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Effects of hormones and growth factors on TGF-β1 expression in bovine mammary epithelial cells

Joanna Zarzyńska, Małgorzata Gajewska and Tomasz Motyl*

Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, Nowoursynowska 159, 02-787 Warsaw, Poland

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The decline of mammary epithelial cell (MEC) number during mammary gland involution in the cow is due to inhibition of proliferation and induction of apoptosis. Transforming growth factor-beta 1 (TGF-β1) belongs to a group of intramammary auto/paracrine inhibitors of bovine MEC growth and inducers of apoptosis. However, the mechanism responsible for the regulation of TGF-β1 expression in MEC is not known. The present study examined the effect of the hormones, growth hormone (GH), somatostatin (STS), 17-β oestradiol (E2), progesterone (P4), as well as the growth factors, insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF), on TGF-β1 expression in the bovine MEC lines, BME-UV1 and MAC-T. The model of apoptosis in bovine mammary gland *in vitro* was applied by reduction of fetal bovine serum (FBS) (from 10% to 2% or 0·5% FBS) in the cell environment to show the relationship between TGF-β1 expression and apoptosis in bovine MEC. RT-PCR, Western blot and laser scanning cytometry (LSC) were used for analysis of TGF-β1 transcript and protein level as well as apoptosis and cell cycle in examined MEC. In this model of apoptosis, FBS deficiency (mimicking the naturally occurring decline in the access of bioactive compounds and nutrients at the end of lactation and dry period) was associated with increased TGF-β1 expression at the level of transcript and protein, induction of apoptosis and inhibition of cell cycle. Exogenous TGF-β1, IGF-I, EGF and GH inhibited FBS-deficiency-stimulated TGF-β1 expression. The suppressive effect of GH was reversed when cells were maintained longer in FBS-deficient medium. In general, STS, E2 and P4 increased TGF-β1 expression. However, this effect was dependent on hormone concentration and cell line. BME-UV1 cells were much more responsive to the peptides, GH, STS, IGF-I and EGF, whereas MAC-T cells were more responsive to the steroid sex hormones: E2 and P4.

Keywords: IGF-I, GH, somatostatin, EGF, 17-β estradiol, progesterone, apoptosis.

Mammary gland remodelling is based on a cyclic growth and involution of secretory tissue regulated by dynamic equilibrium between apoptosis and mitosis of mammary epithelial cells (MEC) (Wilde et al. 1999). Apoptosis decreases cell number during mammary gland involution in rodents (Quarrie et al. 1995, 1996), after weaning in sows (Motyl et al. 2000) and during drying-off in goats (Waręski et al. 2001) and cows (Wilde et al. 1997). MEC apoptosis undergoes intramammary induction by milk stasis (Quarrie et al. 1996, 1995), feedback inhibitor of lactation (FIL) (Wilde et al. 1999), IGFBP-5 (Tonner et al. 1999), IGFBP-3 (Baumrucker et al. 2000), Fas ligand (Song et al. 2000) and TGF-β1 (Motyl et al. 2001). Detachment from extracellular matrix (ECM) (Gilmore et al. 2000) and physical distension of the epithelium (Wilde et al. 1999) also induce MEC apoptosis. Furth et al. (1997) proposed a two stage model of mammary gland involution in rodents. The primary apoptotic process is distinct from the second stage of involution typified by lobular-alveolar collapse. The first stage is reversible if suckling is resumed within a few days and is controlled by local factors, i. e., the loss of survival factors and gain of death factors. The second stage of involution is initiated by loss of systemic hormonal stimulation followed by the activation of matrix metalloproteinases, which are involved in degradation of ECM and basement membrane. The loss of cell connections with the ECM basement at this stage leads to secondary apoptosis (Furth et al. 1997). Mammary involution in dairy ruminants occurs at a slower rate than in rodents; alveolar structure is maintained for

*For correspondence; e-mail: t_motyl@hotmail.com

several weeks and lactation can be reinitiated after 4 weeks or more of involution (Capuco & Akers, 1999). In bovine mammary gland the loss of the MEC population begins after the peak of lactation, when the dynamic equilibrium between mitosis and apoptosis is shifted towards apoptosis. However, the most dynamic induction of MEC apoptosis is associated with cessation of milking at the beginning of the dry period (Wilde et al. 1997, 1999). Several factors affect the rate of MEC apoptosis in dairy animals, including the frequency and efficiency of milking, nutrition, galactopoietic hormones and reproductive status (Stefanon et al. 2002).

Transforming growth factor-beta 1 (TGF-β1) is a multifunctional regulatory peptide (25 kDa), which stimulates growth of most mesenchymal cells and inhibits growth of epithelial, lymphoreticular, haematopoetic and endothelial cells. TGF-β1 inhibits MEC proliferation in an auto/paracrine manner, and is considered an important local regulator of mammary tissue involution. It is secreted in colostrum and first milk, mainly in a latent form (Rogers et al. 1996). Expression of the cytokine and its receptors in MEC increases during involution of the mammary gland in the mouse (Atwood et al. 1995), cow (Plath et al. 1997), goat (Waręski et al. 2001) and sow (Motyl et al. 2001). TGF-β1 inhibits proliferation, differentiation and milk protein synthesis in MEC and increases ECM formation (Sudlow et al. 1994). TGF-β1 effects are strikingly biphasic in mouse MEC: whereas relatively high concentrations of this cytokine inhibit colony formation, lower concentrations stimulate extensive elongation and branching of epithelial cord (Soriano et al. 1998). TGF-β1 is also an apoptogenic agent for murine MEC (Motyl et al. 2000; Kolek et al. 2001, 2003b) and bovine MEC (Kolek et al. 2003a).

