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Expression of the neonatal Fc receptor (FcRn) in the bovine mammary gland

Balázs Mayer[1], Márton Doleschall[1], Balázs Bender[2], János Bartyik[3], Zsuzsanna Bősze[2], László V. Frenyó[1] and Imre Kacskovics[1]*

[1] Department of Physiology and Biochemistry, Faculty of Veterinary Science, Szent István University, PO Box 2, H-1400 Budapest, Hungary
[2] Department of Animal Biology, Agricultural Biotechnology Center, H-2100 Gödöllő, Hungary
[3] Agricultural Company of Enying, H-8155 Kiscséripuszta, Hungary

In ruminants, protective immunoglobulins are transferred to the newborn *via* colostrum to mediate maternal immunity. There is a high selectivity in the transport of immunoglobulins from the maternal plasma across the mammary barrier into the colostrum, and only IgG1 is transferred in large amounts. We have recently analysed the expression of the neonatal Fc receptor (FcRn) in sheep mammary gland around parturition. Re-analysing this issue in bovine confirmed our previous data indicating that FcRn is homogeneously localized in the mammary gland acinar cells before parturition, however a remarkable difference was observed in the pattern after calving, where only the apical side of the cells was strongly stained. The presence of the FcRn in the acinar epithelial cells of the mammary gland and the obvious change in distribution before and after parturition indicate that FcRn plays an important role in the IgG transport during colostrum formation in ruminants.

Keywords: FcRn, mammary gland, non-lactating, parturition, bovine, IgG.

Maternal IgG endows the fetus with protection against congenital infection and also provides adequate immunity for the first weeks of independent life, since at birth the offspring is exposed to a similar antigenic environment as its mother. In mammals, the offspring acquire maternal IgGs either before or after birth.

In ruminants, protective immunoglobulins are transferred to the newborn via colostrum to mediate passive immunity. There is a high selectivity in the transport of immunoglobulins from the maternal plasma across the mammary barrier into the colostrum, and only IgG1 is transferred in large amounts (reviewed in Butler 1999). Upon ingestion of the colostrum, the immunoglobulins are transported across the intestinal barrier of the neonate into its blood. This intestinal passage appears to be non-specific, and later, a large proportion of the absorbed IgG1 has been suggested to be recycled back into several mucosal surfaces, like the intestinal lumen (Newby & Bourne 1976; Besser et al. 1988) and respiratory tract (Wilkie 1982).

*For correspondence; e-mail: Kacskovics.Imre@aotk.szie.hu

Preferential binding of IgG1 to the mammary epithelial cells was previously shown near parturition (Kemler et al. 1975; Sasaki et al. 1977; Barrington et al. 1997) and these cells were reported to stain prepartum with anti-IgG1 serum (Leary et al. 1982). There is a rapid drop in the concentration of all lacteal immunoglobulins immediately postpartum (Butler 1983) and the selectivity of this transfer has led to the speculation that FcRn might be involved in this process.

The neonatal Fc receptor (FcRn) was first identified in rodents as the receptor that transfers maternal immunoglobulins (IgGs) from mother to newborn *via* the neonatal intestine (Rodewald 1976). Since then, this receptor has been detected in different epithelial cells which delivers IgG across these barriers, as well as in endothelial cells which are responsible for the maintenance of serum IgG levels (reviewed in Ghetie & Ward 2000). FcRn binds IgG in a strictly pH dependent manner (binding occurs at pH 6, but not at pH 7·4) and consists of a heterodimer of an integral membrane glycoprotein, similar to MHC class I α-chains, and β2-microglobulin (Simister & Mostov 1989).

One of several functions described for FcRn is the regulation of IgG isotype transport into milk as it was

localized to the epithelial cells of the mammary gland in lactating mice. Analysis of the transfer of Fc fragments and IgG which have different affinities for FcRn indicated that it prevents IgG from being secreted into milk (Cianga et al. 1999). In addition to this finding, the expression of FcRn in the mammary gland of other species like possum (*Trichosurus vulpecula*) (Adamski et al. 2000), swine (Schnulle & Hurley 2003), ruminants (Kacskovics et al. 2000; Mayer et al. 2002) and human (Cianga et al. 2003) were shown, however, how FcRn is involved in the mammary IgG transport in these species has not been directly assessed.

