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Modulation of Expression of mRNA encoding for Proteases and Immunological Factors in the Bovine Mammary Gland

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CONTENT

ABBREVIATIONS

αLA	alpha lactalbumin	(m)RNA	(messenger) ribonucleic acid
Ab	antibody	n	number
bp	base pairs	OD	optical density
cDNA	complementary deoxyribonucleic acid	PA	plasminogen activator
CP	crossing point	PAI	plasminogen activator inhibitor
DMEM-F12	Dulbecco's modified Eagle's medium F12	pIgR	polymeric immunoglobulin receptor
ECM	extracellular matrix	PMN	polymorphonuclear neutrophil leukocytes
FcRn	neonatal Fc receptor	qPCR	quantitative polymerase chain reaction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	SEM	standard error of the mean
GM-CSF	granulocyte monocyte-colony stimulating factor	TGF	transforming growth factor
IFNγ	interferon gamma	TIMP	tissue inhibitor of metalloproteinases
Ig	immunoglobulin	TNF	tumor necrosis factor
IGFBP	insulin growth factor binding protein	tPA	tissue-type plasminogen activator
IL	interleukin	uPA	urokinase-type plasminogen activator
IMI	intramammary infection	(u)PAR	(urokinase-type) plasminogen activator receptor
kDa	kilo dalton		
LPS	lipopolysaccharide		
MEC	mammary epithelial cell		
M-MLV	moloney murine leukemia virus		
MMP	matrix metalloproteinases		

ABSTRACT

During its developmental time course, the bovine mammary gland undergoes critical structural and physiological changes, especially at morphogenesis and involution. Moreover, the udder has to cope with physiological conditions that impair greatly its immune defense and make it highly susceptible to new infection. The hypothesis of work was that a possible relation between matrix proteases which are implied in structural changes and cytokines which coordinate the immune system could explain these phenomena. Therefore the transcriptional activity of various serine proteases, metalloproteases and important proinflammatory cytokines has been studied throughout all the developmental cycle and compared. In addition, the mammary cell types which could be involved in the production of these factors have been investigated. Immunohistochemistry has been used for the proteases while a model has been developed to determine the part played by the mammary epithelial cells in the production of cytokines.

The transcripts of matrix proteases and cytokines showed a clear upregulation during mammogenesis and late involution and were strongly downregulated during lactation and early involution. Thus, both types of factors have a common pattern of gene expression and this pattern was correlated with the biological function of the gland. The induction of matrix proteases coincided with the up regulation of cytokines and this phenomenon closely paralleled the stages when the gland underwent structural changes. On the other hand, down regulation of cytokines and proteases corresponded to the periods where infection incidence was increased.

The leukocytes are the common link between these factors. Cytokines are involved in leukocyte homing and activation into the mammary gland. One property of leukocytes is to induce expression of proteases when activated. It is proposed that an upregulation of cytokines during mammogenesis and involution induce the migration into the gland of a large number of leukocytes. These activated leukocytes likely produce proteases and it is this induction together with the one of the ductal and endothelial cells that explain the increase of proteases transcripts. At the onset of lactation and in early involution, cytokines being down-regulated, the number of leukocytes migrating into the gland diminished; hence a concomitant downregulation of matrix proteases and weaknesses in the immunological protection.

ZUSAMMENFASSUNG

Während der verschiedenen Entwicklungsstadien vollziehen sich in der Milchdrüse der Kuh entscheidende strukturelle und physiologische Veränderungen, insbesondere während der Morphogenese und der Involution. Außerdem hat das Euter mit physiologischen Bedingungen zu kämpfen, die seine Immunabwehr beeinträchtigen und es für neue Infektionen anfällig machen. Die Arbeitshypothese war, dass ein möglicher Zusammenhang zwischen Matrixproteasen, die an strukturellen Veränderungen beteiligt sind, und Zytokinen, die das Immunsystem koordinieren, diese Phänomene erklären könnte.

Deshalb wurde die Regulation der Genexpression einiger wichtiger Vertreter dieser beiden Faktoren untersucht. Die Expression der Transkripte von Metallo-und Serinproteasen und einiger pro-inflammatorischer Zytokine während des gesamten Entwicklungszyklus wurde mit quantitativer RT-PCR nachgewiesen. Zusätzlich wurde untersucht, welche Zellen der Milchdrüse an der Synthese dieser Faktoren beteiligt sein könnten. Immunhistochemie wurde für die Proteasen verwendet, während ein Modell zur Bestimmung der Rolle der Euterepithelzellen bei der Synthese der Zytokine entwickelt wurde. Die Transkripte der Matrixproteasen und Zytokine zeigten eine klare Hochregulation während der Bildung der Milchdrüsen und der späten Phase der Involution. Beide Faktoren zeigten also ein gemeinsames Muster in der Genexpression, das mit der biologischen Funktion der Drüse korreliert werden konnte. Die Induktion der Matrixproteasen fiel mit der Hochregulation der Zytokine zusammen und diese verlief parallel zu den Phasen, in denen die Drüse strukturelle Veränderungen erlebte. Andererseits korrespondierte die Herabregulation der Zytokine und Proteasen mit Perioden, in denen die Infektionshäufigkeit angestiegen war. Die Leukozyten repräsentieren normalerweise die Verbindung zwischen beiden Faktoren. Zytokine sind an der Steuerung und Aktivierung der Leukozyten in der Milchdrüse beteiligt. Eine Eigenschaft von aktivierten Leukozyten ist die Induktion der Expression von Proteasen. Möglicherweise induziert eine Hochregulation der Zytokine während der Mammogenese und der Involution die Migration einer großen Zahl von Leukozyten in die Milchdrüse. Diese aktivierten Leukozyten bilden wahrscheinlich Proteasen und es ist diese Induktion zusammen mit der Induktion der Ductus- und Endothelzellen, die den Anstieg der Protease-Transkripte erklärt. Zu Beginn der Laktationsperiode und in der frühen Phase der Involution werden die Zytokine herabreguliert und die Anzahl der Leukozyten, die in die Milchdrüse

migrieren wird verringert. Deshalb werden eine gleichzeitige Herabregulation der Matrixproteasen und eine Immunschwäche ausgelöst.

