The expression of the IGF family and GH receptor in the bovine mammary gland

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Abstract

To study the involvement of the IGFs in mammary development and lactation of the cow, the temporal expressions of IGF-I and -II, its receptor type 1 (IGFR-1), IGF-binding proteins (IGFBPs)-1 to -6 and GH receptor (GHR) mRNA were examined. This was carried out for different stages of mammatogenesis, lactogenesis, galactopoiesis and involution in the bovine mammary gland of 26 animals. Furthermore, IGF-I was localised by immunohistochemistry. The highest mRNA concentrations for IGF-I were detected in the mammary tissue of late pregnant heifers (days 255–272) and significantly lower expression was detected during lactogenesis and galactopoiesis. Immunohistochemistry of IGF-I revealed only a weak staining in the epithelium of the ducts during mammatogenesis. The epithelium of the alveoli were negative during mammatogenesis, lactogenesis and galactopoiesis but displayed distinct IGF-I activity during involution. In the stroma a distinct staining of the cytoplasm of adipocytes and of vascular smooth muscle cells was observed. A certain percentage of fibroblasts (usually 20–30%) were also immunopositive. In contrast, highest expression for IGFR-1 was detected during galactopoiesis and involution. The lowest mRNA concentration for IGFR-1 was

found during pregnancy (days 194–213). In general, the expression of IGF-II was not regulated during mammatogenesis and lactation, but decreased during involution. The mRNA for the six binding proteins was detected in the bovine mammary gland. The dominant binding proteins were IGFBP-3 and -5. The highest expression of IGFBP-3 was observed during mid-pregnancy and the lowest during late lactation, involution and in non-pregnant heifers. The mRNA for IGFBP-5 increased during late mammatogenesis and lactogenesis followed by a decrease thereafter. In general, the mRNA concentrations for IGFBP-2, -4 and -6 were barely detectable during all stages. In contrast, the expression for IGFBP-1 was up-regulated in the mammary gland of virgin heifers and increased around the onset of lactation. mRNA for GHR was found during all stages examined without outstanding fluctuations.

In conclusion, locally produced IGF-I and -II may mediate mammatogenesis. The high mammary IGFR-1 mRNA during lactation suggests a role for peripheral IGF-I in maintenance of lactation. The role of IGFBPs in the mammary gland needs further evaluation.

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Introduction

The insulin-like growth factor (IGF) and growth hormone (GH) system is important for the regulation of mammalian epithelial cell proliferation and differentiation. IGF may act as mediator of GH during mammary development and differentiation in ruminants (Gluckmann & Breier 1987, Breier & Sauerwein 1995). No GH binding to ruminant mammary tissue *in vitro* has been detected (Hauser *et al.* 1990); however, GH mRNA is expressed in epithelial cells (Glimm *et al.* 1990). IGF-I is believed to mediate galactopoietic effects from exogenous bovine GH in the cow (Karg *et al.* 1988, Sharma *et al.* 1994). IGF-I can act

in a paracrine or endocrine manner (Holly & Wass 1989). IGF-I mRNA was detected in the bovine mammary gland (Glimm *et al.* 1992), although not in the epithelial cells. The liver is supposed to be the primary source of circulating IGF-I, which may affect target tissues such as the mammary gland. IGFs are potent mitogens involved in the regulation of cell proliferation and apoptosis. The action of IGFs seems to be mediated mainly through a specific cell membrane receptor called IGF receptor type 1 (IGFR-1) (Jones & Clemmons 1995). It binds IGF-I with a higher affinity than IGF-II or insulin. The IGFR-2 is more specific for IGF-II than for IGF-I and does not bind insulin (Massague & Czech 1982, Rechler & Nissley

1985). Different IGFR-1 mRNA transcripts have been detected in the bovine mammary gland (Glimm *et al.* 1992).