We have previously shown that the sheep FcRn is expressed exclusively in the epithelial cells of the acini in the mammary gland, and there was a remarkable cellular redistribution of this receptor around parturition with a downward expressional trend postpartum. We also found its presence in the crypt epithelial cells (that secrete IgG1 into the lumen) of the neonatal lamb (Mayer et al. 2002). Others have found that there is a correlation between allotypes of the cattle FcRn alpha-chain and β2-microglobulin and the IgG1 content in their calves (Laegreid et al. 2002; Clawson et al. 2004). In a more recent study we demonstrated bovine FcRn expression in epithelial cells of the lower airways and in the alveoli of the lung, where IgG is the major immunoglobulin in secretion (Mayer et al. 2004). These findings led to the hypothesis, that this receptor was indeed involved in IgG1 secretion in these cells in ruminants.

Although, the function of the sheep mammary gland is considered to be highly similar to its cattle's counterpart, and FcRn transcripts have already been detected in the bovine mammary gland from non-lactating animals (Kacskovics et al. 2000), we decided to analyse the localization of FcRn alpha-chain expression in the bovine mammary gland around the time of parturition. Based on this survey cow FcRn shows similar expression and localization pattern as was observed in ewes.

Materials and Methods

Samples for histology

Mammary gland tissue from a non-lactating 4-year-old Holstein-Friesian cow was sampled at a local slaughterhouse and the specimens were fixed in 4% paraformaldehyde (PFA) in PBS treated with dietyl-pyrocarbonate (Sigma-Aldrich Co., St. Louis, MO).

Biopsies (16 gauge × 10-cm length biopsy needle; Magnum, Bard, Covington, GA) were collected from the mammary gland (length of sample notch: 1·9 cm) of 3-year-old Holstein-Friesian cows 14, 7 and 1 days prepartum, on the day of calving, and 7, 14 days postpartum, under local anaesthesia, as reported previously (Colitti et al. 2000). To prevent local infection, Aureomycin Violet Spray (Fort Dodge Animal Health, Overland Park, KS) was applied. Samples were harvested for reverse transcriptase-PCR into liquid nitrogen or for immunohistochemistry into freshly made 4% PFA.

Biopsy experiments were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Science, Szent István University (Ref: 23/B/2000) and complied with the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Reverse transcriptase-PCR

Total RNA was extracted (by using TRIzol Reagent, Gibco BRL-Life Technologies Inc., Gaithersburg, MD) from frozen mammary gland biopsy samples. Two micrograms RNA were reverse transcribed by using Moloney-murine leukemia virus (M-MLV) reverse transcriptase enzyme (Promega, Madison, WI) and the (dT)17-adapter primer as recommended by the manufacturer. PCR was performed to obtain a 134 bp long bovine FcRn alpha-chain specific amplicon of the cytoplasmic and 3′-untranslated region (914–1047 bp, NM_176657; Kacskovics et al. 2000). The amplified segment was separated by electrophoresis on 1% agarose gel and stained with ethidium bromide.

In situ *hybridization*

After overnight fixation, non-lactating mammary tissue samples were embedded in paraffin. Subsequently, 5 µm thick sections were cut and placed onto Superfrost slides. *In situ* hybridization was performed as described previously (Mayer et al. 2004). Briefly, an antisense fragment of the cytoplasmic and 3′-untranslated region (914 to 1280 bp; NM_176657; Kacskovics et al. 2000) from a bovine cDNA clone was DIG-dUTP labelled by a linear PCR method. After deparaffination, the sections were digested by proteinase K (Boehringer Mannheim, Mannheim, Germany), and postfixed with 4% PFA in order to stop the digestion. Subsequently, the specimens were washed and then the DIG-labelled probe was added (final concentration: 0·25 ng/µl). After the initial 3–5 min denaturation step at 94 °C, the ISH was carried out overnight at 45 °C on an *in situ* block. Detection was performed with DIG Nucleic Acid Detection kit (Boehringer Mannheim) according to the instructions of the manufacturer.