1 INTRODUCTION

1.1 The mammary gland

Mammary glands are accessory reproductive organs that secrete milk as a source of nutrition for the offspring. Lactation is an important part of the reproductive strategy of all mammals. The digestive apparatus of newborn mammals is not sufficiently developed and milk matches with this limited digestive capabilities. Milk has a high caloric value and provides a balanced nutrient source as well as a range of protective and bioactive factors.

Mammary glands are apocrine, tubulo-alveolar skin glands that are located on the ventral surface of the animal. The bovine udder is composed of four separate glands or "quarters". Each gland has its own secretory tissue and cavities and is drained by a separate teat (Wattiaux 1999).

The mammary gland consists of two main tissue types: secretory or parenchymal tissue, and the mammary fat pad or stromal tissue.

The stromal tissue includes fibrous connective tissue of the parenchymal tissue and the fatty tissue of the fat pad. The fat pad is required for correct development of the parenchymal components as the ducts branch and grow into the fat pad. The fat pad of the ruminant mammary gland consists primarily of adipocytes and fibroblasts, as well as mast cells, endothelial cells, and lymphatic cells. Although the fat pad provides structural support to the gland, it also plays an integral role in regulating mammary development. The mammary fat pad produces numerous growth factors and extra-cellular matrix components, which may directly stimulate mammary epithelial proliferation, mediate the effects of systemic hormones, interact with developing epithelium to modulate gene expression, or alter responsiveness to mammogenic stimuli (Hovey et al. 1999).

The secretory tissue is made up of alveoli (acini) settled in a basement membrane. The alveolus is the basic milk secreting structure of the lactating mammary gland and corresponds to a blind-sac consisting of a single layer of secretory epithelial cells (alveolar cells) surrounding a lumen. In turn the epithelial cells are surrounded by a diffuse layer of contractile myoepithelial cells involved in milk ejection (McManaman & Neville 2003).

The fundamental microscopic organization of the mammary tissue is essential to the lactation function of that tissue. If the histological organization is disrupted for any reason, then the tissue ability to produce milk is compromised.

1.2 Developmental cycle

Mammals reproduce more than once, and therefore, lactate more than once. The mammary gland is one of a relatively few structures of the body which can repeatedly undergo cycles of structural development, functional differentiation, and regression. First, the mammary gland develops structurally (mammogenesis); the greatest degree of structural development of the mammary gland occuring during pregnancy (Knight & Sorensen 2001). Then, milk synthesis and secretion begins (lactogenesis); the process of lactogenesis coincides with the formation of colostrum and occurs in coordination with parturition. Milk secretion is maintained until the young no longer needs milk or milk is no longer removed from the gland (galactopoiesis). Then the mammary gland undergoes involution and the cycle can start again with a new pregnancy.

1.2.1 Mammogenesis

Mammogenesis refers to the development of mammary gland structures. This is most often assumed to occur during pregnancy, however mammary development begins when the animal is an early fetus and proceeds beyond initiation of lactation. During puberty, in response to ovarian hormone stimulation and the actions of growth hormone (Topper & Freeman 1980), the ductal epithelium undergoes active proliferation. It ramifies into the surrounding supporting tissue of the fat pad to establish a ductal network that will ultimately support pregnancy-associated alveolar development. In ruminants, the ductal growth is dichotomously branched in association with the formation of terminal lobular units (Hovey et al., 1999). Once established, the ductal network is then positioned to undergo lobulo-alveolar development and differentiation in response to the hormonal stimulation of pregnancy. Pregnancy is often considered to be the period of most extensive mammary growth. It is during this period that the mammary gland epithelium experiences its greatest and most rapid phase of proliferation.

A strong correlation exists between the number of secretory cells and milk yield (Tucker 1966); consequently optimal parenchymal proliferation and development is essential for maximizing future milk yield. Prepubertal and pubertal ductal proliferations provide a foundation for the later development of secretory alveoli during gestation (Akers et al. 1990) and effects on mammary development during this time may later be translated into milk yield.

1.2.2 Lactogenesis

Lactogenesis is the term meaning the initiation of lactation. This is the process of functional differentiation which mammary tissue undergoes when changing from a non-lactating to a lactating state (Sordillo & Nickerson 1988). This process is normally associated with the end of pregnancy and occurs around the time of parturition. Because lactogenesis is particularly dependent upon a specific set of hormones called the Lactogenic Complex of Hormones, mammary tissue from most states of the non-lactating mammary gland also can be made to undergo some degree of lactogenesis by administration of high amounts of those hormones, even in non-pregnant animals (Tucker 2000).

1.2.3 Galactopoiesis

Galactopoeisis is the maintenance of lactation once it has been established. Two key interrelated components contribute to the maintenance of lactation, galactopoietic hormones and removal of accumulated milk. Much of the fundamental knowledge on galactopoietic hormones comes from classical studies demonstrating that inhibition of hormone secretion will inhibit milk production. Conversely, administration of additional amounts of galactopoietic hormones during lactation can enhance milk production, depending on the species, stage of lactation, and particular hormone (Akers 2002). The most widely known example of this has led to the common practice of administration of bovine somatotropin (or bovine growth hormone) to lactating dairy cattle resulting in increased milk yields (Bauman 1999; Capuco et al. 2001)

