Cellular localisation of GH receptor in the bovine mammary gland during mammogenesis, lactation and involution

F Sinowatz, D Schams¹, S Kölle, A Plath¹, D Lincoln²
and M J Waters³

Institute of Veterinary Anatomy, University of Munich, D-80539 Munich, Veterinärstrasse 13, Germany

¹Institute of Physiology, Technical University of Munich, D-8530 Freising, Germany

²Faculty of Allied Health Sciences, University of Kuwait, Kuwait

³Department of Physiology and Pharmacology, University of Queensland, St Lucia, Queensland, Australia

(Requests for offprints should be addressed to F Sinowatz, Lehrstuhl für Tieranatomie II, University of Munich, D-80539 Munich, Germany;

Email: f.sinowatz@anat.vetmed.uni-muenchen.de)

Abstract

We have used immunohistochemistry and non-radioactive *in situ* hybridisation to localise the GH receptor and its transcript in the bovine mammary gland during mammogenesis, lactation and involution. We found a characteristic pattern of immunoreactive GH (irGH) receptor distribution in the epithelial and stromal compartments during the different stages of mammary gland development: The ductular epithelium showed a distinct staining for irGH receptor during most stages, whereas the alveolar epithelium contained a modest amount of GH receptor during pregnancy which increased during lactation and galactopoiesis. In dry cows, the immunostaining for GH receptors in the alveolar epithelium was very weak or

negative. Curiously, the amount of GH receptor mRNA appeared relatively constant during mammogenesis and lactation. The epithelial cells of the alveoli and ducts as well as the endothelial cells showed a distinct signal in our *in situ* hybridisation studies. The predominant localisation of GH receptors in the epithelium of ducts and alveoli is supportive of a role for GH in epithelial differentiation and maintenance. Furthermore, the increased intensity of immunostaining in bovine mammary tissue post partum suggests a direct role for GH receptor in mediating the effect of GH in milk production and secretion.

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Introduction

The development of the mammary gland during pregnancy and lactation is a complex process brought about by the interaction of many hormonal and paracrine influences (Forsyth 1982). To achieve a high milk yield at parturition, a major cell proliferation occurs in the mammary gland during pregnancy as a result of the combined actions of oestrogen, progesterone, adrenal corticoids, growth hormone (GH), prolactin (Sheffield & Anderson 1989) and in some species, like the bovine, placental lactogen (Byatt *et al.* 1994). The finding that GH injection in the bovine markedly increases milk production (Knight *et al.* 1992) suggests that GH plays a key role in lactation in this species. However, the mechanisms used by GH to elicit these actions on mammogenesis and galactopoiesis have not been fully established, and the relative contributions of metabolic repartitioning, elevated endocrine insulin-like growth factor-I (IGF-I), elevated blood flow and direct GH action remain to be established.

The results of several GH receptor-binding studies using bovine mammary membrane preparations have led to the now widely accepted view that GH receptors are not present in the bovine mammary gland (Gertler *et al.* 1984, Akers 1985, Kazmer *et al.* 1986, Keys & Djiane 1988) and that GH has only indirect effects on mammary gland functions. Several significant conceptual problems arise from this postulated absence of GH receptors in mammary tissue. (1) In other species (such as rodents) where GH is reported to have *in vivo* as well as *in vitro* actions, such as increasing IGF-I mRNA (Kleinberg *et al.* 1990) and casein synthesis, GH receptor mRNA and protein have been reported in mammary gland ductal and alveolar epithelia (Lincoln *et al.* 1995, Tiong & Herrington 1991, Feldman *et al.* 1993). (2) Many cell types, which are significant components of the bovine mammary gland, such as fibroblasts, adipocytes, T-lymphocytes and smooth muscle cells, have been shown to possess GH receptors (Isaksson *et al.* 1985). (3) GH receptor mRNA has been demonstrated and characterised in lactating bovine tissue

using Northern blots, *in situ* hybridisation (Glimm *et al.* 1992) and RT-PCR (Röpke *et al.* 1994).

In this extensive study with 27 heifers and cows at defined stages of mammary gland development, we have investigated the cellular localisation of the GH receptor and its transcript, using immunocytochemistry and non-radioactive *in situ* hybridisation.