IGF-binding proteins (IGFBPs) regulate the interaction between IGFs and their receptors. At least six IGFBPs have been described (Ballard *et al.* 1989) and several (IGFBP-2 to -5) have been detected also in cows' milk (Campbell *et al.* 1991, reviewed in Baumrucker *et al.* 1993). Bovine mammary cells in culture produce and secrete at least four species of binding proteins (McGrath *et al.* 1991).

Published data point towards a role of the IGF system during developmental or apoptotic processes in the mammary gland. However, very little is known about the different stages of these processes in ruminants. The aim of this descriptive study was to examine the expression pattern of IGF-I and -II and IGFR-1 and GH receptor (GHR) during defined stages of mammary growth and lactation in the cow. Furthermore, the expression of the six binding proteins was studied to gain insight into their possible functions in the bovine mammary gland. Finally, the localisation of IGF-I was evaluated by immunohistochemistry. All together the data should help in more experimentally oriented future studies to discover the possible role of the IGF system in the mammary gland.

Materials and Methods

Animals, tissue sampling and preparation

The mammary gland from German Brown Swiss cows ($n=26$) was removed within 20 min after slaughter during defined stages. Small pieces (1–2 g) of mammary tissue and liver were frozen in liquid nitrogen and stored at -80°C . Slices of the mammary tissue for immunohistochemistry were prepared as described later on. The classification of the animals was established as follows. (i) Ductal growth (18-month-old virgin heifers, $n=4$); lobuloalveolar development during first pregnancy. (ii) Days 194–213 of pregnancy ($n=4$) or (iii) days 255–272 of pregnancy ($n=4$). (iv) Lactogenesis (onset of secretion during days 5–11 post-partum, $n=4$); galactopoiesis. (v) Peak lactation ($n=4$). (vi) Late lactation ($n=3$). (vii) involution ($n=3$); 3–4 weeks after dry-off and non-pregnant.

RNA extraction

Total RNA was isolated from bovine tissues using an adapted guanidinium thiocyanate-phenol method as described (Plath *et al.* 1997). The RNA was dissolved in diethylpyrocarbonate-treated water and quantified by absorbance at 260 nm. Agarose gel electrophoresis and ethidium bromide staining verified quantity and quality.

RT-PCR

Four micrograms of RNA were used for the cDNA synthesis as described previously (Plath *et al.* 1997).

Conditions for the enzymatic amplification were optimised for all the factors studied. For every PCR amplification the linear range was verified by introducing increasing cDNA amounts as well as cycle numbers. Each PCR contained 8 mM Tris-HCl, 4 mM KCl, 1.5 mM MgCl_2 , 0.08% Triton X-100, 0.6 μM of each primer (except for ubiquitin: 1.3 μM), 0.5 units Taq Polymerase PrimeZyme (Biometra, Goettingen, Germany) and cDNA derived by reverse transcription of 0.33 μg RNA in a final volume of 25 μl . Amplification conditions included 30 (IGF-I and -II, GHR), 35 (IGFR-1, IGFBP-3), 37 (IGFBP-1, -4 and -5) and 40 cycles (IGFBP-2 and -6) of denaturation at 94°C for 1 min, annealing at 60°C (55°C IGFR-1, 63°C IGF-I, IGFBP-5 and GHR) for 1 min. A single denaturation step at 94°C for 2 min and a final extension step at 72°C for 2 min were performed, except for ubiquitin: 19 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C 45 s. The primers encoding the bovine sequences were as followed: for IGF-I 5'-GGG CTG AGT TGG TGG ATG-3', rev 5'-CTC CAG CCT CCT CAG ATC AC-3'; for IGF-II 5'-TAT GCT GCT TAC CGC CCC AG-3', rev 5'-ACA TCC CTC TCG GAC TTG GC-3'; for IGFR-1 5'-TTA AAA TGG CCA GAA CCT GAG-3', rev 5'-ATT ATA ACC AAG CCT CCC AC-3'; for IGFBP-1 5'-AGC CCA GAG AAT GTG TCC C-3', rev 3'-TTC TTG TTG CAG TTT GGC AG-3'; for IGFBP-2 5'-ACT GTC ACA AGC ATG GCC TG-3' (Winger *et al.* 1997), rev 5'-CCT CCT GCT GCT CAT TGT AGA-3'; for IGFBP-3 5'-TGC GCC CTT ACC TGC TAC-3', rev 5'-CAG TTG GGA ATG TGG ATG C-3'; for IGFBP-4 5'-GCC CTG TGG GGT GTA CAC-3', rev 5'-TGC AGC TCA CTC TGG CAG-3'; for IGFBP-5 5'-TGC GAG CTG GTC AAG GAG -3', rev 5'-TCC TCT GCC ATC TCG GAG -3'; for IGFBP-6 5'-AGA AAG AGG ATT TGC CTT TGC-3', rev 5'-TCC GGT AGA AGC CCC TAT G-3'; for GHR 5'-AGA TGG GAT GGA TAA TCC TGG-3', rev 5'-TCT TTG GAA CTG GAA CTG GG-3'; for ubiquitin 5'-ATG CAG ATC TTT GTG AAG AC-3' (Gabler *et al.* 1997), rev 5'-CTT CTG GAT GTT GTA GTC-3'.