It is not known how TGF-β1 expression in bovine mammary gland is regulated. Studies on other animal species (mainly rodents) and human mammary carcinoma cells implicate the following hormones and growth factors in inhibition or stimulation of TGF-β1 expression in MEC: prolactin (Atwood et al. 1995; Motyl et al. 2000), growth hormone (GH) (Graichen et al. 2002; Huynh et al. 2000), somatostatin (STS) (Huynh et al. 2000); steroid sex hormones (Sathyamoorthy et al. 1998), EGF (Motyl et al. 2000) and IGF-I (Huynh et al. 2000). Expression of TGF-β1 gene is negatively regulated by ECM. Transcription of TGF-β1 is high in the absence of ECM and is considerably lower in the presence of endogenously synthesized basement membrane (Streuli et al. 1993). Lack of information on the regulation of TGF-β1 expression in the bovine mammary gland prompted us to explore the effect of endocrine and auto/paracrine factors on cytokine content in bovine MEC. The model of apoptosis in bovine mammary gland *in vitro* was applied to show the relationship between TGF-β1 expression and bovine MEC death. Laser scanning cytometry (LSC), Western blot, and RT-PCR were used for analysis of TGF-β1 expression, cell cycle and apoptotic cell number.

Materials and Methods

Media and reagents

DMEM with Glutamax, phosphate buffer saline, pH 7·4 (PBS), fetal bovine serum (FBS), penicillin-streptomycin, fungizone and gentamycin sulphate were obtained from Gibco BRL (Paisley, Scotland, UK). DME/F-12, RPMI-1640, NCTC 135 powdered medium, α-lactose, lactoalbumin hydrolysate, glutathione, bovine holo-transferrin, hydrocortisone, L-ascorbic acid, porcine TGF-β1, bovine GH, insulin-like growth factor I (IGF-I), bovine insulin, epidermal growth factor (EGF), STS and all the other reagents were obtained from Sigma Chemicals (St. Louis, MO, USA). Polyclonal chicken anti-bovine TGF-β1 antibody was supplied by R&D Systems, Inc. (Minneapolis, MN, USA). Horseradish peroxidase-conjugated goat anti-chicken IgG was supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Alexa Fluor 488 secondary antibody was purchased from Molecular Probes (Eugene, OR, USA). Reagents for Western blotting were purchased from BioRad (Hercules, CA, USA) and Western blotting detection reagents and Hyperfilm ECL were supplied by Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). Plastic cell culture Petri dishes were purchased from Corning Glass (Corning, NY, USA). Sterile conical flasks, disposable pipettes were supplied by Nunc Inc. (Naperville, IL, USA).

Cell culture

Bovine mammary epithelial BME-UV1 cell line was established by Professor B Zavizion (Dept of Animal and Food Sciences, University of Vermont, USA) and was kindly provided by Professor Antonella Baldi (Animal Nutrition Institute, Faculty of Veterinary Medicine, University of Milan, Italy).

Cells were cultured in a routine culture medium (mixture of DME/F-12, RPMI-1640 and NCTC 135 in proportions of 5 : 3 : 2 by volume) enriched with α-lactose (0·1%), glutathione (1·2 mM), bovine insulin (1·0 μg/ml), bovine holo-transferrin (5·0 μg/ml), hydrocortisone (1·0 μg/ml), L-ascorbic acid 10 μg/ml), 10% (v/v) heat-inactivated FBS, penicillin-streptomycin (50 i.u./ml), fungizone (2·5 μg/ml), gentamycin (50 μg/ml) in an atmosphere of 5% CO_2/95% humidified air at 37 °C and routinely subcultured every 2 d.

The second bovine mammary epithelial cell line, MAC-T, was kindly provided by Dr Imre Kacskovics (Dept of Physiology and Biochemistry, Faculty of Veterinary Science, Szent Istvan University, Budapest). Cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 0·2% (w/v) L-glutamine, gentamycin (50 mg/ml), penicillin-streptomycin (50 i.u./ml), fungizone (2·5 mg/ml), bovine insulin (5 mg/ml) in an atmosphere of 5% CO_2/95% humidified air at 37 °C and routinely subcultured every 2 d.

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Experimental procedure and immunofluorescence staining for Laser scanning cytometry

Exponentially growing cells were seeded to Lab-Tek four-chamber slides (NUNC Inc.) and cultured until confluency in optimal conditions (see above). The medium was then removed and replaced with:

1. 0·5% FBS medium for 24 and 48 h
2. 0·5% FBS medium containing IGF-I (50 ng/ml), EGF (10 ng/ml) or both of these factors for 24 h
3. 10% FBS medium containing three different concentrations of: GH (1; 5; 10 μg/ml), progesterone (P4) (0·1; 0·5; 1 ng/ml), STS (1; 10; 50 ng/ml) and 17-β oestradiol (E2) (1×10^{-10} mol/ml; 1×10^{-7} mol/ml; 1×10^{-4} mol/ml) for 24 h
4. 10%, 2%, 0·5% and 0% FBS medium with addition of TGF-β1 (2 ng/ml) for 24 h. The concentration of TGF-β1 used in the experiments was established after screening tests and on the basis of previous observations on HC11 mouse MEC and BME-UV1 cells, which showed both antiproliferative and apoptotic effects of TGF-β1 at 2 ng/ml (Kolek et al. 2003a, b). As a control to the experiment, cells not exposed to TGF-β1 were used.

Cells cultured in routine 10% FBS medium were used as a control to the experiments. For each experiment at least three replicates were performed.

Cells were fixed in 0·25% formaldehyde for 15 min, washed twice with PBS, suspended in ice-cold 70% methanol and stored at 4 °C for 30 min. Finally, the methanol was aspirated and samples were stored at −80 °C until staining. At the time of analysis, cells were washed twice with PBS-1% bovine serum albumin (BSA) and incubated for 1 h with primary antibodies diluted 1 : 100 with PBS-1% BSA. After primary incubation the cells were washed twice with PBS-1% BSA and incubated for 1 h with 1 : 500 Alexa Fluor 488 secondary antibodies. The cells were then washed twice in PBS-1% BSA and finally incubated with 5 μg/ml solution of 7-aminoactinomycin D (7-AAD) for 30 min. to counterstain the DNA. Finally, the coverslips were mounted on microscope slides using ICN mounting medium (ICN Biomedicals Inc, Aurora, OH, USA).