Materials and Methods

Animals, tissue sampling and preparation

Mammary tissue was obtained from 27 German Brown Swiss heifers and cows at distinct periods of mammary gland development immediately after slaughter. The material studied comprised mammary tissue from non-pregnant and primigravid heifers, cows during lactogenesis, early and late lactation and dry, non-pregnant cows. The following classification of animals was established. Mammogenesis; mammatogenesis – ductal growth (non-pregnant heifers); mammatogenesis – lobuloalveolar development during first pregnancy (days 194–213 of pregnancy and days 255–272 of pregnancy); lactogenesis: days 5–11 post partum; galactopoiesis (peak lactation and late lactation); involution (3–4 weeks dried off, non-pregnant).

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 a graded series of ethanol, cleared in xylene and embedded in paraffin. Serial sections (5 µm) were cut on a Leitz microtome and mounted on glass slides.

Production and characterisation of monoclonal antibodies (MAbs)

The monoclonal antibodies MAb 7 and MAb 43 to the rabbit GH receptor were produced by application of hybridoma technology to splenic lymphocytes from mice immunised with human GH affinity-purified preparation of rabbit liver GH receptor. These antibodies recognise independent epitopes on the extracellular portion of the rabbit GH receptor, do not cross-react with insulin or prolactin receptors in the appropriate receptor assays, and do not react with bovine GH receptor (Amit *et al.* 1992). MAb 263, which recognises a cross-species determinant with high affinity (Barnard *et al.* 1984), was similarly prepared by immunisation of mice with purified rat and rabbit GH receptor. MAb 263 is reactive against the GH receptor in a number of species including the bovine (Leung *et al.* 1987, Kölle *et al.* 1997). These antibodies have been used previously to detect GH receptor in the bovine uterus and placenta and in bovine ovaries (Kölle *et al.* 1997, 1998). The MAbs used in this study recognise epitopes shared by both the GH receptor extracellular

domain and the GH-binding protein (GHBP), since the GHBP is the extracellular domain of the GH receptor. Thus no distinction between the location of GH receptor and GHBP was possible.

Immunohistochemistry

Serial 5 µm sections were cut from paraffin blocks on a Leitz microtome and collected on gelatine/chrom alum-coated slides. Following deparaffinisation, the presence of GH receptors 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 phosphate-buffered saline (PBS) for 1 h at 20 °C. Sections were then incubated as follows: (1) for 18 h at 4 °C with mouse anti-GH receptor (MAb 7 at 100 µg/ml, MAb 43 at 8 µg/ml and MAb 263 at 12.5 µg/ml) or *Brucella* MAb at 25 µg/ml in PBS–1% bovine serum albumin (BSA), (2) for 1 h with biotinylated goat anti mouse IgG (Amersham International plc, Amersham, Bucks, UK), diluted 1:200 in PBS–1% BSA) and (3) 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 (DAB) in PBS containing 1% H₂O₂. 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, dehydrated, cleared and mounted.

Controls were performed by (1) replacing the MAb 263 with unrelated primary antibodies (*Brucella abortus* MAb, MAb 50.8 (Agen, Brisbane, Australia) of the same isotype (IgGK1) at the same or greater concentration or (b) substituting MAb 263 for the species-specific GH receptor antibody MAb 7, which does not react with bovine receptor (Amit *et al.* 1992).

In situ hybridisation

For *in situ* hybridisation, pieces of tissue (0.5 × 0.5 cm) were fixed in 4% paraformaldehyde (PFA) in PBS for 12 h at 4 °C. Subsequently, specimens were washed in 0.5 M sucrose in PBS and in PBS. After snap-freezing in liquid nitrogen, the tissue was embedded in freezing medium (Shandon Southern Products, Cheshire, England). Sections (7 µm) were mounted on 3-aminopropylene-ethoxysilane-coated slides, dried at 50 °C and stored at –70 °C until use.

The antisense oligonucleotide probe based on the cDNA of the bovine GH receptor (Hauser *et al.* 1990) was

used. The sequence of the biotin-labelled antisense probe was 5'-ACC CAG TGG AAA ATG GAC CCT T-3'.

