reduced to clumps of 10–30 cells. Digestion was terminated by filtration of the suspension through a 150 μm-pore-size nylon mesh (Lockertex, Warrington, Cheshire, U.K.). The acini were harvested by centrifugation at 80 *g* for 5 min, and resuspended in HBSS supplemented as described above but without BSA or collagenase, and with 14 mM Hepes, 0.04 mg/ml DNAase (Boehringer Mannheim Ltd., Lewes, East Sussex, U.K.) and 0.1 mg/ml soybean trypsin inhibitor. The cells were washed three times and filtered through a 53 μm-pore-size nylon mesh. The filtrate was centrifuged and the acini were resuspended in culture medium comprising 50% (v/v) Medium 199, 50% (v/v) Ham's F12 supplemented with 5 μg/ml insulin, 0.01 μg/ml cortisol, 2.5 mg/ml BSA, 0.1 μg/ml prolactin, 0.01 μg/ml epidermal growth factor, 0.001 μg/ml tri-iodothyronine, 12 mM glucose, 14 mM Hepes and 30 mM $NaHCO_3$. The acini suspension was diluted with culture medium to give an absorbance of 0.015 A_{450} units/ml, equivalent to 7×10^5 cells/ml.

Incorporation of [^{35}S]methionine into cellular proteins

In a pulse–chase experiment, acini were incubated for 1 h with 20 μCi/ml L-[^{35}S]methionine (cell-labelling grade; sp. radioactivity > 1000 Ci/mmol; Amersham p.l.c., Aylesbury, Bucks., U.K.). An equal volume of ice-cold culture medium was added to the acini and the suspension was centrifuged at 80 *g* for 5 min. The pellet was washed in ice-cold culture medium. In a continuous labelling experiment, acini were incubated with 10 μCi/ml L-[^{35}S]methionine for up to 24 h. Lactating acini were kept in 24-well tissue-culture plates (1 ml/well final volume) at 37 °C under a humidified atmosphere of air/CO_2 (19:1). Acini were incubated for various times with recombinant human TGFβ1 (Gibco) or the equivalent volume (100 μl) of vehicle [50 mM sodium acetate (pH 4.5)/1% (w/v) BSA]. Incorporation of [^{35}S]methionine into cell-associated and secreted trichloroacetic acid-precipitable protein was measured by liquid-scintillation counting as previously described by Turner et al. (1992).

Immunoblotting of secreted β-casein

Incubations of lactating acini were terminated by centrifugation at 5000 *g* for 1 min. An equal volume of cold methanol was added to each 500 μl aliquot of supernatant. After 60 min at −70 °C, precipitated protein was pelleted by centrifugation at 5000 *g* for 2 min. The pellet was washed by centrifugation with cold methanol, solubilized in 100 μl of SDS dissociation buffer [1.25% (w/v) SDS/2 mM EDTA/125 mM Tris/10% (w/v) sucrose/1% (v/v) 2-mercaptoethanol/10% (v/v) glycerol/0.001% (w/v) Bromophenol Blue, pH 6.8] and heated to 80 °C for 2 min. Samples were loaded on to 12.5%-(w/v)-polyacrylamide slab gels and separated by electrophoresis. Purified mouse caseins were run as controls. After electrophoresis, proteins were transferred to nitrocellulose membrane, blots were washed in PBS (138 mM NaCl/3 mM KH_2PO_4/20 mM Na_2HPO_4, pH 7.4), incubated for 1 h in PBS with 1% (w/v) BSA, washed with PBS and incubated for 1 h in polyclonal rabbit anti-(mouse β-casein) antiserum diluted 1:10000 in PBS. Blots were washed with PBS and incubated for 1 h in peroxidase-labelled anti-rabbit IgG diluted 1:400 in PBS with 1% (w/v) BSA and 0.5% Tween-20. After extensive washing in PBS with 0.5% Tween-20, immunoreactive protein was revealed by enhanced chemiluminescence (ECL; Amersham).

Measurement of [^{35}S]methionine incorporated into proteins secreted from mammary-tissue explants from lactating mice

The inguinal mammary glands were removed from a mid-lactation (10 days *post partum*) CD1 mouse and rinsed in Dulbecco's modified Eagle's (DME)/Ham's F12 mixed medium containing 5 μg/ml gentamycin sulphate. The tissue was minced and 20 explants, weighing 70–80 mg, were placed on to the surface of microfoil plinths in 35 mm-diameter plastic Petri dishes. Each Petri dish was filled with 4 ml of culture medium with 20 μCi/ml L-[^{35}S]methionine, and TGFβ1 or vehicle added. Explants were incubated for 3 h at 37 °C under a humidified atmosphere of air/CO_2 (19:1). At hourly intervals, 1 ml of medium was removed from each dish and replaced with an equivalent volume of fresh medium (supplemented as described above). Any medium removed was centrifuged at 5000 *g* for 1 min, and the amount of [^{35}S]methionine incorporated into trichloroacetic acid-precipitable protein measured by liquid-scintillation counting as described above.