1.2.4 Involution

Involution begins with the cessation of periodic milk removal, either by drying off a cow or by weaning the young. Cessation of milk removal leads to rapid changes in the mammary tissue. This period is characterized by cell loss that starts during lactation. In spite of continued milk removal, milk yield in dairy cattle declines as lactation progresses. Mammary tissue function declines after peak lactation and this is at least in part due to a decrease in mammary cell number (Knight & Peaker 1984; Wilde & Knight 1989). The cell loss during the declining phase of lactation in the goat and cow apparently is the result of programmed cell death, also called apoptosis (Quarrie et al. 1994; Wilde et al. 1997). This process of cell loss does not seem to be as dramatic as that observed in the mouse. Despite the loss of cells, bovine mammary alveoli retain general structural integrity throughout involution (Holst et al. 1987). Involution-associated ultrastructural changes in bovine mammary cells begin within 48 hours after cessation of milk removal. The most apparent change is the formation of large stasis vacuoles in the epithelial cells, formed largely as a result of intracellular accumulation of milk fat droplets and secretory vesicles (Hurley 1989). Alveolar lumenal area declines during this period, while interalveolar stromal area increases. By day 28 of involution the collapsed alveolar structures remaining are considerably smaller than during lactation, with a very small lumen. General alveolar structure is maintained throughout involution in the cow, a period characterized by loss of secretory activity, decreased size of alveoli, and renewal of senescent epithelial cells (Capuco & Akers 1999). Involution is essential for proper re-development of the gland and for optimizing milk yield during the next lactation (Swanson 1965; Sorensen & Enevoldsen 1991). Theses changes that occur in the mammary gland during the dry period greatly influence mammary cell proliferation and mammary function in the subsequent lactation.

1.3 Extracellular matrix-degrading proteases

These different stages of normal mammary development require a finely controlled degradation and remodeling of the extracellular matrix (ECM). Several proteases are implicated in the turnover of the ECM. Among them, the matrix metalloproteinases (MMPs) are considered the key enzymes in this process.

Since ECM proteases have been associated with tumor growth and metastasis

(Egeblad & Werb 2002), considerable body of research exists concerning the expression and role of the MMPs and inhibitors in mammary gland in murine models (Benaud et al. 1998; Rudolph-Owen & Matrisian 1998; Green & Lund 2005).

Another protease family, the plasminogen activator (PA) system plays also an important role in degrading the ECM. Working hand in hand together, the MMPs and the PA systems can degrade all ECM components.

1.3.1 Matrix metalloproteinase family

The matrix metalloproteinases (MMPs) comprise a family of zinc-dependent endopeptidases that consist of at least 25 proteolytic enzymes. They are synthesized as inactive zymogens (proenzymes) and activated by proteinase cleavage.

As a family, MMPs can degrade most components of the extracellular matrix (ECM) but their targets include also other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor–binding proteins, cell surface receptors, cell-cell adhesion molecules (Table 1). Thus MMPs are able to regulate cell behavior in several ways (Sternlicht et al. 2000). Regulation of most of the MMP family members is tightly controlled. The activity of the protases is regulated by the balance between MMPs and their natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Activation of the proenzymes and the function of the mature MMPs can be inhibited by the binding of TIMPs.

Table 1. Summary of the nomenclature and actions of the studied MMPs.

MMP	Common name	Collagen	Additional substrates
MMP-1	Collagenase-1, interstitial collagenase, fibroblast collagenase	I, II, III, VII, VIII, X, XI	Aggrecan/gelatin/fibrin/MMP-2/ MMP-9/Pro-TNF-α/IGFBPs/IL-1ß
MMP-2	72kDa gelatinase, gelatinase A, 72 kDa type IV collagenase	I, III, IV, V, VII, X, XI	Aggrecan/elastin/fibronectin/ gelatin/laminin/IGFBPs/IL-1ß/MMP-9/MMP-13/ProTGF-ß/ProTNF-α /substance P
MMP-14	Membrane type 1 metalloproteinase, MT1	I, II, III, IV	Aggrecan/elastin/fibronectin/ gelatin/laminin/MMP-2/MMP-13/ProTNF-α
MMP-19	RASI-1	I, IV	Fibronectin/gelatin

1.3.2 Plasminogen activator family

The plasminogen activator (PA) system belongs to the serine protease family, the second main family of matrix-degrading proteases. It consists of plasminogen, the inactive form of plasmin, the tissue and urokinase-type plasminogen activator (tPA and uPA), a cell surface plasminogen activator receptor (uPAR) and two plasminogen activator inhibitors (PA-1 and PA-2).
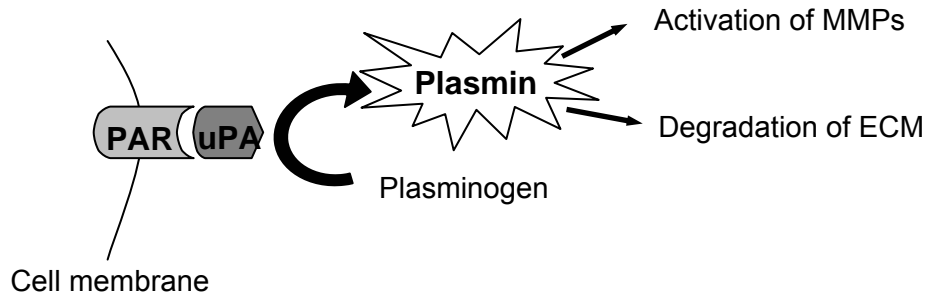
Figure1. *Production of plasmin by the plasminogen activator system.*

tPA and uPA transform plasminogen into its active form plasmin in the extracellular space.

uPA is able to bind the uPAR to build a stable complex whose purpose is thought to focus the plasmin activity on the cell surface. Plasminogen activation is limited by the action of the PAIs.

Plasmin directly degrades matrix proteins such as fibrin and laminin and also activates MMP zymogens (Sternlicht & Werb 2001).