Slides adjusted to room temperature were hydrated in a graded series of ethanol (100% to 50%), washed in PBS and incubated in 0.2 M HCl for 10 min. After washing in PBS, specimens were treated with proteinase K (2 µg/ml; Sigma-Aldrich, Deisenhofen, Germany) at 37 °C for 30 min. Enzyme reaction was stopped by incubation in PBS-glycine (2 mg/l) and post-fixation was performed in 4% PFA in 0.1 M PBS. Samples were washed again in PBS-glycine and incubated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. After washing in distilled water treated with diethylpyrocarbonate, the slides were prehybridised at 42 °C for 2 h. Both the prehybridisation and the hybridisation were performed in humid chambers containing filter papers saturated with 4 × sodium chloride-sodium citrate (SSC) and 50% formamide. The prehybridisation solution comprised 0.5 M NaCl, 12 mM Tris, pH 7.5, 1 mM EDTA, 5 × Denhardt's solution, 0.5 µg/ml heparin, 0.5 µg/ml salmon sperm DNA and 50% formamide. The biotinylated probes were diluted to a concentration of 20 ng/µl. Hybridisation was carried out at 43 °C overnight. After having washed the slides in 0.2 SSC for 1 h, the hybridisation signals were visualised using the avidin-streptavidin-biotin complex (DAKO, Hamburg, Germany) according to the manufacturer's instructions. The slides were incubated in 0.05 mg/ml DAB containing 1% H₂O₂ for 10 min. After washing in distilled water, the slides were dehydrated in a graded series of ethanol (50% to 100%) and xylene and mounted with Eukitt (Riedel-de-Haen, Seelze, Germany).

For controls, specificity of hybridisation was monitored in control experiments carried out with the sense probe 5'-biotin-GGC TAT GTG AGC ACA GAC CA-3'. In order to check background staining, hybridisations were also performed without oligonucleotides. Bovine liver, known to be rich in GH receptor, was used as control organ.

Results

The GH receptor displayed immunoreactivity both in the cytoplasm and the cellular membrane. No nuclear immunostaining was seen in any of our sections, regardless of the stage of mammary development. In the different phases of mammary gland development (mammarygenesis, lactogenesis, galactopoiesis and involution), a characteristic immunostaining pattern of the epithelial and stromal components of the gland was obtained, which will be described later. The specificity of GH receptor localisation in this study was verified by a negative immunoreaction when control MAb 7 (Fig. 1 h) and 43 (only reactive with rabbit GH receptor) and MAb 50.8 (against the unrelated *Brucella abortus* antigen) were used as primary antibodies. Only incubation with MAb 263, which recognises a

cross-species determinant with high affinity and which has been reported to be expressed in the bovine (Kölle *et al.* 1998), resulted in a positive immunostaining. With *in situ* hybridisation, distinct amounts of GH receptor mRNA were found in the glandular epithelium, in the epithelium of the duct and in the vascular endothelium during all stages of mammarygenesis and lactation (Fig. 1a). Labelled sense oligonucleotide revealed no reaction (Fig. 1b).

Mammarygenesis

Mammarygenesis in non-pregnant heifers Mammarygenesis in non-pregnant heifers was mainly characterised by ductal growth. The mammary gland was characterised by well-developed adipose tissue in which small groups of ducts could be 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. The ductular epithelium was surrounded by three to four layers of condensed connective tissue. Between the ducts, areas of loose connective tissue were seen which contained comparatively few cells. The luminal layer of the ductal epithelium displayed distinct staining for GH receptor (Fig. 1c) and its transcript. The basal layer comprising the myoepithelial cells was negative or only moderately positive. Ten to twenty per cent of the fibroblasts in the interlobular stroma were immunopositive, whereas the circular arranged layers of fibroblasts around the ductular epithelium were usually negative for GH receptors. The vascular endothelium was moderately to distinctly stained for GH receptor and its transcript, whereas the endothelium of lymph vessels always appeared negative. The smooth muscle cells of blood and lymph vessels were distinctly immunopositive. A fair number of macrophages in the stroma showed strong immunoreactivity but no obvious staining for GH receptor mRNA. The fat cells displayed a distinct perinuclear staining of their cytoplasm.

Days 194–213 of pregnancy of primigravid heifers

Even at the beginning of this period (day 194) the alveoli were arranged in lobuli. The alveoli were small, roundish and lined by a cuboidal epithelium. The flat myoepithelial cells were difficult to see and were usually negative for immunoreactive GH receptor. The adipose tissue was well developed. Later in this period, the adipose tissue was reduced. At day 204, about 60–70 alveoli per section were seen to be surrounded by one to two layers of fat cells followed by connective tissue. The glandular epithelium was generally moderately immunopositive but, in alveoli with flat distended epithelium, no positive immunoreaction was seen. In several alveolar epithelial cells small lipid droplets occurred. The ductular epithelium was moderate to distinctly immunopositive and showed distinct amounts of GH receptor mRNA. In the stroma no