Immunoblot analysis of proteins secreted from mammary tissue explants from pregnant mice

Tissue explants were prepared from the inguinal mammary glands of 15-day-pregnant CD1 mice as described above. Explants were incubated in 4 ml of culture medium comprising DME/Ham's F12 supplemented with 5 μg/ml each of gentamycin sulphate, aldosterone, and the lactogenic hormones insulin, cortisol and prolactin (see Robinson et al., 1993). The explants were incubated with TGFβ1 or vehicle for 4 days at 37 °C under a humidified atmosphere of air/CO_2 (19:1). Medium was replaced every 24 h and centrifuged at 5000 *g* for 1 min. Protein present in the medium was precipitated with cold methanol and separated by electrophoresis on 12.5% gels for immunoblotting as described above.

RESULTS

In the present study we examined the effect of TGFβ1 on casein synthesis and secretion using three experimental preparations: isolated mammary cell acini from lactating mice, explant cultures of mammary tissue from lactating mice, and mammary-tissue explants from pregnant mice that were allowed to differentiate in culture. Two doses of TGFβ1 were chosen. TGFβ1 at 5 ng/ml has been shown to inhibit maximally casein secretion from mouse mammary explants in a previous study (Robinson et al., 1993), and this concentration, which is equivalent to 200 pM, is within the range of concentrations of TGFβ1 (112–236 pM) recently detected in mouse milk (Letterio et al., 1994). TGFβ1 was also used at a supramaximal dose of 50 ng/ml (2 nM).

To examine any possible acute effect of TGFβ1 on casein secretion from lactating mammary cells, we made use of a preparation of polarized acini from lactating mouse mammary gland. This preparation has been used extensively in the past to study the characteristics of protein (essentially casein) secretion from mammary epithelial cells (Turner et al., 1992, 1993; Renninson et al., 1993). A pulse–chase labelling protocol allowed the effect of TGFβ1 on secretion of preformed caseins from the lactating acini to be assessed. Previous studies have shown that a 1 h labelling time is sufficient to allow incorporation of label into newly synthesized caseins and some packaging into secretory vesicles, such that secretion begins without a lag during the chase period (Turner et al., 1992). Over a chase period of 3 h, at

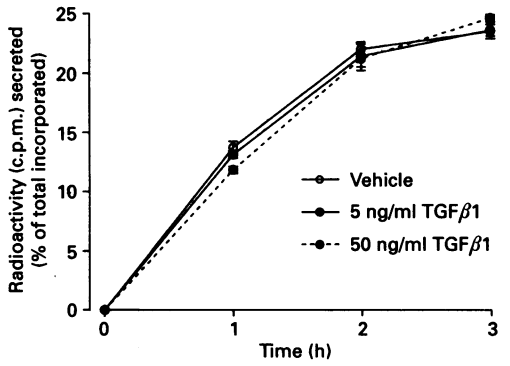

Figure 1 Effect of TGFβ1 on secretion of pre-formed protein from lactating mouse mammary acini

Mammary acini were pulse-labelled with 20 μCi/ml [^{35}S]methionine for 1 h, then washed and incubated with TGFβ1 (5 or 50 ng/ml) or vehicle. The extent of protein secretion over the following 3 h was determined by measuring radioactivity incorporated into cell-associated or medium trichloroacetic-acid-precipitable protein. Results show the percentages of total radioactivity that were secreted and are expressed as means ± S.E.M. ($n = 4$).

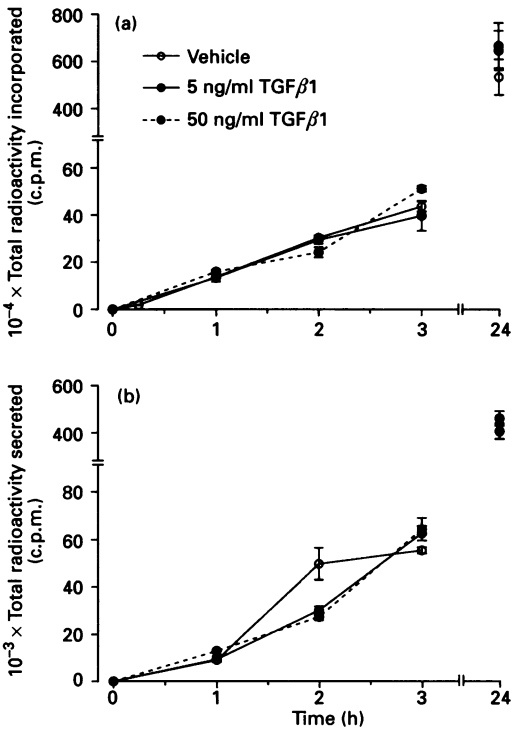

Figure 2 Effect of TGFβ1 on protein synthesis and secretion by lactating-mouse mammary acini