PCR products were analysed by agarose gel electrophoresis, and double-strand sequencing (Sequiseive, Vaterstetten, Germany) was done to verify the obtained amplification products. However, RT-PCR and the evaluation technique used is relative and not a strictly quantitative method. The cDNA levels of the house-keeping gene ubiquitin were almost equal throughout the different stages with a tendency to decrease during lactation and involution (data not shown).

RNase protection assay (RPA)

The RPA was performed and validated as described (Plath *et al.* 1996). Briefly, for IGF-I, pooled total mammary

RNA (20 µg) was introduced into a commercial RPA (Ambion, Austin, TX, USA); the RNase dilution employed was 1:100 and hybridisation was carried out overnight (16 h) at 45 °C. Precast 10% polyacrylamide gels were supplied with 7 M urea (Cleangels; Pharmacia, Freiburg, Germany). Sense and radiolabelled antisense riboprobes were generated by subcloning of the desired homologous gene products (PCR cloning and RNA-transcription kit; Stratagene, La Jolla, CA, USA). Increasing amounts of *in vitro*-synthesised sense RNA for IGF-I were hybridised with the respective labelled riboprobe and compared with a standard mammary RNA sample to allow a quantification of the mRNA.

Immunohistochemistry

For histology and immunohistochemistry, tissue samples (approximately 15 mm long and 5 mm thick) were fixed in Bouin's solution or in methanol/glacial acid (ratio 1:1, w/v) for 24 h, dehydrated in graded series of ethanol, cleared in xylene and embedded in paraffin. Serial sections (5 µm) were cut on a microtome and mounted on glass slides.

Following deparaffinisation, the presence of IGF-I was demonstrated immunohistochemically by the streptavidin-biotin horseradish peroxidase complex technique (Hsu *et al.* 1981). Endogenous peroxidase activity was eliminated by incubation with 0.5% (v/v) hydrogen peroxide solution in absolute methanol for 15 min at 20 °C. Non-specific protein binding was eliminated by incubation with 20% normal goat serum in PBS for 1 h at 20 °C. Sections were then incubated (i) for 18 h at 4 °C with a rabbit polyclonal antibody to IGF-I (KI801, dilution 1:1600; A. Skottner Kabi Pharmacia, Stockholm, Sweden), (ii) for 1 h with biotinylated goat anti-rabbit IgG (Amersham-Pharmacia, Freiburg, Germany, diluted 1:200 in PBS-1% BSA) and (iii) for 1 h at 20 °C with streptavidin horseradish peroxidase complex, diluted 1:250 in PBS-1% BSA.