Figure 1 Bovine mammary gland at the following stages is shown. (a) Non-pregnant heifer, *in situ* hybridisation. The mRNA encoding GH receptor is mainly focalised in the cytoplasm of the ductular epithelial cells. In the stroma, positive fibroblasts and endothelial cells are seen. Bar: 30 μ m. (b) Lactogenesis, *in situ* hybridisation, control section. In the controls performed by using the sense oligonucleotide no signal is visible. Bar: 30 μ m. (c) Non-pregnant heifer. The luminal layer of the ductular epithelial cells displays distinct immunostaining for GH receptor. Positive immunostaining is also seen in a subset of fibroblasts. Bar: 30 μ m. (d) Mammogenesis, day 257 of pregnancy. The intensity of GH receptor immunoreactivity in the alveolar epithelium is generally weak. The epithelium of the ducts appears moderately stained for irGH receptor. Bar: 150 μ m. (e) Lactogenesis, day 11 post partum. The alveolar epithelium displays a distinct reaction for irGH receptor. Bar: 100 μ m. (f) Galactopoiesis, week 8 of lactation. Secretory cells of the alveoli show a strong reaction for irGH receptor. The secretory material within the lumen of the alveoli is always negative. Bar: 30 μ m. (g) Involution. In cows which have been dry for 3 weeks the epithelium of the small alveoli shows only weak staining for GH receptor. Bar: 20 μ m. (h) Non-pregnant heifer. Control section for immunostaining. The specific antibody MAb 263 was substituted for the species-specific (rat, rabbit) GH receptor antibody MAb 7, which does not react with bovine receptor. Bar: 30 μ m.

significant changes were seen. Ten to twenty per cent of the fibroblasts were GH receptor positive. The vascular endothelium and the smooth muscle cells of the vessel wall were distinctly stained for GH receptor and its mRNA, fat cells were positive in their perinuclear cytoplasm. A fair number of strongly immunostained macrophages was seen in the interlobular stroma.

Days 255–272 of pregnancy of primigravid heifers

During late pregnancy, the amount of adipose tissue around the lobuli was further reduced, but the adipocytes still displayed perinuclear immunoreactivity. The height of the alveolar epithelium had increased and varied in form between cuboidal and columnar. Many lipid droplets were seen in the alveolar cytoplasm. The intensity of GH receptor immunoreactivity in the alveolar epithelium was generally weak (Fig. 1d). Occasionally, a weak positive immunoreaction appeared in the luminal secretion of the alveoli. No immunostaining was seen in the myoepithelial cells. The epithelium of the ducts was moderately to distinctly positive for the GH receptor. No significant changes were seen in the stroma. Some moderately immunostained fibroblasts and strongly positive macrophages occurred in the interlobular stroma. Endothelium and smooth muscle cells of blood vessels stained weakly for GH receptor. *In situ* hybridisation showed the same distribution pattern of the GH receptor mRNA as in days 194–213 of pregnancy.

Lactogenesis (days 5–11 post partum)

The lobuli of the mammary gland consisted of numerous alveoli with wide lumina separated by only small amounts of connective tissue. Within the alveoli, a large amount of secretion occurred. The glandular epithelial cells of the distended alveoli contained numerous lipid droplets of varying size. The epithelium displayed a distinct staining for immunoreactive GH (irGH) receptor (Fig. 1e). The myoepithelial cells were always negative. The amount of intralobular stroma appeared to be very reduced. Some fat cells were seen in the periphery of the lobuli. Only a few, strongly stained macrophages were located in the interlobular stroma, whereas most of the fibroblasts were immunonegative. Endothelium and smooth muscle cells of the blood vessels stained weakly for GH receptor. GH receptor mRNA was distinctly expressed in the cytoplasm of the glandular epithelial cells. The controls performed by using the sense oligonucleotide for hybridisation regularly showed no signal.

Galactopoiesis

Peak lactation In week 10 of lactation the secretory cells of the alveoli were distinctly stained for GH receptor (Fig. 1f) and its transcript. Myoepithelial cells, which were difficult to see, appeared immunonegative. Some of

the fibroblasts in the interlobular stroma were weakly positive. The number of macrophages had increased significantly. They were also seen in the intralobular connective tissue and could be discerned easily by their strong reaction for irGH receptor. Blood vessel endothelium and smooth muscle remained moderately immunoreactive. The endothelium of the wide lymph vessels was always negative for the GH receptor and its transcript.