Mammary acini were incubated in a continuous-labelling protocol with 10 μCi/ml [^{35}S]-methionine and TGFβ1 (5 or 50 ng/ml) or vehicle. The extent of protein synthesis and secretion over the following 3 h and 24 h later was determined by measuring the radioactive counts incorporated into cell-associated protein (**a**) or into medium trichloroacetic-acid-precipitable protein (**b**). Results are means ± S.E.M. ($n = 4$).

concentrations of 5 or 50 ng/ml, TGFβ1 did not alter protein secretion from mammary epithelial cells in comparison with cells incubated with vehicle (Figure 1). This result indicated a lack of any direct acute effect of TGFβ1 on protein secretion from

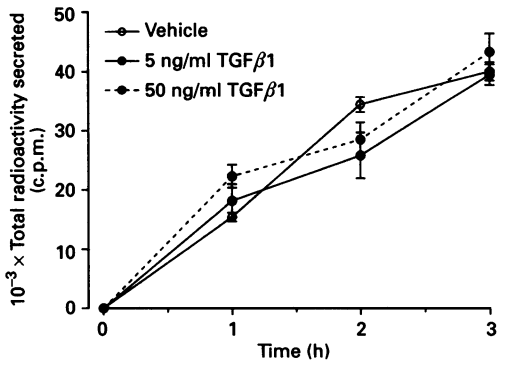

Figure 3 Effect of TGFβ1 on protein secretion from lactating-mouse mammary tissue explants

Mammary-tissue explants from lactating mice were incubated in a continuous-labelling protocol with 20 μCi/ml [^{35}S]methionine and TGFβ1 (5 or 50 ng/ml) or vehicle. The extent of protein secretion over the following 3 h was determined by measuring the radioactivity incorporated into medium trichloroacetic-acid-precipitable protein. Results are means ± S.E.M. ($n = 3$).

lactating acini, but did not preclude the ability of TGFβ1 to affect casein synthesis. In a continuous-labelling experiment, isolated mammary acini remained viable in suspension culture and continued to synthesize and secrete protein over a 24 h period. Neither 5 nor 50 ng/ml TGFβ1 affected incorporation of label into newly synthesized protein in comparison with cells incubated with vehicle (Figure 2a). Protein secretion from the same cells followed a similar pattern to synthesis (Figure 2b) and was unaffected by TGFβ1 over this time. One explanation for the lack of effect of TGFβ1 on this preparation could be receptor loss during isolation of the acini. Therefore the effect of 5 and 50 ng/ml TGFβ1 on protein secretion from mammary-tissue explants from lactating mice, in which receptors should remain intact, was also examined. TGFβ1 did not affect protein secretion over a 3 h period (Figure 3), ruling out this possibility.

Immunoblotting of methanol-precipitable protein secreted (over 3 h) from lactating mammary acini, with a polyclonal anti-(mouse β-casein) antiserum detected a single band corresponding to β-casein (Figure 4a). Quantification by scanning densitometry, using purified mouse milk caseins (39% of which was β-casein) as the standard, revealed that, over 3 h, lactating mammary acini secreted 774 ± 96 ng of β-casein/10^6 cells.

Caseins represent a small proportion of the total protein secreted from undifferentiated pregnant-mouse mammary tissue; therefore, rather than measuring total protein secreted (as with lactating mammary cells), anti-(β-casein) antiserum was used to immunoblot protein secreted from mammary-tissue explants from pregnant mice. Results from previous studies suggest that exposure of pregnant-mouse mammary tissue to TGFβ1 *in vitro* over 4 days can inhibit the onset of lactation and subsequent secretion of caseins (Robinson et al., 1993). The effect of the recombinant human TGFβ1 preparation used in the present study on β-casein secretion from pregnant-mouse mammary explants was examined. Mammary explants were incubated with lactogenic hormones and TGFβ1 (5 or 50 ng/ml) or vehicle for 4 days. Culture medium was replaced every 24 h and the medium immunoblotted for β-casein. After 1 or 2 days in culture, immunoblotting of medium from explants detected no, or very small amounts of, β-casein respectively (results not shown). After 3 or 4 days in culture, β-casein was detected in medium from explants incubated with vehicle ('−'), but not in medium from explants incubated with 5 or 50 ng/ml TGFβ1