Visualisation was achieved by incubation for 5 min in 0.5 mg/ml diaminobenzidine in PBS containing 1% hydrogen peroxide. Between each step sections were washed three times in PBS and once in PBS-1% BSA. All incubations were carried out in humidified chambers to prevent evaporation. Sections were left un-counterstained or nuclei were lightly counterstained in Mayer's haematoxylin, and were dehydrated, cleared and mounted.

Controls were performed by (i) replacing the IGF-I antibody with unrelated primary antibodies (*Brucella abortus* Mab, Mab 50-8; Agen, Brisbane, Austria) at the same or greater concentration, (ii) substituting the anti-IGF-I antibody with normal rabbit serum at different concentrations and (iii) omission of the primary antibody.

Statistical analysis

When the data subjected to one-way ANOVA showed significant differences, a Bonferroni test was performed.

Results

Expression of IGF family members during different developmental and functional stages

The obtained partial PCR sequences for IGF-I (155 bp), IGF-II (215 bp), IGFBP-1 (275 bp), IGFBP-2 (168 bp), IGFBP-3 (346 bp), IGFBP-4 (342 bp), IGFBP-5 (257 bp), IGFBP-6 (324 bp), IGFR-1 (314 bp) and GHR (366 bp) were 100% homologous to the known bovine sequences (see Fig. 1 for the different PCR products and the corresponding sizes).

IGF-I mRNA expression was analysed by RT-PCR and RPA. The highest IGF-I mRNA concentration was found in the mammary tissue of the virgin and pregnant heifers when compared with the even higher concentration in the liver. The expression decreased significantly ($P < 0.05$) during lactogenesis and galactopoiesis followed by an increase during involution (Figs 2 and 3). Furthermore, comparison between increasing amounts of *in vitro*-synthesised sense RNA and mammary RNA during RPA analysis showed that approximately 0.5 pg IGF-I mRNA/µg RNA was contained in non-lactating mammary tissue (data not shown).

The densitometric analysis of the RT-PCR of IGF-I and -II, IGFR-1 and IGFBP-1, -3 and -5 during the different stages examined are shown with the most representative bands in Fig. 2. The expression pattern presented for IGF-I was confirmed by RPA (Fig. 3). Due to the higher sensitivity of the PCR, the lower mRNA expression throughout lactation was detectable. The relative IGF-I mRNA concentration of the virgin heifers was slightly lower when compared with the RPA analysis.

Relative expression of IGF-II mRNA (Fig. 3) showed no significant differences during mammogenesis, lactogenesis and lactation and decreased significantly during involution ($P < 0.05$).

The highest IGFR-1 mRNA concentration was detected during late lactation and involution and the lowest during mammogenesis (days 194–213 of pregnancy). In the liver sample no IGFR-1 expression was detected. The IGFBP-3 mRNA concentrations were statistically different only during early mammogenesis and decreased thereafter to significantly lower levels during late lactation and involution. The IGFBP-5 mRNA concentration showed a clear upregulation during late mammogenesis and lactogenesis ($P < 0.05$), followed by a decrease thereafter.

IGFBP-1 showed an upregulation in virgin heifers ($P < 0.05$, when compared with all the other stages) and during early lactation. IGFBP-4 mRNA (data not shown) was increased only weakly during late mammogenesis and lactogenesis.

For IGFBP-2 and -6 only very weak non-regulated signals were detected. A signal of GHR mRNA was found without any significant change throughout all stages of the mammary gland examined (data not shown).

Figure 1 Specific RT-PCR products for IGF-I and -II, IGFR-1, IGFBP-1, -2, -3, -4, -5 and -6 and GHR separated by agarose gel electrophoresis in the bovine mammary gland of virgin heifers as an example for the expression of these factors. M=100 bp marker.