1.4 Mastitis susceptibility

The inflammation of the mammary gland is called mastitis. It is usually developed in response to intramammary bacterial infection, although many other microorganisms including fungi, mycoplasmas and viruses can cause inflammatory reactions (Bradley 2002).

Mastitis can occur in the mammary gland at all stages of the cow's life but two particular periods in the lactation cycle present increased incidence of mastitis.

1.4.1 Dry period

The early dry period (first two weeks) is the time of the highest incidence of new intramammary infection (IMI).

A number of factors contribute to the elevated susceptibility to infection during the early dry period: as the milk is no longer periodically removed from the gland, there is an accumulation of a large volume in the gland (for the first few days) and milk is an excellent growth medium for bacteria. In addition teat-end disinfections are stopped and leakage from the teats may have a potential influence.

Moreover, the other defense mechanisms represented by the somatic cells (leukocytes) have to cope with some difficulties as they have a tendency to ingest milk fat, casein and debris. This decreases their phagocytic capability, making them less efficient (Paape et al. 1992). However, immunoglobulins are increasing, but their level is still low.

1.4.2 Prepartum period

This phase of the dry period marks the transition from the non-lactating state to the lactating state. It starts at about 3 to 4 weeks prepartum. The selective transport of IgG1 is a major activity of the epithelial cells in the two weeks prior to parturition (Guidry et al. 1980). Concentrations of the major milk components start to increase at about two weeks prepartum, and then increase markedly 3-5 days prepartum. The potential for IMI is increased again because the gland is undergoing the opposite changes from those during early involution since fluid accumulates in the udder as calving approaches without being removed (Fox et al. 1995). Relatively few leukocytes are present in the secretion and they are confronted with increasing milk fat and casein concentrations

1.4.3 Inflammation actors

Inflammation is the cellular reaction of innate immunity against invading microorganisms or their components such as lipopolysaccharide (LPS), toxin exposure or cell injury. During mastitis, the inflammatory response is initiated by macrophages together with epithelial cells by production and release of tumor necrosis factor alpha (TNF-α), interferons (IFN) and interleukins (IL) (Sordillo 1997).

These cytokines elicit a rapid influx of leukocytes (neutrophils, mononuclear phagocytes) from peripheral blood through the mammary epithelium into milk and activate them to eliminate the infectious agent (Persson et al. 1993).

IL-1, IL-6, IL-8, TNF-α and IFN-γ are particularly known to be important to orchestrate the acute phase response. They contribute to the febrile response, the induction of hepatic synthesis of acute phase proteins, the leukocyte recruitment, changes in vascular permeability (Riollet et al. 2000). Of these, IL-1 (α and β) and TNF-α are extremely potent inflammatory molecules (Feghali & Wright 1997; Bannerman et al. 2005). Others like the granulocyte monocyte-colony stimulating factor (GM-CSF) are known to cause a rapid increase in the number of neutrophils and macrophages when infused in a recombinant form into the gland (Wedlock et al. 2004).

However, little is known about the role played by MEC in their recruitment or production of cytokines. Whether the MEC can produce or enhance action of cytokines has become lately an object of interest.

2 OBJECTIVES

The mammary gland undergoes dramatic morphological and functional changes during its development, lactation and subsequent involution cycles characterized by successive phases of growth, morphogenesis, differentiation, high metabolic activity and apoptosis. At the ultrastructural level this includes dramatic changes in tissue architecture, involving ductal epithelial branching and morphogenesis, invasion of tissue compartments, vascularization and subsequent organized remodeling. These structural changes must take place accurately to allow an optimal performance of the udder throughout one cycle. On the other hand, immune cells that are present throughout all the developmental cycle are increased at specific stages although the udder has to cope at least twice with physiologic conditions that impair its defense mechanisms and make it highly vulnerable to intramammary infection. This study was designed to determine which relationship exists between matrix proteases and immunological mediators in the bovine mammary development cycle and the possible association with the phenomenon just mentioned.

3 MATERIAL AND METHODS

Material and methods are described in detail in the attached papers (Appendix). A general description with special comments is presented in the following sections.

3.1 Animals

Two sets of animals were used for the present work.

The first set encompasses 38 non-pregnant German Fleckvieh and Holstein Frisian cows. They were selected at well-defined stages of development (Table 2) and small pieces of mammary glands tissue were taken maximum 20 minutes after slaughter. Tissue was frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Table 2. Classification of the first set of investigated animals.

Mammogenesis	i	Ductal development; non-pregnant heifers (approx. 18 months old)	n=4
Lactogenesis	ii	Onset of milk secretion; 4-8 days post partum	n=5
Galactopoiesis		Maintenance of milk secretion	
	iii	Peak lactation; 2-8 weeks post partum	n=5
	iv	Mid lactation; 4-5 months	n=4
	v	Late lactation; 8-12 months	n=4
Involution		Dry off	
	vi	Early involution; 24-48h	n=5
	vii	Mid involution; 96-108h	n=3
	viii	Late involution; 14-28 days	n=8

The other set of material includes explants taken from mammary glands of eight lactating cows. An incision was made in one of the quarter through the connective tissue to expose the secretory tissue and the explants were taken with a biopsy tool within 20 min after slaughter.

3.2 Explant culture

The explants were taken off the mammary gland with a biopsy tool. This results in a highly rapid and reproducible sampling since the dimensions of the needle are

constant. The tissue pieces obtained were very slim and long, similar in shape and weight, having the advantage to offer a large contact surface to absorb the medium. An additional asset is that they conveniently did not require further manipulation. Explants were transferred in 12 well plates, one explant per well, in 1 ml of medium, at 37°C. The basal medium consisted of DMEM-F12 medium supplemented with insulin (10 µg/ml), hydrocortisone (0.5 µg/ml), penicillin G (100 µg/ml) streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). Explants were stimulated with LPS at a final concentration of 10 µg/ml for 3 hours or 6 hours. Non treated explants served as control. All chemicals were purchased from Sigma Chemicals, Seelze, Germany.