Late lactation In cows lactating for 13 months, the appearance of the mammary parenchyma had significantly changed. The alveoli were irregular in their outline. They were lined by a secretory epithelium which varied between flat and columnar in height. In many lumina which contained a large amount of secretory material, corpora amylacea were seen quite frequently. At the periphery of the lobuli, small alveoli with a columnar epithelium and a small lumen occurred. The number of fat cells in the stroma was low. Staining for the GH receptor and its transcript was distinct in the epithelium of many alveoli and in the ductular epithelium. Few fibroblasts of the interalveolar stroma appeared weakly positive for GH receptor and its mRNA. A significant number of macrophages were now seen in the intralobular stroma and contained a high amount of irGH receptor. As in the previous stages, blood vessels were positive for the GH receptor and its mRNA, whereas lymph vessels did not show any staining in their endothelium. In big lymph vessels, the smooth muscle cells of the cell wall are distinctly positive for the GH receptor.

Involution of the mammary gland

In cows which were dry for 3 weeks, the epithelium of the small alveoli showed only a weak staining for the GH receptor (Fig. 1g). The expression of GH receptor mRNA was also reduced. The epithelium of the ducts was weakly to distinctly stained. The density of the intralobular stroma had increased and many macrophages could be easily discerned by their pronounced staining for GH receptor. No apparent GH receptor staining was seen in the adipose tissue. The amount of GH receptor and its transcript in vascular endothelial cells showed no change compared with previous stages.

Discussion

In the bovine, GH is the most abundant of all anterior pituitary hormones. It is responsible for the growth of skeletal and soft tissues and for a number of metabolic actions (Kelly *et al.* 1994). Along with prolactin and placental lactogen, GH forms a family of hormones which have been shown to be derived by duplication of an

ancestral gene (Forsyth 1982). Of the many hormones involved in the regulation of mammary growth and lactation (Plath *et al.* 1997), GH is considered to play a key role (Knight *et al.* 1992). When administered to young peripubertal heifers, GH stimulates a significant increase in mammary gland development (Seyrsen *et al.* 1986). Similarly, administration of GH to lactating dairy cows induces a well-documented rise in milk production (Peel & Bauman 1987). Despite the numerous reports of important physiological actions of GH in cattle, the mechanisms by which GH exerts its influence on mammary development, galactopoiesis and lactogenesis are unclear. It was hypothesised that GH action may involve partitioning of nutrients to target tissues such as the mammary gland (Bauman & Currie 1980), a GH-induced increase in IGF-I concentrations in plasma (Peel & Bauman 1987) and in mammary epithelial cells (Glimm *et al.* 1988) or paracrine interactions with GH-responsive cells within the target tissue, including the stroma of the mammary gland (Hauser *et al.* 1990).

Previous studies using ^{125}I -labelled bovine GH failed to demonstrate GH receptor in bovine mammary gland tissue (Akers 1985, Keys & Djiane 1988), and *in vitro* experiments (Gertler *et al.* 1984) could not demonstrate an influence of exogenous GH administration on the casein, fat or α -lactalbumin synthesis of isolated bovine mammary cells. In contrast, more recent work using Northern blot and *in situ* hybridisation (Glimm *et al.* 1992), PCR and ribonuclease protection assay (Hauser *et al.* 1990, Röpke *et al.* 1994) demonstrated GH receptors in bovine mammary gland. Hauser *et al.* (1990) cloned cDNA for the bovine GH receptor out of a cDNA library prepared from the liver of a pregnant Holstein heifer. The cDNA clone hybridises to a single 4.5 kb mRNA species and shares a high degree of sequence homology with GH receptor cloned from other species. Utilising the bovine GH receptor cDNA as a probe, a high level of bovine GH receptor mRNA was detected in bovine liver and lower concentrations in bovine kidney, anterior pituitary and mammary gland. From the sequence data obtained, Hauser *et al.* (1990) concluded that the GH receptor protein is most likely 616 amino acids in length. The bovine GH receptor shows a high degree of amino acid homology to other reported GH receptors displaying 82% identity to the rabbit, 76% to the human and 71% to rat and mouse receptor sequence (Hauser *et al.* 1990).

From the studies of Hauser *et al.* (1990) it became obvious that the GH receptor mRNA is present in the bovine mammary gland and several suggestions were made to explain the differences between these results and previous radioligand-binding studies, which failed to demonstrate GH receptor in the bovine mammary gland. It was suggested that the bovine GH receptor mRNA may not be translated in the mammary gland. Alternatively, GH receptor mRNA and its protein may be

present in only a small subpopulation of mammary cells, resulting in dilution of GH-specific binding to whole tissue extracts.