Immunohistochemistry

Mammogenesis *Mammogenesis in non-pregnant heifers.* Mammogenesis in non-pregnant heifers was mainly characterised by ductal growth. The mammary gland was characterised by a well-developed adipose tissue in which small groups of ducts were seen. The comparatively wide lumina of the ducts were lined by a two-layered epithelium with cuboidal luminal secretory cells and a basal layer of myoepithelial cells. Three or four layers of condensed connective tissue surrounded the ductular epithelium. Between the ducts, areas of loose connective tissue were seen, which contained comparatively few cells. As shown in Fig. 4a and b the luminal layer of the ductular epithelium was negative or displayed only weak immunostaining for IGF-I. The basal layer comprising the myoepithelial cells was negative. Ten per cent of the fibroblasts in the interlobular stroma appeared positive for immunoreactive IGF-I. The endothelium of blood and lymph vessels was always negative, whereas smooth muscle cells of blood and lymph vessels were distinctly immunopositive. Many of the adipocytes (about 80%) showed a distinct perinuclear IGF staining in their cytoplasm.

Days 194–213 of pregnancy of primigravid heifers. At the beginning of this period (day 194) the alveoli were well developed to lobuli. The alveoli were small, roundish and lined by a cuboidal epithelium which appeared negative for immunoreactive IGF-I. The flat myoepithelial cells under the secretory epithelial cells were difficult to see and were usually negative for immunoreactive IGF-I. The adipose tissue was well developed at the beginning and displayed a distinct IGF-I staining. Later in this period the

fat cells were reduced. At day 204 about 60–70 alveoli per section were seen to be surrounded by one or two layers of fat cells followed by connective tissue. The glandular epithelium was always negative. In several alveolar epithelial cells small lipid droplets occurred. In the stroma no significant changes were seen. About 20–30% of the fibroblasts were IGF-I positive. The vascular endothelium was not immunostained, but the smooth muscle cells were generally distinctly IGF-I positive. Adipocytes were generally IGF-I positive in their perinuclear cytoplasm.

Days 255–272 of pregnancy of primigravid heifers. During late pregnancy the amount of adipose tissue around the lobuli was further reduced, but the remaining fat cells appeared still immunoreactive for IGF-I in the cytoplasmic area around their nuclei. The height of the alveolar epithelium increased and varied between cuboidal and columnar. Many lipid droplets were seen in the alveolar cytoplasm. No immunostaining for IGF-I was seen in the secretory epithelial and myoepithelial cells. The epithelium of the ducts was always IGF-I negative. No significant changes were seen in the stroma. Some moderately immunostained fibroblasts occurred in the interlobular stroma. Endothelium of blood and lymph vessels was negative whereas smooth muscle cells of the vessels were moderately to distinctly stained.

Lactogenesis (days 5–11 post-partum) The lobuli of the mammary gland consisted of numerous alveoli with wide lumina separated by small amounts of connective tissue (Fig. 4c). Within the alveoli a large amount of secretion occurred. Within the glandular epithelial cells of

Figure 2 Densitometrically analysed RT-PCR of IGF-I and -II, IGFBP-1, -3 and -5 and IGFR-1 from virgin heifers (column 1), primigravid heifers between days 194 and 213 of pregnancy (column 2), primigravid heifers between days 255 and 272 of pregnancy (column 3), during lactogenesis (column 4), during peak lactation (column 5), during late lactation (column 6) and during involution (column 7). Data from densitometric analysis are shown as means $\pm$ S.E.M.; for IGF-I $n=3, 3, 3, 3, 3, 3, 3$; for IGF-II $n=4, 4, 4, 3, 4, 3, 4$; for IGFBP-3 $n=4, 4, 3, 4, 3, 4$; for IGFBP-1 $n=4, 4, 4, 3, 4, 3, 4$; for IGFR-1 $n=4, 4, 3, 3, 4, 3, 3$. Significant differences ($P<0.05$) are indicated for IGF-I and -II, IGFR-1 and IGFBP-1, -3 and -5. ND=not detected. The upper panel depicts the most representative bands during each stage.