3.3 Total RNA extraction and first strand cDNA synthesis

RNA was extracted whether with an adapted guanidinium thiocyanate/phenol method (Plath et al., 1997) or with Trifast isolation reagent (Peqlab, Erlangen, Germany). The quantity of the extracted RNA was determined with a photometer at a wavelength of 260 nm, corrected by the 320 nm background absorption. RNA integrity was verified by the OD_{260}/OD_{280} nm absorption ratio which was consistently above 1.80.
Total RNA was reverse transcribed to cDNA in a volume of 40 µl containing 1 µg of total RNA and 200U of M-MLV reverse transcriptase (Promega, Madison WI, USA).

3.4 Quantitative PCR

Quantitative PCR was performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) by a standard protocol in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). PCR primers used to amplify the transcripts are described in table 3. Crossing point (CP) values were acquired using the comparative quantification method (Rotor-Gene software version 5.0, Corbett Research). Relative mRNA levels were calculated by normalization of the CP (= ΔCP) of the target gene to a single reference gene (β-actin) or the arithmetic mean of the CP of several reference genes (β-actin and GAPDH). In some cases, the mRNA expression was compared to a control and presented then as ΔΔCP.

Table 3. Sequences of the PCR primers.

Genes		Sequence (5'→3')	Product size (bp)	Accession number
ß-actin	For	AACTCCATCATGAAGTGTGACG	234	U39357
	Rev	GATCCACATCTGCTGGAAGG		
GAPDH	For	GTCTTCACTACCATGGAGAAGG	197	U85042
	Rev	TCATGGATGACCTTGGCCAG		
MMP-1	For	CAAACCAGGTGCAGGTATCG	232	NM174112
	Rev	AAGGTCCGTAGATGGCCTG		
MMP-2	For	CCCAGACAGTGGATGATGC	248	NM174745
	Rev	TTGTCCTTCTCCCAGGGTC		
MMP-14	For	ACTTGGAAGGGGGACACC	236	AF144758
	Rev	AGGGGGCATCTTAGTGGG		
MMP-19	For	TTTCAAGGGGGACTATGTGTG	240	X92521
	Rev	CAATAGAGAGCTGCATCCAGG		
TIMP-1	For	CATCTACACCCCTGCCATG	231	AF144763
	Rev	CAGGGGATGGATGAGCAG		
TIMP-2	For	GGGTCTCGCTGGACATTG	256	AF144764
	Rev	TTGATGTTCTTCTCCGTGACC		
tPA	For	GGGGAAGCACAACCACTG	263	X85800
	Rev	AGCTGATCAGGATCCCCC		
uPA	For	TGCAGCCATCTACAGGAGG	240	X85801
	Rev	TGGTGAGCAAGGCTCTCC		
uPAR	For	TGTTTCCAGAAACCGCTACC	234	S70635
	Rev	AAGTGGAAGGTGTGGTTGTTG		
PAI-1	For	CAGCGACTTACTTGGTGAAGG	231	X52906
	Rev	TCCAGGATGTCGTAGTAACGG		
αLA	For	ACCAGTGGTTATGACACACAAGC	233	M18780
	Rev	AGTGCTTTATGGGCCAACCAGT		
IL1-β	For	TTCTCTCCAGCCAACCTTCATT	214	M37211
	Rev	ATCTGCAGCTGGATGTTTCCAT		
TNF-α	For	TAACAAGCCGGTAGCCCACG	277	AF011926
	Rev	GCAAGGGCTCTTGATGGCAGA		
IL-6	For	GCTGAATCTTCCAAAAATGGAGG	188	NM173923
	Rev	GCTTCAGGATCTGGATCAGTG		
IL-8	For	ATGACTTCCAAGCTGGCTGTTG	200	BC103310
	Rev	TTGATAAATTTGGGGTGGAAAG		
GM-CSF	For	ATGTGGCTGCAGAACCTGCTTC	316	U2385
	Rev	CTGGTTCCCAGCAGTCAAAGGG		
FcRn	For	TGAACGGCGAGGAGTTCATG	288	AJ313190
	Rev	GCTCAGGTGGGTAGAAGGAGA		
pIgR	For	ATGTGAGCCTGGAGGTCAGCCA	353	AJ313189
	Rev	CTCCAGCACCTGGAGGTCAA		

3.5 Immunohistochemistry

The immunochemistry was carried out in cooperation with Prof. Sinowatz (Institute of Veterinary Anatomy, Munich, Germany).

All proteins were localized on paraffin wax embedded mammary gland tissue previously fixed in Bouin's solution.

For many of the matrix proteases there are no commercially available antibodies (Ab). Therefore immunochemistry was performed for only few of the proteases investigated with qPCR. The primary Ab were from mouse (anti-MMP-2) or rabbit (anti-MMP-1, anti-MMP-14, anti-TIMP-2), all raised against human protein epitopes. Their reactivity against bovine had not been tested so far. The second Ab was biotinylated and detected with the streptavidin-biotin horseradish peroxidase complex technique.

4 RESULTS AND DISCUSSION

Determining which role ECM-degrading proteases and cytokines may play during normal development and functioning of bovine mammary gland requires having a basal level of information on these factors in the normal biology of the gland, characterization studies were therefore undergone. Important information is brought by the study of the gene expression of the target factors. The expression pattern of major members of the MMP and serine protease families as well as their inhibitors on the one hand and of proinflammatory cytokines on the other hand has been investigated with qPCR at several time points which cover all stages of mammary development (Appendix 1). This characterization study showed that almost all of the tested genes for matrix proteinases and their inhibitors are expressed in bovine mammary gland and change according to the stage of development or function of the mammary gland. Although some studied proteases such as MMP-1 and tPA did not show any regulation, most of them exhibit changes in their expression. MMP-2, -14, -19, TIMP-1, -2, uPA, uPAR and PAI transcripts are upregulated during the mammogenesis and lactogenesis phases, decrease during galactopoiesis to be markedly downregulated during early involution. Then in late involution, they recover the level they had during mammogenesis. As expected matrix proteases are expressed during processes involving tissue remodeling events such as ductal development and involution and certainly during lobulo-alveolar development that occurs at pregnancy.