Laser scanning cytometry

Probes were analysed by LSC (CompuCyte Corp., Boston, MA, USA). At least 5×10^3 cells per slide were analysed. Fluorescence excitation was provided by a 488-nm argon laser beam. Combination of dichroic mirrors and filters transmitting light at a 520±20 nm wave length for green fluorescence of Alexa Flour 488 antibodies, and >650 nm for far red fluorescence of 7-AAD were used. Green fluorescence was measured separately over the nucleus (NF) and the cytoplasm (CF). NF was measured within an area outlined by the 'integration contour', located 2 pixels outside the 'threshold contour' triggered by the far red fluorescence of 7-AAD. CF was measured within the rim of cytoplasm 10 pixels wide, located outside the 'integration contour'. The background green fluorescence was automatically measured within a 2 pixels range outside 'peripheral contour' and subtracted from both nuclear and cytoplasm green fluorescence, to obtain the final values of NF and CF respectively.

Another parameter measured was maximal pixel (MP) corresponding to the highest value of measured fluorescence in the cell, regardless of cellular compartment. DNA MP was used for evaluation of chromatin condensation as a characteristic feature of apoptosis (Bedner et al. 1999).

Quantitative evaluation of apoptotic cells and cell number in respective phases of the cell cycle was analysed from DNA histograms using WinCyte software (CompuCyte Corp.). Apoptotic cells with lowered DNA content were gathered in sub-G1 region of DNA histogram.

The results were analysed using Microsoft® Excel 2000 software (Microsoft Corporation, Redmond, WA, USA) and Prism version 3.00 software (GraphPad Software, San Diego, CA, USA).

Western blot analysis

Cells were cultured on Petri dishes until confluency in a routine culture medium (see above). The medium was then removed and replaced with 0·5% FBS medium containing IGF-I (50 ng/ml), EGF (10 ng/ml) or both of these factors for 24 h. Cells cultured in a routine 10% FBS medium were used as a control to the experiment. Three replicates of the experiment were performed.

Cultured cells were pelleted by centrifugation at 400 ***g*** at 4 °C for 2 min, and then frozen at −20 °C. At the time of analysis the cells were suspended in ice-cold PBS. Fractionation of cells into nuclear and cytosolic fractions was performed according to Del Buffalo et al. (1996) with modifications as described. After centrifugation at 400 ***g*** at 4 °C for 2 min, the supernatant was removed and the cell pellet was suspended in 0·5 ml Lysis Buffer I (pH 7·4) (10 mM-Tris–HCl, 10 mM-NaCl, 10 mM-EDTA, 3 mM-$MgCl_2$, 1% Triton X-100, 4 mM-PMSF, 20 μg leupeptin/ml as the protease inhibitor and 2 mM-sodium orthovanadate as the alkaline/tyrosine phosphatases inhibitor) and incubated at 4 °C for 30 min. The cell suspension was centrifuged at 500 ***g*** at 4 °C for 30 min and the supernatant (containing cytoplasm with mitochondria and microsomes) was carefully removed and passed 6 times through a 20-gauge syringe needle. Protein concentration in the cytosolic fraction was determined by Bradford's method (1976). Lysates were mixed 1 : 2 (v/v) with Laemmli sample buffer (BioRad) containing 2·5% 2-mercaptoethanol and boiled for 3 min.

Samples containing identical quantities of proteins were subjected to SDS-PAGE (10% gel) together with Kaleidoscope Marker (BioRad). Electrophoresis was performed for 2 h at 100 V using a Mini Protean II™ apparatus (BioRad)

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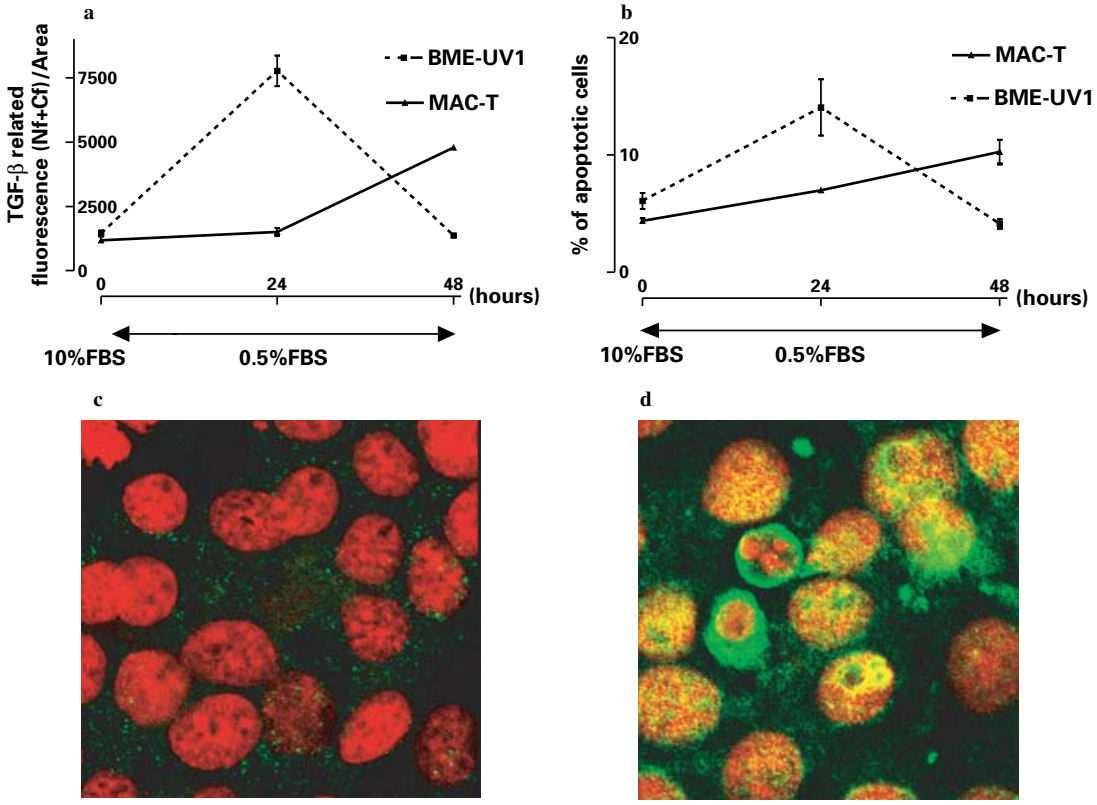