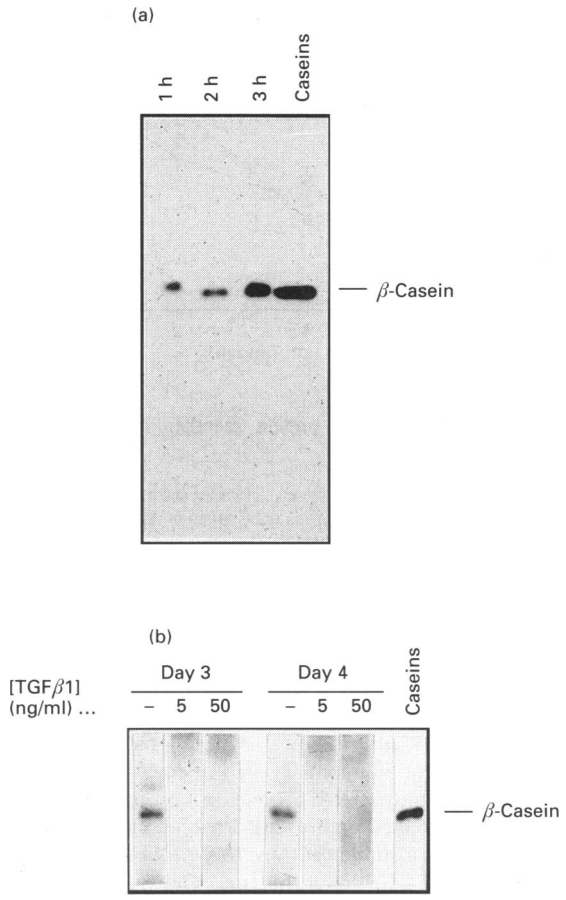

Figure 4 Effect of TGFβ1 on β-casein secretion assessed by immunoblotting

(**a**) An immunoblot which demonstrates that a 1:10000 dilution of polyclonal anti-(mouse β-casein) antiserum detects only β-casein in methanol-precipitable protein secreted from isolated lactating mammary acini at various times over 3 h. Purified total mouse caseins were included as a control. (**b**) Immunoblot of methanol-precipitable protein secreted from mammary-tissue explants from pregnant mice. Explants were incubated with lactogenic hormones and TGFβ1 (5 or 50 ng/ml) or vehicle (—) for 4 days. Culture medium was replaced every 24 h and methanol-precipitable protein immunoblotted with a 1:10000 dilution of anti-(mouse β-casein) antiserum. Results from 3- and 4-day incubations are shown here with total mouse caseins included on the same immunoblot as a control.

(Figure 4b). Thus TGFβ1 appeared to inhibit the onset of β-casein production by mammary explants in culture.

DISCUSSION

We have demonstrated that, despite the presence of lactogenic hormones, incubation of mammary-tissue explants from mid-pregnant mice with 5 or 50 ng/ml TGFβ1 for 3 or 4 days prevents the appearance of β-casein in the culture medium. This is in agreement with the observations of Robinson et al. (1993), who report that exposure of explants to 5 ng/ml TGFβ1 (or TGFβ2 and TGFβ3) for 4 days inhibits casein synthesis and release at the post-transcriptional level. This latter observation raises the possibility that TGFβ1 may also exert inhibitory effects on production of casein by lactating mammary cells. This hypothesis, however, is not supported by the results presented here. Using a pulse–chase protocol, we have shown that, over a 3 h incubation period, neither 5 nor 50 ng/ml TGFβ1 inhibited secretion of preformed caseins from lactating-mouse mammary epithelial cells. In addition, these concentrations of TGFβ1 were also found not to inhibit casein synthesis or secretion from lactating mammary acini (over 24 h) or casein secretion from lactating mammary-gland explants (over 3 h).

Studies have shown that a milk-protein, FIL, suppresses mammary-gland differentiation (Wilde et al., 1990). In addition, FIL acutely inhibits constitutive casein synthesis in, and secretion from, lactating mouse mammary epithelial cells (Renninson et al., 1993), indicating a possible role for FIL in rapid feedback inhibition of casein secretion *in vivo*. Like FIL, TGFβ1 is present in mouse milk (Letterio et al., 1994) and has been shown to modulate mammary-gland development (Robinson et al., 1991; Jhappan et al., 1993). The results obtained in the present study support the notion that TGFβ1 suppresses differentiation of mammary tissue and the subsequent production of β-casein. The results presented here, however, indicate that an acute role for TGFβ1 in inhibition of constitutive synthesis or secretion of caseins from differentiated lactating mammary epithelial cells is unlikely.

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