Figure 3 Densitometrically analysed RPA of IGF-I. Columns represent pooled mammary RNA (20 µg, $n=4$) from virgin heifers (column 1), from primigravida heifers between days 194 and 213 of pregnancy (column 2), from primigravida heifers between days 255 and 272 of pregnancy (column 3), during lactogenesis (column 4), during peak lactation (column 5), during late lactation (column 6), during involution (column 7) and in the liver. nd=not detected. The upper panel depicts original results of the RPA.

the distended alveoli numerous lipid droplets of varying size were seen. The secretory epithelial cells showed a weak staining for immunoreactive IGF-I. The myoepithelial cells were always negative. A few fat cells, which showed distinct IGF-I immunoreactivity, were seen in the periphery of the lobuli. In the interlobular stroma most of the fibroblasts were immunonegative. Endothelium of blood and lymph vessels was negative and smooth muscle cells of the blood and large lymph vessels stained weakly for IGF-I.

Galactopoiesis *Peak lactation.* In the 10th week of lactation the secretory cells and myoepithelial cells of the alveoli were negative for immunoreactive IGF-I (Fig. 4d). Some of the fibroblasts in the interlobular stroma were weakly positive but a fair number of distinctly IGF-I-positive macrophages appeared, which were also seen in the intralobular connective tissue. No significant changes were seen in the blood vessels. Their endothelium was

negative and their smooth muscle layer appeared weakly to distinctly IGF-I immunopositive.

Late lactation. In cows lactating for 13 months the appearance of the mammary parenchyma changed significantly. The alveoli were irregular in their outline and were lined by a secretory epithelium, which varied between flat and columnar in its height. Many lumina containing a high amount of secretory material corpora amylacea were seen. At the periphery of the lobuli small alveoli with a columnar epithelium and a small lumen occurred. No IGF-I immunoreactivity was seen in either the secretory or myoepithelial cells of any of the acini or ducts. The number of weakly IGF-I-positive fat cells in the stroma was low. A few fibroblasts of the interalveolar stroma were weakly IGF-I immunoreactive. A significant number of macrophages were seen in the intralobular stroma and contained a high amount of immunoreactive IGF-I. As in the previous stages, endothelium of blood and lymph vessels did not show any immunostaining for IGF-I.

Involution of the mammary gland

In non-pregnant cows which were dry for 3 or 4 weeks the size and outline of the acini showed a wide variation. Lobuli with small and roundish acini were distributed between areas with large acini with an irregular outline and a folded epithelium. The cubic epithelium of the small acini as well as that of the large ones displayed a distinct immunoreactivity for IGF-I (Fig. 4e). In the lumina of the acini neutrophilic granulocytes frequently occurred with a varying IGF staining (weak to distinct) in their cytoplasm. The epithelium of the ducts was also distinctly stained. The density of the intralobular stroma had increased considerably and it stained weakly for IGF-I. Many macrophages were easily discerned by their pronounced staining for IGF-I, whereas groups of lymphocytes were consistently seen in the stroma, and were always negative. A weak to distinct IGF-I immunoreactivity occurred in the adipose tissue and the vascular smooth muscle cells.

Figure 4 (Opposite) Immunohistochemistry of IGF-I. (a) Bovine mammary gland, non-pregnant heifer. No immunoreactive IGF-I is seen in the ductular epithelium. (b) Bovine mammary gland, non-pregnant heifer. The endothelium of blood and lymph vessels appears always negative whereas smooth muscle cells of blood and lymph vessels are distinctly immunopositive for IGF-I. Many of the adipocytes (about 80%) show a distinct perinuclear IGF staining in their cytoplasm. (c) Bovine mammary gland, day 6 post-partum. The alveolar epithelium displays a weak reaction for immunoreactive IGF-I. (d) Bovine mammary gland, galactopoiesis, 4th week of lactation. The secretory cells and myoepithelial cells of the alveoli are negative for immunoreactive IGF-I. Some of the fibroblasts in the interlobular stroma are weakly positive but a fair number of distinctly IGF-I-positive macrophages appear. (e) Bovine mammary gland, 4th week of dry period. The cubic epithelium of the small acini, which contains large lipid droplets, as well as that of the large ones displays a distinct immunoreactivity for IGF-I. In the lumina of the acini frequently neutrophilic granulocytes occur with a varying IGF staining (weak to distinct) in their cytoplasm. (f) Bovine mammary gland, 8th week of dry period. The epithelium of the ducts and some remaining small alveoli is distinctly IGF-I immunopositive. The density of the intralobular stroma has considerably increased and it stains weakly for IGF-I. Many macrophages can be easily discerned by their pronounced staining for IGF-I, whereas groups of lymphocytes also regularly seen in the stroma are always negative. A weak to distinct IGF-I immunoreactivity occurs in the adipose tissue and the vascular smooth muscle cells. Bars are 100 µm throughout.