Fig. 1. Effect of reducing FBS concentration (from 10% to 0·5%) in the incubation medium on (a) TGF-β1 expression and (b) apoptotic cell number in bovine mammary epithelial BME-UV1 and MAC-T cells. TGF-β1-related fluorescence (Nf+Cf/ Area) and percentage of cells in sub-G1 region (apoptotic cells) were measured using LSC (see Materials and Methods). Each sample analysed contained at least 5000 cells. Values are means ± SD for $n=3$. Intensive TGF-β1-related green fluorescence is shown on confocal images of BME-UV1 cells maintained in (d) 0·5% FBS medium for comparison with (c) 10% FBS medium.

after which, the separated proteins were electroblotted on a nitrocelulose membrane (Amersham Pharmacia Biotech) for 70 min at 110 V using a Mini Protean II™ apparatus. Membranes were blocked with a 5% solution of non-fat dried milk in TBST (pH 7·5) overnight at 4 °C. On the next day they were washed 3 times for 5 min in TBST at room temperature and incubated for 1·5 h at room temperature with primary polyclonal chicken anti-bovine TGF-β1 antibodies, diluted 1 : 500. Membranes were washed 3 times for 5 min in TBST and incubated with diluted 1 : 5000 horseradish peroxidase-conjugated goat anti-chicken secondary antibodies for a further 1 h at room temperature. Finally the membranes were washed 3 times for 5 min in TBST and labelled proteins were visualized using the ECL Western blotting detection reagents and Hyperfilm ECL high performance chemiluminescence (Amersham Pharmacia Biotech).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cells were cultured on Petri plates until confluency in a routine culture medium (see above). The medium was then removed and replaced with 2% FBS medium. Cells were harvested from plates, centrifuged (300 ***g*** at 4 °C for 10 min) and washed twice with ice-cold PBS. RT-PCR used to measure TGF-β1 RNA transcripts. Actin served as a reference gene. Isolation of mRNA was performed using mRNA Capture Kit (Boehringer Mannheim, Germany). A cell pellet containing 3×10^5 cells was suspended in 200 µl lysis buffer. This was followed by hybridization of mRNA with a biotin-labelled oligo $(dT)_{20}$ probe and immobilization of poly (A^+)-RNA in streptavidin-coated PCR tubes (0·2 ml). The reverse transcription mix containing 13 µl dH_2O, 4 µl 5 × first strand buffer, 2 µl 0·1M-DTT, 1µl 10 mM-dNTP and 1 µl Super Script reverse transcriptase was added to the streptavidin-coated PCR tube and incubated at 37 °C for 1 h. The RT mix was removed and the tube was carefully washed with 250 µl washing buffer. PCR was performed for TGF-β1 according to the methods described previously (Kojima et al. 1996). Then, 3 µl aliquots of PCR products were separated on the polyacrylamide gel (12·5%) using Phast System® (Pharmacia Biotech, Uppsala, Sweden) and 100 bp DNA ladder as a marker. PCR products were stained with Past Gel®DNA Silver Staining Kit (Pharmacia). The optical density (OD) and area of DNA bands was measured using Kodak 1D Image Analysis Software (Eastman Kodak

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Company Scientific Imaging Systems, Rochester, NY, USA).

Statistical analysis

Results were statistically analysed using ANOVA and Tukey's multiple range tests with Prism version 3.00 software (GraphPad Software, San Diego, CA, USA); $P \leqslant 0{\cdot}05$ was regarded as significant and $P \leqslant 0{\cdot}01$ as highly significant.

Results

Expression of TGF-β1 in the model of apoptosis in bovine mammary gland in vitro

In the present study apoptosis in the bovine mammary gland *in vitro* was modelled by decreasing the availability of FBS for bovine MEC. The reduction of FBS content in the culture medium for bovine MEC mimicked the naturally occurring deprivation of bioactive compounds (hormones, growth factors and cytokines) and nutrients in mammary gland at the end of lactation and during drying off. In both bovine mammary epithelial cell lines, BME-UV1 and MAC-T, reducing FBS content in the medium from 10% to 0·5%, evoked a significant increase in TGF-β1 expression, measured at the level of protein by LSC (Fig. 1a). The response of BME-UV1 cells was faster and more dynamic, occurring after 24 h, whereas in MAC-T cell cultures it appeared after 48 h of FBS deficiency. The pattern of TGF-β1 expression was very similar to the pattern of apoptosis in examined cultures (Fig. 1b), indicating a positive relationship between the expression of cytokine and the induction of apoptosis in bovine MEC. The drop in TGF-β1 expression and apoptotic cell number in BME-UV1 culture after 48 h of FBS deficiency was probably due to detachment of cells undergoing apoptosis. A higher TGF-β1 protein content in FBS-deficient bovine MEC was also shown on confocal images, where the intense TGF-β1-related green fluorescence was visible in cells maintained in 0·5% FBS-containing medium, including apoptotic cells (Fig. 1, compare c and d). RT-PCR analysis revealed that the higher TGF-β1 protein content in FBS-deficient cells was associated with increased transcription of this cytokine gene (Table 1). The ratio of TGF-β1 mRNA/actin mRNA (where actin was used as a reference gene), was higher in the culture of BME-UV1 cells maintained in 2% FBS, than in 10% FBS-enriched medium. The higher TGF-β1 transcript level was accompanied by a significant increase of both TGF-β1 protein–related fluorescence and apoptotic cell number at 2% FBS in the incubation medium (Table 1). The results suggest that FBS contains bioactive compounds, e.g., hormones, growth factors or cytokines, which exert a suppressive effect on TGF-β1 expression in bovine MEC.