ECM proteases have been investigated intensively in mouse since a correlation with tumor progression has been established (Lochter et al. 1998; Deryugina & Quigley 2006; VanSaun & Matrisian 2006). One intriguing observation is that several discrepancies exist between the bovine and the murine model. Murine ECM proteases are strongly up-regulated during morphogenesis of the mammary gland as well as in the second phase of involution but unlike the bovine, inhibitors are downregulated at these same periods (Figure 2) (Rudolph-Owen & Matrisian 1998; Fata et al. 2004). Moreover, the main producers of ECM proteases in mouse are the MEC and certainly due to a short cycle, proteases are upregulated with much more magnitude (Wiseman et al. 2003). In bovine, proteases inhibitors are regulated in the same way than the proteases themselves. These data suggest that the activity of matrix proteases in mouse is rather regulated by the downregulation of protease

expression together with the presence of inhibitors (Talhouk et al. 1992) while in bovine, it is rather a subtle ratio between proteases and their inhibitors that controls the protease activity. The alteration of mRNA levels is probably not the most important factor in the regulation of the activity of protease in bovine and the process is likely a very complex system. A possible regulation at the translational or post-translational levels arose from our study.
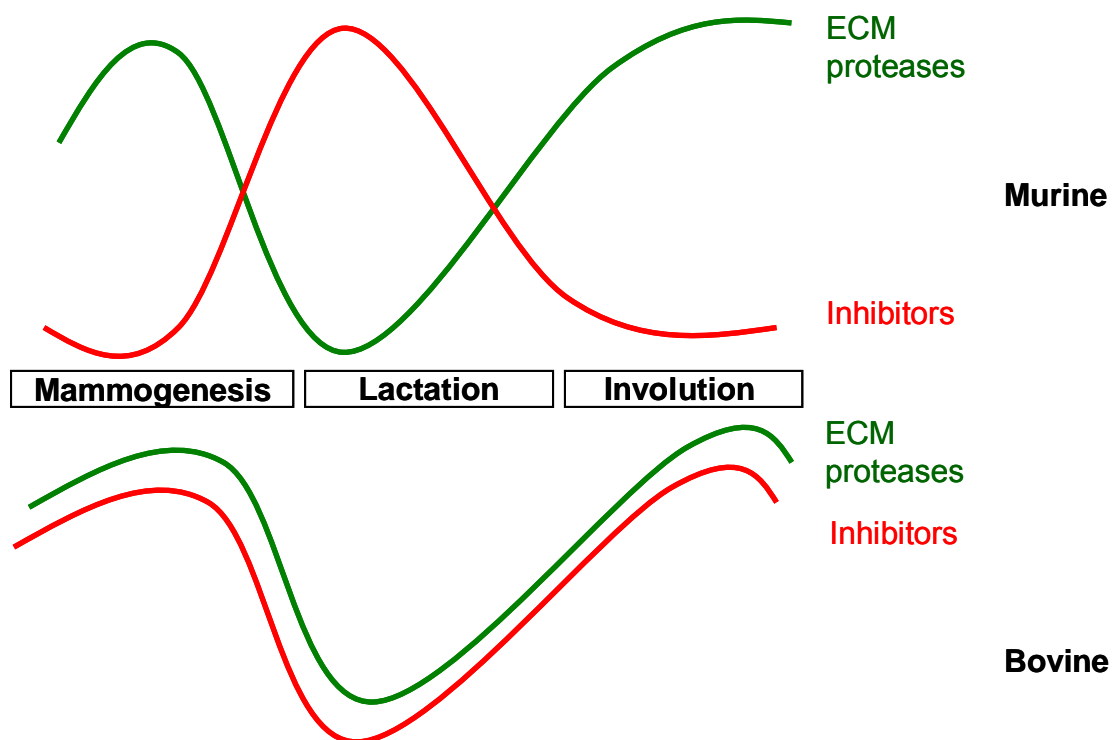
Figure 2. *Schematization of the expression profiles of matrix proteases and their inhibitors during the development of the mammary gland in murine and bovine models.*

The gene expression for the proinflammatory cytokines IL-1β and TNF-α was examined during the normal development of bovine mammary gland. The same samples than for the matrix proteases experiment were used in order to compare exactly with the expression of the proteases. Proinflammatory cytokines are also detectable at all stages of development of healthy mammary gland and are moreover regulated in the same way than matrix proteases (Figure 3) exhibiting maximum of gene expression at mammogenesis and late involution and being strongly