Discussion

The mRNA of the investigated factors was detected in the bovine mammary gland, suggesting paracrine and endocrine functions.

In general, the higher mRNA expression of IGF-I during mammogenesis when compared with lactation indicates a potential proliferative role throughout development. Nevertheless, the possibility of a dilution effect due to increased milk protein mRNA expression during lactation must be considered. The changes in ubiquitin mRNA concentrations were considerably smaller than those observed for IGF mRNA. This fact, and different expression patterns observed for the different factors, corroborate the notion of a specific control of the expression in the bovine mammary gland. Neither the GHR nor the IGFR-1 mRNA concentration decreased with the onset of lactation. This decrease of the IGF-I mRNA concentration in the bovine mammary tissue may be species specific, since it was not observed in the sow (Lee *et al.* 1993). As expected, the mammary IGF-I mRNA expression was lower as in the liver, although the mammary IGF-I mRNA concentration of the virgin heifers was relatively high. Histologically the mammary gland was characterised by a well-developed adipose tissue and many adipocytes (about 80% show a distinct perinuclear IGF-I staining). We assume that most of the IGF-I mRNA was expressed by adipocytes. The participation of IGF-I during ductal development by a paracrine mechanism is possible, since IGF-I expression was relatively high in virgin heifers. Locally produced IGFBP-1 may modulate IGF actions as suggested by the higher IGFBP-1 mRNA expression. The mechanism for an inhibitory or stimulatory activity of IGF-I by IGFBP appears to be a competition for binding to the IGFR (Cohick 1998). During pregnancy the immunohistochemical localisation of IGF-I did not change. The lower expression of mRNA for IGF-I by RPA (days 194–213 of pregnancy) suggests the action of other local modulating factors. This decrease was followed by an increase during advanced pregnancy (days 255–272). Placental oestrogen could be an important competence factor for expression changes and correlates with increasing oestradiol-17 β levels in blood plasma (Theyerl-Abele *et al.* 1990). A similar increase of IGF-I expression during pregnancy was described in the sow (Lee *et al.* 1993), although the regulation may be species specific, because oestradiol seems to downregulate the IGF-I and IGFR-1 expression in swine (Lee *et al.* 1993). In mice the IGF-I expression was reinitiated at the end of pregnancy (Richert & Wood 1999) and this agrees with the cow data. IGF-I stimulates the proliferation of bovine mammary epithelial cells *in vitro* (McGrath *et al.* 1991) and *in vivo* (Collier *et al.* 1993). These observations may indicate a role for endocrine or paracrine IGF-I during late pregnancy lobuloalveolar development, modulated by increased oestradiol levels.

The IGFBP-3 mRNA expression decreased significantly during late lactation when compared with mammogenesis. Additionally, mammary IGFBP-3 protein secretion was higher during the late pre-partum period and involution than during lactation (Baumrucker & Erondur 2000). Interestingly, lactoferrin specifically binds to IGFBP-3 and causes competitive displacement of IGF from IGF-IGFBP-3 complexes, thereby providing free IGF for the receptor (Baumrucker & Erondur 2000). Dehoff *et al.* (1988) and Hadsell *et al.* (1990) described an increase of IGF-I binding during lactogenesis. The weak immunohistochemical staining for IGF-I protein and the lower mRNA expression of IGF-I during lactogenesis and galactopoiesis may indicate a minor function of local IGF-I during that period. Alternatively, high expression of IGFR-1 may suggest an increased importance for the endocrine IGF-I (produced by the liver) and may explain the well-known galactopoietic effect of exogenous GH (Schams *et al.* 1991). For goats, endocrine IGF-I inhibits apoptosis in mammary epithelial cells rather than affecting the secretory activity of epithelial cells (Knight & Wilde 1993). Moreover, treatment of cows with somatotrophin did not increase the expression of IGF-I in the mammary gland (Sharma *et al.* 1994).