Table 1. Expression of TGF-β1 and apoptotic cell number in bovine mammary epithelial BME-UV1 cells cultured in growth-promoting (10% FBS) and FBS-deficient (2% FBS) medium. TGF-β1 expression was evaluated at the level of transcript, TGF-β1 mRNA/actin mRNA (where actin was used as a reference gene) and protein, TGF-β1-related fluorescence (Nf+Cf)

Values are means ± SD for $n = 3$

	Type of incubation medium	
	Growth promoting	FBS-deficient
TGF-β1 mRNA/actin mRNA (IOD)	2·93 ± 0·02	8·43** ± 0·1
TGF-β1-related fluorescence (Nf+Cf)	29651 ± 352	60812* ± 432
Apoptosis (%)	6·7 ± 0·6	10·8* ± 0·8

Statistical significance of the effect of incubation medium: * $P \leqslant 0{\cdot}05$; ** $P \leqslant 0{\cdot}01$

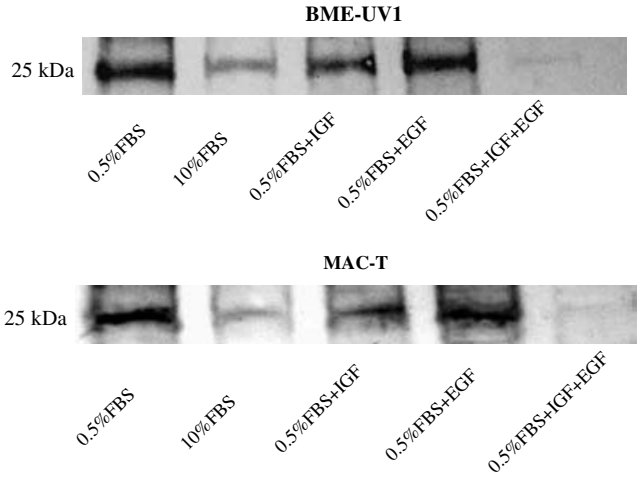

Fig. 2. Western-blot analysis of TGF-β1 level in bovine mammary epithelial BME-UV1 and MAC-T cells cultured for 24 h in growth-promotoring (10% FBS) or deficient (0·5% FBS) medium supplemented with EGF (10 ng/ml), IGF-I (50 ng/ml) or a combination of these factors (IGF-I+EGF).

Effects of growth factors on TGF-β1 expression in bovine MEC

The list of putative TGF-β1 suppressors includes EGF and IGF-I, which are known to inhibit TGF-β1 expression, at least in the mouse MEC (Huynh et al. 2000; Motyl et al. 2000). Moreover, EGF and IGF-I stimulate bovine mammary epithelial BME-UV1 cell proliferation (Zavizion et al. 1996). The results of the present study clearly show that IGF-I, and in particular the combination of IGF-I and EGF, prevent FBS-deficiency-induced TGF-β1 expression, which was shown by both Western blot (Fig. 2) and LSC (Table 2) analyses. BME-UV1 and MAC-T were both reactive to IGF-I, but only BME-UV1 was reactive to EGF (Table 2). EGF potentiated the suppressive effect of IGF-I on TGF-β1 protein level, which was particularly evident on the blots

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Table 2. Effects of IGF-I (50 ng/ml), EGF (10 ng/ml) and a combination of these growth factors (IGF-I+EGF) administrated to 0·5% FBS-containing medium for 24 h on TGF-β1 expression and apoptosis in BME-UV1 and MAC-T cells. Cells cultured in growth-promoting medium (10% FBS) were used as a control. TGF-β1 expression and apoptosis were analysed by LSC (see Materials and Methods) and are shown as a percentage of cells with a high MP and a percentage of apoptotic cell number, respectively

Values are means ± SD for $n=3$

		Type of incubation medium				
			0·5% FBS			
Parameter	Cell line	10% FBS	none	+IGF-I	+EGF	+IGF-I+EGF
TGF-β1-related fluorescence (% HMP)	BME-UV1	$24·7^{a}\pm0·5$	$63·8^{b}\pm6·6$	$35·6^{c}\pm6·3$	$48·6^{c}\pm3·1$	$21·6^{a}\pm2·7$
	MAC-T	$7·7^{a}\pm0·7$	$15·3^{b}\pm5·0$	$8·6^{a}\pm3·4$	$15·7^{b}\pm0·5$	$5·6^{a}\pm1·3$
Apoptosis (%)	BME-UV1	$4·8^{a}\pm1·7$	$15·0^{b}\pm0·7$	$1·3^{c}\pm0·01$	$5·5^{a}\pm0·5$	$2·6^{ac}\pm0·5$
	MAC-T	$3·5^{a}\pm0·1$	$5·7^{b}\pm0·5$	$2·6^{a}\pm0·9$	$5·2^{b}\pm2·2$	$2·7^{a}\pm0·5$

Means without a common superscript letter differ significantly ($P\leqslant0·05$)

(Fig. 2). The anti-apoptotic effect of IGF-I and EGF (EGF only in BME-UV1 cells) resulted in a lower percentage of cells undergoing apoptosis (Table 2). Analysis of cell cycle revealed that in BME-UV1 cells cultured in growth promoting medium (10% FBS) the average number of cells in G1, S and G2/M was: 47·3%; 18·2%; 29·8%, respectively. FBS-deficiency evoked the inhibition of cell cycle shown as the increase of cell number in G1 (61·2%) and the drop in G2/M (14·0%) phases. The number of cells in S phase remained unchanged (17·7%). IGF-I protected against FBS-deficiency-induced inhibition of cell cycle, which was manifested by the decrease of the cell number in G1 (49%) and simultaneous increase in S (30·2%) and G2/M (19·0%) phases. The response of cell cycle to EGF was less pronounced (54·3%; 22·5%; 16·6% for G1, S and G2/M, respectively) in comparison with IGF-I. There were no differences in cell cycle phases in the case of MAC-T cell line after 24 h of incubation.