Immunohistochemical staining

Sections from biopsies were prepared similarly as completed on non-lactating mammary gland tissue samples by *in situ* hybridization. For immunohistochemistry, an affinity purified antiserum (raised against the peptide CLEWKEPPSMRLKAR representing the highly conserved 173–186 aminoacid residues of bovine FcRn alpha-chain plus an N terminal Cys for conjugation) was used at final concentration of 120 µg/ml. Non-lactating mammary gland and mammary biopsy sections were incubated with affinity-purified anti-FcRn at 4 °C overnight and for 1 h at

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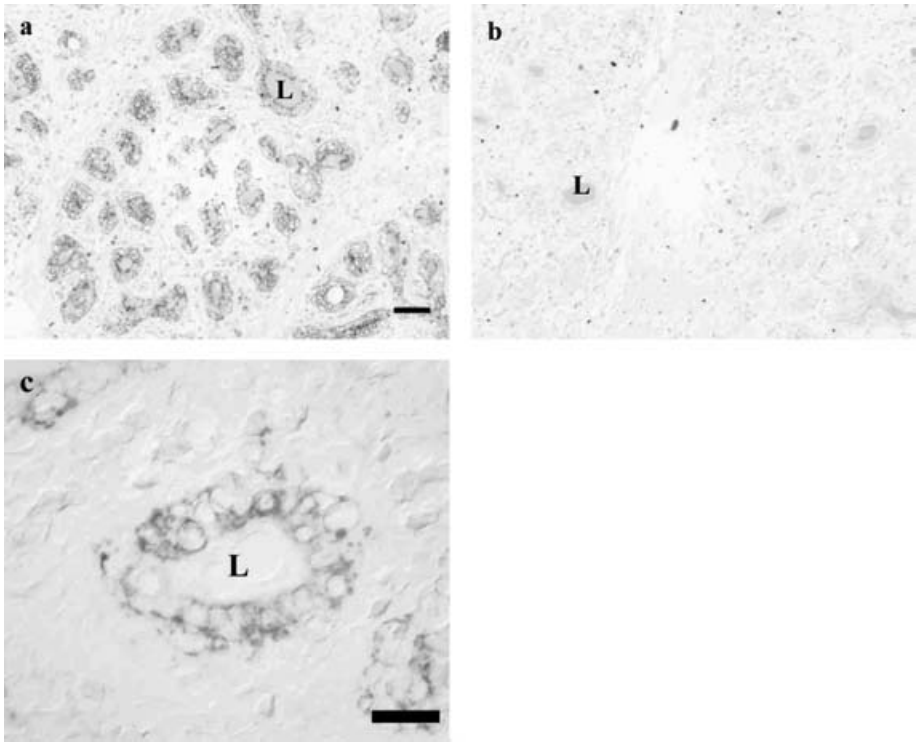

Fig. 1. *In situ* hybridization and immunohistochemistry on a non-lactating mammary gland section. (a) Mammary tissue was hybridized with an anti-sense bovine FcRn specific, digoxigenin-labelled probe, scale bar indicates 50 µm; (b) negative control hybridized with a sense probe derived from the same fragment; (c) immunostaining of non-lactating mammary gland with affinity purified anti-FcRn rabbit serum, scale bar indicates 20 µm. L, lumen of the acini.

room temperature and then with biotinylated goat anti-rabbit IgG for 30 min at room temperature. The secondary antibody was detected using peroxidase-labelled avidin (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The production of antisera and the immunohistochemical protocol have been published previously (Mayer et al. 2002).

Results and Discussion

In the frame of this study, we intended to analyse the expression and localization of the FcRn in the bovine mammary gland in non-lactating animals and also around parturition. First we analysed FcRn heavy chain expression in non-lactating mammary gland sections by *in situ* hybridization and immunohistochemistry. In order to detect the FcRn heavy chain transcripts, a digoxigenin-labeled probe complementary to a segment of the transmembrane, cytoplasmic and 3′-untranslated regions of the bovine FcRn alpha-chain cDNA was used, as it shows low degree of similarity to the bovine MHC class I molecule (Kacskovics et al. 2000). The anti-sense probe detected FcRn mRNA in the acinar and ductal epithelial cells. We noticed scattered staining in the interstitium (which could possibly be due to staining of macrophages), but could not detect FcRn expression in vascular endothelium (Fig. 1a). Sections hybridized with a sense probe derived from FcRn alpha-chain cDNA exhibited weak non-specific background signal (Fig. 1b). Immunohistochemistry using affinity purified anti-FcRn rabbit sera confirmed our *in situ* hybridization data as we could observe diffuse signal over acinar epithelial cells (Fig. 1c). Consistent with the *in situ* hybridization data there was no signal in endothelial cells (Fig. 1c). This finding is in good agreement with our previous result which indicated the presence of the bFcRn heavy chain transcripts in bovine non-lactating mammary gland (Kacskovics et al. 2000), and presents similar result to a more recent publication which demonstrated FcRn expression in the epithelial but not in the endothelial of the human mammary gland. Noteworthy, that the localization of the human FcRn was primarily intracellular, too (Cianga et al. 2003). Detecting FcRn in bovine mammary gland confirms earlier data, which demonstrated FcRn expression in other species (Cianga et al. 1999; Adamski et al. 2000; Kacskovics et al. 2000; Schnulle & Hurley 2003). After having established that our immunohistological method is reliable for detection of FcRn in bovine mammary tissue sections, a series of mammary gland biopsy sections collected around parturition were incubated with the anti-FcRn sera. We found the most significant change of the FcRn localization in the acini.