downregulated in early involution. The analysis of GM-CSF expression completes the study of proinflammatory cytokines (Appendix 3). The question to know whether this cytokine is a prevalent one for the development of mammary gland as well as for the defense of mammary gland in case of infection arose. Transcription was then examined in healthy and mastitis mammary gland of lactating cows. It seems there is a trend of up regulation of this cytokines during the two critical stages i.e. mammogenesis and involution as well as during mastitis but we could not determine a significant change in the expression of this cytokine (Figure 3c).
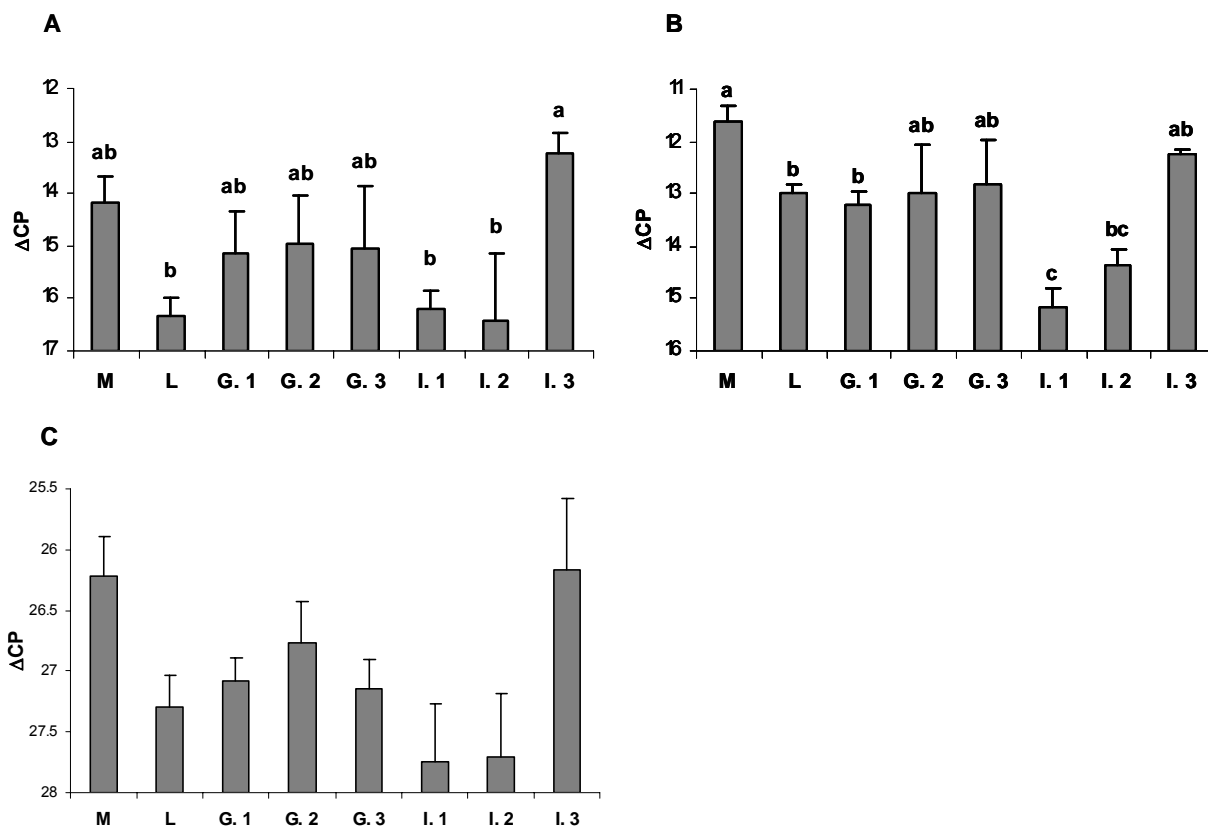

Figure 3. Expression of IL1-β (A), TNF-α (B) and GM-CSF (C) mRNA in bovine mammary gland during its development. M: pubertal mammogenesis (18-month-old heifers, n = 4); L: lactogenesis (onset of secretion during days 4-8 days post partum, n = 5); G.1: galactopoiesis peak lactation (2-8 weeks post partum, n = 4); G.2: galactopoiesis mid lactation (4-5 months); G.3: galactopoiesis late lactation (8-12 months); I.1: early involution (24-48h after end of last milking); I.2: mid-involution (96-108h); I.3: late involution (14-28 days). Results represent $\Delta CT \pm SEM$ from n = 3-8/group. Different superscript letters indicate significant different means (P < 0.05). No statistically significant difference was detected for GM-CSF transcript (P = 0.090).

Another important information was brought by the determination of the cell types involved in this expressional changes. Since modulation of transcripts does not necessarily correspond to the presence and even less to a change of the protein, immunolocalization for some of the factors was performed to check for the functional relevance of the mRNA expression patterns obtained (Appendix 1). Moreover, immunohistochemistry allows detecting which mammary cellular types are involved in their synthesis. Interestingly, the immunohistochemistry performed with MMP-1, -2, -14 and TIMP-2 antibodies revealed different patterns than the ones of mRNA expression. Thus, MMP-1 seems to be up-regulated during late involution in cytoplasm of the ductal epithelial cells. MMP-2 is up-regulated in a same manner but is present in the endothelial cells only. As for MMP-14 and TIMP-2 they are both present in ductal epithelial cells and do not seem to be regulated throughout the different stages of development and function of the mammary gland. Although, the protein has been detected in different cell types in other models, in bovine their expression seems to be very cell type-specific. Metalloproteases are mainly expressed in ductal and endothelial cell. Some proteases only show a weak signal in MEC. The immunohistochemistry data were important to check the functional significance of the change in mRNA expression. It seems that in this case the alteration of mRNA levels is probably not the most important factor in the regulation of the activity of proteins.

In addition, an experimental model was specially developed to delimit the part played by the epithelial cells in the expression of cytokines (Appendix 2). This explant culture model allows studying the response of epithelial cells in "*in vivo*-like" configuration where the tissue-specific functions are maintained, while guaranteeing a homogenous inflammatory pressure. As an intact and functional piece of the original mammary gland, the explant might contain resident macrophages, endothelial cells and other cell types than epithelial cells that could produce cytokines. Thus, the possibility that cytokines may also be produced by other cell types present in the explant cannot be formally ruled out but the cytokine expression by leukocytes that migrate at the place of the infection can be excluded. Leukocytes are known to express massively a large range of cytokines, especially when stimulated (Sordillo & Streicher 2002). Furthermore, the sheer number of epithelial cells in the explants gives them the potential to be a major contributor to the cytokine secretion. *In vivo*, several kinds of cells can produce cytokines in the mammary