In the subsequent experiment, the effect of exogenous TGF-β1 on its expression in BME-UV1 cells was investigated. Progressive reduction of FBS concentration in the incubation medium evoked a linear, significant increase of TGF-β1-related fluorescence in examined cell cultures (Fig. 3), which is concordant with the results presented in Fig. 1 and Tables 1 and 2. Administration of TGF-β1 (2 ng/ml) significantly diminished TGF-β1 expression at all applied FBS concentrations. The concentration of TGF-β1 applied in the experiment was established in a previous study in this laboratory (Kolek et al. 2003a, b), showing both antiproliferative and antiapoptotic effects of 2 ng/ml TGF-β1.

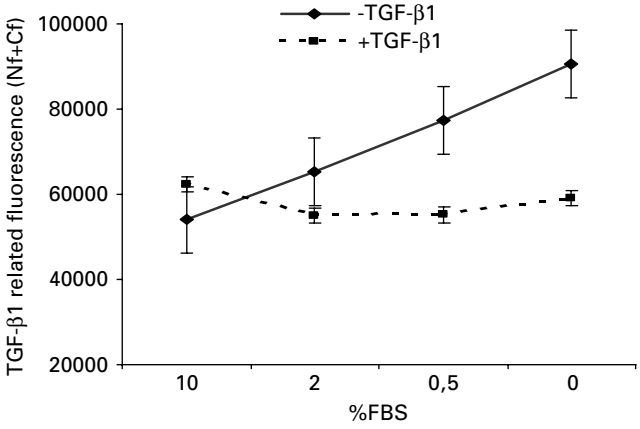

Fig. 3. Effect of progressive FBS deprivation and exogenous TGF-β1 (2 ng/ml) on TGF-β1 expression in BME-UV1 cells. TGF-β1 expression was measured using LSC and shown as TGF-β1-related fluorescence (Nf+Cf) (see Materials and Methods). Each sample analysed contained at least 5000 cells. Values are means ± SD for $n=3$.

Effects of hormones on TGF-β1 expression in bovine MEC

High reactivity of bovine MEC to IGF-I with regard to the decrease of TGF-β1 expression, inhibition of apoptosis and activation of cell cycle prompted us to explore the effect on TGF-β1 expression in bovine MEC of other members of the somatotropic axis, i.e., GH and STS. GH at a concentration of 1 μg/ml significantly decreased TGF-β1 expression measured both as the percent of cells with high expression of the cytokine and concentration of TGF-β1 in the cell (Table 3). The higher GH concentration in the medium (5 and 10 μg/ml) did not increase its suppressive effect on TGF-β1 expression. GH-induced suppression of TGF-β1 was evident only in well-nourished cells, cultured in the medium containing 10% FBS. In poorly fed cells (0·5% FBS-supplemented medium) the effect of GH was opposite and manifested with dose-dependent increase of TGF-β1 expression (Table 3, in brackets). Application of graded concentration of STS increased both the number of cells with high expression of TGF-β1 and the concentration of the cytokine in the cell (Table 3). The effect of GH and STS on MAC-T cell cultures was statistically non significant (results not shown).

In the final step of the study, the effect of the steroid sex hormones, E2 and P4 on TGF-β1 expression was investigated. The E2-induced increase in the number of cells with

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Table 3. Effects of graded concentrations of growth hormone (GH) and somatostatin (STS) administrated to growth-promoting medium (10% FBS) for 24 h on TGF-β1 expression in BME-UV1 cells. Additionally, the effect of 24-h GH supplementation to deficient medium (0·5% FBS) on TGF-β1 expression was investigated (in brackets). Cells were maintained in the deficient medium for 24 h prior to GH administration. TGF-β1 expression was analysed by LSC (see Materials and Methods), and is shown as a percentage of cells with a high MP and the ratio of TGF-β1-related fluorescence within the cell to the cell area

Values are means ± SD for $n=3$

	TGF-β1-related fluorescence	
Treatment	% High MP	Nf+Cf/Area
Growth hormone (μg/ml)		
0	$20{\cdot}5^{a}\pm3{\cdot}0$ ($5{\cdot}8^{a}\pm2{\cdot}9$)	$3432^{a}\pm262$ ($1321^{a}\pm244$)
1	$4{\cdot}9^{b}\pm1{\cdot}6$ ($11{\cdot}9^{b}\pm5{\cdot}6$)	$1697^{b}\pm164$ ($1568^{a}\pm457$)
5	$11{\cdot}3^{c}\pm5{\cdot}8$ ($20{\cdot}2^{c}\pm7{\cdot}3$)	$2418^{c}\pm458$ ($1800^{a}\pm244$)
10	$9{\cdot}3^{c}\pm1{\cdot}8$ ($23{\cdot}3^{c}\pm8{\cdot}4$)	$1664^{b}\pm125$ ($2671^{b}\pm286$)
Somatostatin (ng/ml)		
0	$6{\cdot}0^{a}\pm4{\cdot}2$	$624^{a}\pm118$
1	$8{\cdot}0^{a}\pm4{\cdot}2$	$703^{a}\pm123$
10	$13{\cdot}5^{b}\pm2{\cdot}1$	$1021^{b}\pm127$
50	$11{\cdot}0^{ab}\pm5{\cdot}6$	$1180^{b}\pm209$

Means without a common superscript letter differ significantly ($P\leqslant0{\cdot}05$)

high TGF-β1 expression was dose-dependent, which was evident in both examined cell lines (Table 4). A similar stimulatory effect of E2 on the concentration of TGF-β1 in bovine MEC was observed (Table 4). Generally, the response of MAC-T cell line to E2 was more pronounced than in the case of BME-UV1 cell line. In the case of P4 the tendency to an increase of TGF-β1 expression was observed with concentrations up to 0·5 ng/ml, particularly in a MAC-T cell line (Table 4). The highest P4 concentration applied in this experiment (1 ng/ml) appeared to inhibit TGF-β1 expression.