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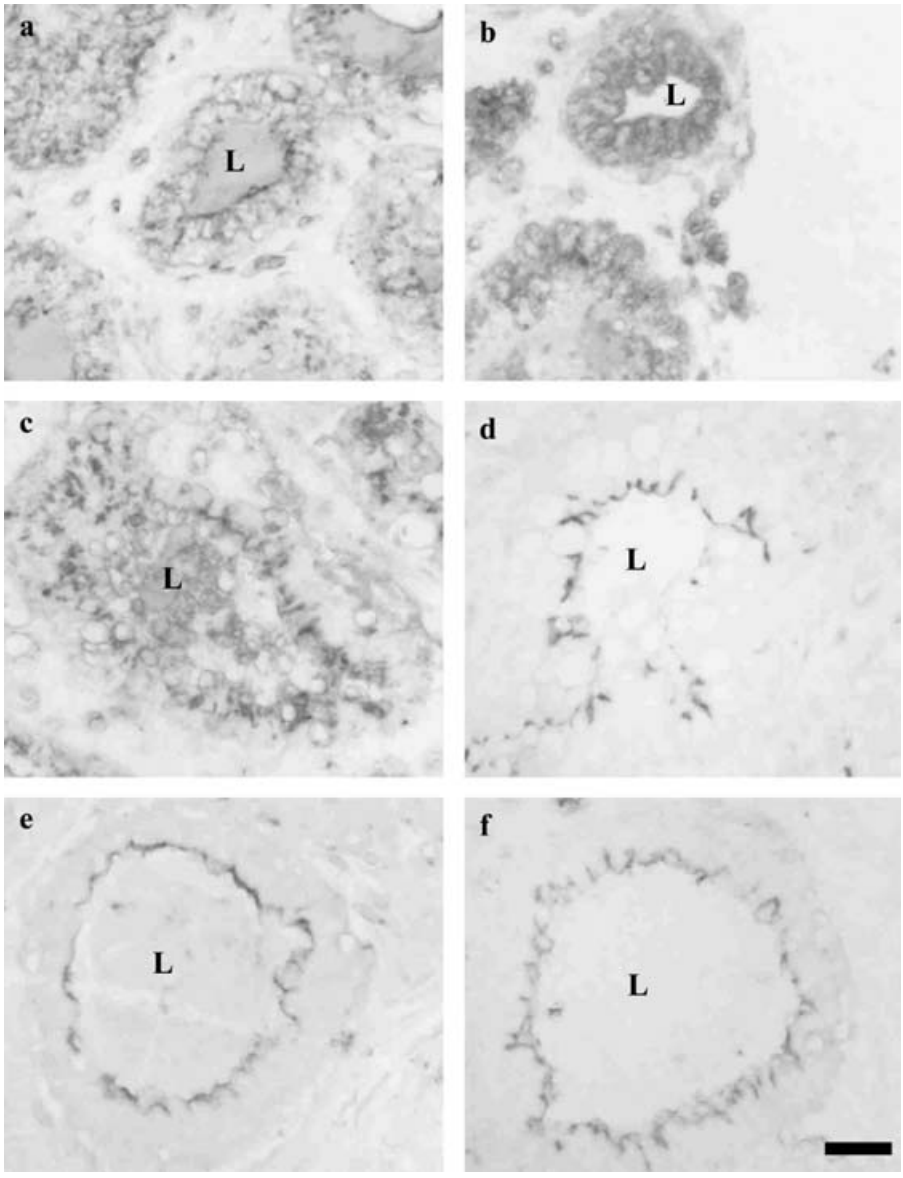

Fig. 2. Immunohistochemical analyses of bovine mammary gland biopsies around parturition. Diffuse FcRn expression was detected 14 (a) and 7 (b) days prepartum in the acinar cells. Transition from diffuse to apical staining was observed in these cells 1 day prepartum (c), whereas apical signal appeared on the day of calving (after parturition) (d), 7 (e) and 14 (f) days postpartum. L, lumen of the acini. Scale bar indicates 20 μm.