During involution, IGF-I expression increased again in the mammary gland. Immunohistochemical analyses revealed that during this phase, in contrast to previously examined stages, IGF-I was distinctly localised in the cubic epithelium of small and large acini, in the epithelium of ducts and weakly of the intralobular stroma. This localisation of the IGF-I protein correlates with the measured increase of mRNA expression, suggesting local production. The results lead us to conclude that IGF-I is probably an important local factor for remodelling of the bovine mammary gland during involution. This may be a direct effect of IGF-I or of synergistic actions with other growth factors. A synergistic interaction between IGF-I and transforming growth factor- α (TGF- α) has been suggested by Forsyth *et al.* (1998), and significant expression of TGF- α mRNA in the bovine mammary gland (Plath *et al.* 1997) supports this notion.

Throughout mammogenesis and lactation the IGF-II expression in the mammary tissue was relatively high and constant when compared with lower expression during involution. IGF-II stimulates the proliferation of mammary epithelial cells *in vitro* (Collier *et al.* 1993), although with a lower potency than IGF-I (Peri *et al.* 1992). IGF-II mRNA was also detected in ovine mammary epithelial cells (Forsyth *et al.* 1995). It is interesting to note that in sheep foetal mammary tissue IGF-II was more highly expressed than IGF-I (Forsyth *et al.* 1999). IGF-II may be more important during embryonic development (Adams 1983). Nevertheless, in the adult we assume that there is a significant role of IGF-II during mammogenesis, because in a mouse mammary cell line IGF-II seemed to increase cellular growth and reduce differentiation (Lykos *et al.* 2000).

The distinct upregulation of IGFBP-1 and -5 during specific periods suggests physiological functions for these periods. In contrast to our results with non-detected levels in the bovine mammary tissue, the IGFBP-5 mRNA concentration is increased during involution in rat mammary tissue. It has been proposed that IGFBP-5 inhibits IGF-I-mediated cell survival (Tonner *et al.* 1997). We do not have a clear explanation for the species differences observed. One explanation could be the timing of material collection. The bovine mammary gland tissue was collected relatively late (3–4 weeks after dry-off). Therefore further studies with a better timing of material collection (starting shortly after dry-off) are needed. The other possibility are species differences in IGFBP expression as indicated in the literature (Albiston *et al.* 1995, Flint *et al.* 2000). Because IGFBP-2 and -6 expression in the bovine mammary gland was very weak, we conclude that it is not regulated and does not have a significant physiological role in the bovine mammary gland. Nevertheless, the IGFBP-2 mRNA expression in the ovine mammary tissue seems to be regulated, because it was significantly lower in lactating than in pregnant sheep (Klempt *et al.* 1993).

The GHR mRNA was highly expressed in all stages examined. The receptor protein was also demonstrated in the same mammary gland samples by immunohistochemistry and *in situ* hybridisation in the epithelial and stromal compartments (Sinowatz *et al.* 2000). Therefore, a direct effect of GH on development and function of the bovine mammary gland seems to be indicated.

In conclusion, locally produced IGF-I and -II may play a role during mammary development in virgin and pregnant heifers. A high IGFR expression compared with a lower IGF-I and IGFBP-3 expression during galactopoiesis suggests a function for the endocrine IGF-I during this stage. The increase of IGF-I mRNA during involution and immunohistochemistry indicates that it may be functionally important during this stage of remodelling.

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