Table 4. Effects of graded concentrations of 17-β-oestradiol and progesterone administered to growth-promoting (10% FBS) medium for 24 h on TGF-β1 expression in BME-UV1 and MAC-T cells. TGF-β1 expression was analysed by LSC (see Materials and Methods) and is shown as a percentage of cells with a high MP and the ratio of TGF-β1-related fluorescence within the cell to the cell area

Values are means ± SD for $n=3$

	TGF-β1-related fluorescence			
	% High MP		Nf+Cf/Area	
Treatment	BME-UV1	MAC-T	BME-UV1	MAC-T
17-β oestradiol (mol/ml)				
0	$8{\cdot}4^{a}\pm0{\cdot}7$	$3{\cdot}0^{a}\pm0{\cdot}0$	$1187^{a}\pm157$	$632^{a}\pm102$
1×10^{-10}	$9{\cdot}0^{a}\pm2{\cdot}8$	$13{\cdot}5^{b}\pm0{\cdot}7$	$973^{a}\pm333$	$1775^{b}\pm237$
1×10^{-7}	$15{\cdot}0^{b}\pm1{\cdot}4$	$22{\cdot}5^{c}\pm0{\cdot}7$	$753^{a}\pm219$	$2042^{b}\pm433$
1×10^{-4}	$36{\cdot}5^{c}\pm0{\cdot}7$	$46{\cdot}0^{d}\pm1{\cdot}4$	$1786^{b}\pm261$	$3124^{c}\pm123$
Progesterone (ng/ml)				
0	$10{\cdot}4^{a}\pm5{\cdot}7$	$10{\cdot}3^{a}\pm3{\cdot}2$	$806^{a}\pm248$	$4741^{a}\pm972$
0·1	$11{\cdot}7^{a}\pm1{\cdot}9$	$14{\cdot}1^{b}\pm4{\cdot}4$	$847^{a}\pm171$	$6378^{b}\pm1000$
0·5	$14{\cdot}1^{b}\pm3{\cdot}3$	$16{\cdot}1^{b}\pm3{\cdot}7$	$1070^{b}\pm166$	$7713^{b}\pm1540$
1	$9{\cdot}1^{ab}\pm4{\cdot}5$	$10{\cdot}7^{a}\pm5{\cdot}1$	$790^{a}\pm172$	$4272^{a}\pm394$

Means without a common superscript letter differ significantly ($P\leqslant0{\cdot}05$)

Discussion

Involution of the bovine mammary gland is a result of reduced proliferation and stimulation of MEC apoptosis (Wilde et al. 1997, 1999). However, the physiological mechanism of apoptosis induction is poorly understood. A recent study in this laboratory revealed for the first time that TGF-β1 is not only antiproliferative, but is also an apoptotic factor in bovine MEC (Kolek et al. 2003a). Apoptosis induced by TGF-β1 was manifested by: 1) increased cell number with lower DNA content and condensed chromatin, 2) aggregation of Bax (promoter of apoptosis) on organellar membranes and its interaction with voltage-dependent anion chanel-1 (VDAC-1), 3) enhanced expression of caspase-3 and m-calpain as executors of apoptosis, 4) elevated content of 89 kDa PARP degradation product. The main morphological features of TGF-β1-induced apoptosis in bovine MEC were: cell shrinkage, chromatin condensation, nuclear pycnosis, fragmentation of the nucleus, and formation of apoptotic bodies (Kolek et al. 2003a). Our recent unpublished observations indicate that TGF-β1-induced apoptosis in bovine MEC could be mediated by IGF binding proteins, which suppress IGF-I-dependent cell survival.

There is little published on the pattern of TGF-β1 expression in the mammary tissue of the cow during the lactation cycle. TGF-β1 mRNA level is higher in involuting than in lactating mammary tissue of the cow (Plath et al. 1997). Involution of the mammary gland is controlled by a

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mechanism involving a complex of neuroendocrine and auto/paracrine regulations. For this reason, it is not currently possible to model all aspects *in vitro* even with the models based on primary cultures growing on matrigel-coated basements. The model of involution *in vitro* has already been described for the mouse mammary gland, where a whole organ culture can be induced to involute by the withdrawal of the lactogenic hormones, prolactin, aldosterone and hydrocortisone (Atwood et al. 1995). The simplified model of apoptosis presented in this study was used to verify the relationship between TGF-β1 expression and apoptosis in bovine MEC. The following advantages of BME-UV1 and MAC-T cell lines prompted their use in the model described here: 1) both cell lines express TGF-β1 (Woodward et al. 1995; and the present study), 2) they are reactive to antiproliferative and apoptogenic actions of TGF-β1 with characteristic morphological and biochemical features of apoptosis (Kolek et al. 2003a; Woodward et al. 1995), 3) they are grown on plastic Lab-Tec slides, making them useful for individual cell by cell analysis with LSC (see Materials and Methods). MAC-T cells are also regarded as a good model for nutrition-induced changes in mammary growth, because they can mimic the responses of primary MEC to mammary tissue extracts from heifers fed at low and high levels (Knowles Berry, 2002). The *tgf-β1* gene in MEC is under the suppressive influence of galactopoietic hormones and growth factors, manifested with: 1) inhibition of TGF-β1 expression by prolactin, EGF and IGF-I (Huynh et al. 2000; Motyl et al. 2000) and 2) increase of TGF-β1 expression at the end of lactation and drying-off (Motyl et al. 2001; Waręski et al. 2001), when secretion of galactopoietic hormones declines. It was judged in this study that lowered availability of FBS, which is the natural and species-specific source of hormones, growth factors and cytokines for bovine MEC, would be able to release the *tgf-β1* gene from the suppressive effects of FBS-derived bioactive compounds. The model of apoptosis applied in this study was associated with induced TGF-β1 expression in examined bovine MEC, which was evident both at the transcript and protein level (Fig. 1a, d; Table 1). Elevated TGF-β1 expression was accompanied by a significant increase in apoptotic cell number (Fig. 1b; Table 2), indicating a close relationship between the expression of cytokine and the induction of apoptosis in bovine MEC. These results agree with our previous findings in the mammary gland of goats (Waręski et al. 2001) and sows (Motyl et al. 2001), showing elevated expression of TGF-β1 and its receptor as well as an increase of apoptotic cell number in the course of involution. It could be put forward that the elevated TGF-β1 concentration in the bovine MEC environment may inhibit its synthesis (Fig. 3), suggesting the control of TGF-β1 expression by negative feedback. It is known that preincubation of MAC-T cells with TGF-β1 or TGF-β2 markedly downregulates TGF-β1 receptors (Woodward et al. 1995). The feedback hypothesis requires confirmation, because the impaired TGF-β1 expression under the influence of exogenous TGF-β1 could also result from apoptogenic action of this cytokine. In our previous study on bovine mammary epithelial BME-UV1 cells, the antiproliferative and apoptotic effect of exogenous TGF-β1 was evident at a concentration of 2 ng/ml (Kolek et al. 2003a).