Biopsies 14 and 7 days prepartum showed diffuse signal in the acinar cells (Fig. 2a–b), however on the day of calving (after parturition) and 7 and 14 days postpartum the distribution of FcRn alpha-chain dramatically changed and only the apical/luminal sides of the epithelial cells were stained (Fig. 2d–f). Noteworthy, we found a transient state situation one day before parturition, where a continuous shift of FcRn from the intracellular to the apical side was visible (Fig. 2c). The presence of bovine FcRn alpha-chain transcripts was detected throughout the whole examined period (data not shown), which supports our immunohistological data and exclude the possibility of some staining artifact after parturition.

As concerning the endothelial cells, we could find weak FcRn expression before parturition which may increase the efficacy of the transport of IgG from circulation toward the epithelial cells. However, the lack of FcRn expression in endothelial cells can not completely inhibit IgG accumulation in the interstitium, since a previous study has indicated the presence of both IgG1 and IgG2 in the stroma of the mammary gland during lactation (Leary et al. 1982), when we could not detect FcRn in endothelial cells.

Immunohistochemical data around parturition in the cows underlines our previous results in ewes (Mayer et al. 2002). Based on these findings, and given that we have

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also found FcRn expression in other mucosal epithelial cells which have been previously shown to secrete IgG1 (Mayer et al. 2002; Mayer et al. 2004), along with the fact that FcRn is considered as the only receptor able to transport monomeric IgG through polarized epithelial cells (reviewed in Rojas & Apodaca 2002), it is tempting to speculate that FcRn secrete IgG1 at these sites in ruminants.

However, it should be mentioned, that FcRn was originally considered as a receptor transporting IgG from the apical to basolateral direction in rodents and human (Simister & Mostov 1989; Story et al. 1994). Furthermore, previous analysis suggested that in the lactating mice FcRn appears to play a role in recycling rather than secreting IgG in the mammary gland (Cianga et al. 1999). Since then much evidence has been accumulated indicating significant basolateral-apical transport even in these species (Dickinson et al. 1999; Ramalingam et al. 2002; Schlachetzki et al. 2002; Yoshida et al. 2004). These findings point to the fact that the FcRn mediated IgG transcytosis is a cell type and possibly a species specific process, hence it can not be excluded that FcRn in the bovine mammary gland secretes IgG into the colostrum/milk rather than recycles it to the circulation. This question emphasizes the need of an established polarized monolayer system that allows studies on FcRn mediated IgG transcytosis in cattle.

Our ongoing experiments, in collaboration with others, are focused on the binding affinity of the bovine IgG1, IgG2 and IgG3 to the bovine FcRn. We also study the role of the FcRn in endothelial cells both in the mammary gland which may contribute to the IgG secretion into colostrum and also systematically, where it may regulate IgG homeostasis, as is the case in rodents and human (reviewed in Lobo et al. 2004). The receptor which is involved in IgG1 secretion in the bovine mammary gland is under strict hormonal regulation (Barrington et al. 1999, 2001). In order to better understand how the expression of the FcRn genes is regulated around calving we analysed its promoter in reporter gene assays. Furthermore, we created and started to characterize three transgenic mouse lines, which carry the bovine FcRn alpha-chain on a BAC clone (Bender et al. 2004). The BAC construct was selected for this purpose because it is expected to ensure high level and position-independent expression in transgenic animals. Preliminary RT-PCR and Northern analyses revealed bovine FcRn alpha-chain expression in the intestine and liver of newborn transgenic animals. In summary, our data revealed that FcRn is expressed in the bovine acinar epithelial cells of the mammary gland and shows different intracellular localization during colostrogenesis, lactogenesis, lactation and in non-lactation, where it is hypothesized to mediate IgG transfer into colostrum and later into milk. Since we do not have information if the altered localization of the FcRn receptor does influence IgG transport during these phases of lactation, further studies are awaited to clarify this.

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