In view of the disagreement between conventional radioligand-binding studies (Gertler *et al.* 1984, Akers 1985, Kazmer *et al.* 1986, Keys & Djiane 1988) and the unequivocal demonstration of the GH receptor mRNA by several molecular biological techniques, such as Northern blotting and PCR (Röpke *et al.* 1994), we have sought to demonstrate and localise the bovine mammary gland GH receptor. We found a widespread distribution of GH receptor in the bovine mammary gland cells, including glandular epithelium and stromal components. The ductular epithelium showed a distinct staining for irGH receptor during all stages of gland development except during involution of the gland, when immunostaining was reduced. The alveolar epithelium contained a modest amount of irGH receptor during pregnancy, which increased during lactation and galactopoiesis. In dry cows, the immunostaining for GH receptor was weak again. The localisation of GH receptor to the epithelial compartment of the gland was supported by our *in situ* hybridisation studies, which demonstrated a distinct signal for GH receptor mRNA in the alveolar and ductal epithelium in most stages of mammary gland development except involution. The predominant localisation of GH receptor to the epithelium of ducts and alveoli is in concordance with a supposed role for GH in epithelial differentiation and maintenance (Lobie *et al.* 1990). The immunoreactivity to GH receptor was confined to the cytoplasm and the cellular membrane. No nuclear immunostaining was seen in any stage of mammary gland development in the bovine. This was somewhat surprising, because previous studies in the rat mammary gland gave clear evidence of nuclear immunostaining for GH receptor (Lincoln *et al.* 1995). It was speculated there that the presence of GH receptor in both the nucleus and the cytoplasm might be part of a mitogenic mechanism (Lincoln *et al.* 1998). Nuclear staining is not seen consistently across tissues or species, and even within tissues some nuclei are immunopositive and some negative (Lobie *et al.* 1992, 1994). So it is not possible to draw conclusions from the differences in nuclear localisation between species at this time.

The role of GH in growth and differentiation of the mammary gland is emphasised by the finding that GH receptor is synthesised by the glandular epithelium and the surrounding mesenchymal cells already during foetal development (Knabel *et al.* 1998). Recent studies in adult cows have shown that daily injections of recombinant bovine GH result in higher yields of milk, fat and protein (Jammes *et al.* 1996). Deficiencies in GH and prolactin cause a marked decrease in milk volume (Guyette *et al.* 1979). As the intensity of immunostaining in bovine mammary tissue increases post partum we assume a direct role of GH receptor in mediating the effect of GH in milk production and secretion.

In the stroma of the mammary gland, a certain set of cells (fibroblasts, vascular endothelial cells, smooth muscle cells) consistently showed immunoreactivity for GH receptor. Strong staining, irrespective of the stage of mammary gland development, was seen in the cytoplasm of macrophages. Their number and location varied significantly in the different periods. The highest number of macrophages was seen in late lactation and involution. During these stages macrophages protruded in the interlobular stroma, whereas during the mammaryogenesis and early lactation stages they were usually confined to the interlobular ducts, where they were localised near to the blood vessels. Contrary to immunohistochemistry, macrophages showed no staining by non-radioactive *in situ* hybridisation, implying that these cells might contain phagocytosed fragments of GH receptors which are then recognised by our antibody.

A certain percentage of interlobular fibroblasts (up to 20%) showed weak immunoreactivity for GH receptor. The immunostaining of blood vessels and lymph vessels differed. Whereas the endothelium of blood vessels appeared always moderately to distinctly immunopositive, the endothelium of the lymph vessels was consistently immunonegative. Smooth muscle cells in both types of vessels were usually distinctly immunoreactive for GH receptor. Occurrence of GH receptors in macrophages, fibroblasts and in the wall of blood vessels has also been previously described by several authors (Lobie *et al.* 1990, Kölle *et al.* 1998). Our *in situ* hybridisation experiments did not reveal any transcripts of GH receptor in the cytoplasm of macrophages. As in other tissues (Kölle *et al.* 1998), immunostaining of macrophages in the mammary gland cannot be regarded as specific. In our study, no significant changes in the immunoreactivity for GH receptors were seen in the stromal cells during the different stages of mammary gland development, although considerable changes in the amount and arrangement of the stroma occurred.

In conclusion, the existence of irGH receptor in cells of the bovine mammary gland involved in milk production suggests that local GH action accounts for and, at least partly explains, the strong galactopoietic effect during lactation when recombinant GH is administered in this species.

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