Our model of apoptosis in bovine mammary gland *in vitro* supports the claim that TGF-β1 in bovine MEC is under the influence of suppressive factors present in FBS. The next experiment revealed that IGF-I may make a major contribution to TGF-β1 inhibition and the protection of bovine MEC against apoptosis (Table 2) and partial cell cycle arrest, evoked by limited FBS availability. A similar suppressive effect on TGF-β1 expression was observed in the case of GH action, but only in well-nourished cells (Table 3). These results indicate that GH and IGF-I, apart from their crucial role in the maintenance of lactation in the cow, are important regulators of mammary tissue remodelling through the suppression of TGF-β1 synthesis. Switching from inhibition to induction of TGF-β1 by GH in poorly fed bovine MEC (Table 3) may suggest a new physiological mechanism controlling the expression of TGF-β1. Deprivation of bioactive compounds and nutrients reaching mammary gland at the end of lactation can change (through an unknown mechanism) MEC responsiveness to GH from increased synthesis of milk constituents towards TGF-β1-mediated growth inhibition and apoptosis. The suppressive influence of IGF-I and GH on TGF-β1 expression has also been described in the mouse mammary gland (Huynh et al. 2000). Administration of IGF-I to intact mouse suppresses TGF-β1 mRNA levels in a dose-dependent manner to ~20% of control levels. Transgenic mice overexpressing GH express TGF-β1 in the mammary gland at only 12% of the level of control animals (Huynh et al. 2000). It has also been shown that autocrine hGH decreases transcription of the *PTGF-β* (p53-regulated placental transforming growth factor-beta) gene with consequent decreases in its protein product and accompanying cellular effects, which include cell cycle arrest and apoptosis in human mammary carcinoma cells (Graichen et al. 2002). The present study revealed that STS, apart from its well known inhibitory effect on GH secretion, can directly stimulate synthesis of TGF-β1 in bovine MEC (Table 3). These results are compatible with those from the mouse mammary gland that show that the administration of somatostatin analogue, octreotide increases mammary TGF-β1 expression ~3 fold (Huynh et al. 2000). Overall, our results clearly indicate that hormones of the somatotropic axis are important regulators of TGF-β1 expression in bovine MEC. In general, BME-UV1 cells were more responsive to GH, IGF-I and STS than MAC-T cells. On the other hand, the responsiveness of MAC-T cells to steroid sex hormones was higher than BME-UV1 cells.

It is commonly accepted that oestrogens and progesterone are essential for mammary gland development and remodelling. Circulating oestradiol in the cow is very low until the fourth to sixth month of pregnancy, increases

gradually until the last month of gestation, and then rises rapidly to a peak value (Cowie et al. 1980). Plasma progesterone concentration during pregnancy is elevated to the value found during the luteal phase of the oestrus cycle (Cowie et al. 1980). Most of the genomic actions of oestrogens and gestagens expressed in bovine mammary gland are mediated by the oestrogen receptors ERα and Erβ and progesterone receptor (PR), respectively (Schams et al. 2003). Injections of E2 and P4 to virgin heifers induces TGF-β1 mRNA expression in the mammary tissue (Plath et al. 1997). Moreover, E2 injection for several days before drying-off accelerates mammary involution (Athie et al. 1996). These data from the literature suggest a causative relation between TGF-β1 expression and involution of the bovine mammary gland. A stimulatory effect of E2, which was less than that of P4, on TGF-β1 protein level in bovine MEC cultures (particularly in MAC-T cells) was observed (Table 4). Our results may indicate that a high production of steroid hormones in the pregnant dairy cow together with a higher expression of ERα, ERβ and PR (Schams et al. 2003) may facilitate TGF-β1 synthesis in MEC, which in turn, through its antiproliferative and apoptotic action, participates in mammary tissue involution.

In conclusion, TGF-β1 expression in bovine MEC undergoes complex endocrine and auto/paracrine regulation by hormones of the somatotropic axis and sex steroids. STS, E2 and P4 enhance TGF-β1 expression, whereas the main bovine lactogenic hormones, GH and IGF-I, suppress TGF-β1 synthesis. The suppressive effect of GH on TGF-β1 expression depends on the nourishment level of bovine MEC and becomes stimulatory in poorly fed cells. TGF-β1 expression may also be controlled by a local inhibitory action of EGF and through negative feedback of elevated extracellular TGF-β1 concentration. These effects are dependent on the cell line, because BME-UV1 are more reactive to hormones of the somatotropic axis and EGF, whereas MAC-T cells are more reactive to sex steroids.

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