gland, including resident macrophages, endothelial cells, and leukocyte that migrate into the gland in response to the infection. It is known that mammary epithelial cells (MEC) are able to produce cytokines in normal and infected conditions and several evidences suggest that MEC might participate actively in the immune response during infection. Indeed, normal MEC demonstrated a basal production of IL-1β and IL-1α, IL-6, IL-10, TNF-α and GM-CSF (Okada et al. 1997) and this production is upregulated when the MEC are challenged with bacterial cell wall agent element such as lipopolysaccharide (LPS) (Boudjellab et al. 1998). MEC seems able to mount a response on their own and might participate or enhance the action of the specialized immune cells. The model developed responded to challenge with LPS by up regulation of mRNA expression for a range of cytokines and immunoglobulin receptors. The sheer number of epithelial cells in the explants gives them the potential to be an important contributor to the cytokine secretion in infected conditions and certainly the major one in healthy gland. To support this idea, healthy mammary gland where leukocytes are present in low number exhibited a strong expression of cytokine transcripts (Figure 3).
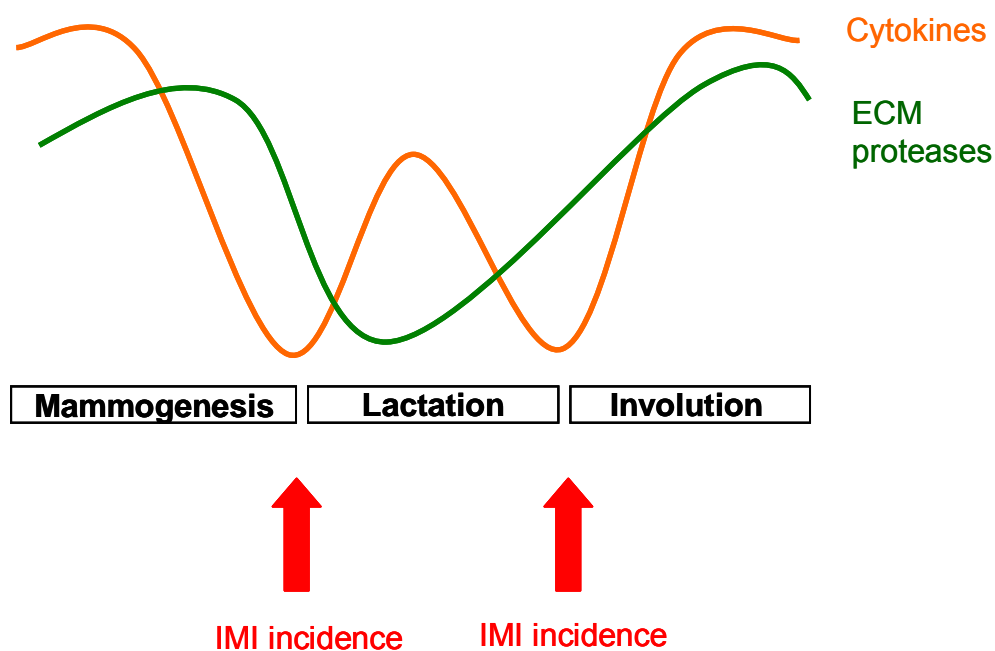
Figure 4. *Schematization of the expression profiles of cytokines and matrix proteases during the development of the bovine mammary gland. Periods when the incidence of mastitis is increased are represented with the red arrows.*

Thus, the immunological changes observed during the normal mammary development coincide with the one of proteases responsible for modeling. Furthermore, a strong statistical relationship exists between upregulation of these two types of factors and mammogenesis and involution periods (Figure 4).

The significant regulation of cytokines genes in healthy mammary gland confirms a role for these factors in normal development of bovine mammary gland. As leukocytes constantly migrate into the normal sterile bovine mammary gland, cells of the immune system are present in the gland during all stages of development (Paape et al. 2000) and their number rises abruptly at involution. It has been suggested that leukocytes and monocytic macrophages in particular are recruited to the mammary gland to supercede phagocytic epithelial cells in the clearance of residual milk and cellular debris during the later stages of involution (Monks et al. 2002). It can be assumed that it is the constant expression of cytokines by mammary cells observed in this study that induce this migration. Cytokines have already been shown to be required for maintaining the physiological baseline trafficking of leukocytes in healthy tissue (Outteridge & Lee 1981). Thus, at involution one expected outcome of the upregulation of cytokines expression is the infiltration of inflammatory cells in a greater number. Inversely, the downregulation in cytokine expression occurring at early involution may be at the origin of fewer leucocytes guarding the mammary gland. It is likely that this lack leads to the weakness of the mammary immune defense and consequently to an increased incidence of mastitis during the drying off period. This down regulation of cytokines is also observed at lactogenesis, another state where new IMI are common (Figure 3).

The concomitant upregulation of matrix proteases may also be the results of different events provoked by this elevation of cytokines. It is possible that mammary epithelial cells increase the expression of proteases upon stimulation by proinflammatory mediators such as Il-1β and TNF-α as it has been described for MMP-9 (Mehrzad et al. 2005). Neutrophils were found to be responsible for the release and activation of MMP and many other proteases (Raulo et al. 2002). Moreover, cytokines induced by *Escherichia coli* challenge could also stimulate neutrophils to express more MMP-9 (Long et al. 2001). Thus, another origin for elevated protease expression at late involution may be due to an increase in neutrophil population of the mammary tissue since neutrophils are recruited consecutively to the increase of cytokines at this

period. Leukocytes such as macrophages utilize matrix proteases to penetrate the basement membrane and reach the epithelium and luminal space. It should not be forgotten that late involution is characterized by an increase of the interalveolar space and a regrowth of the stromal adipose tissue (Monks et al. 2002). So it is normal to see an increase in factors that affect tissue architecture. Similar phenomena could explain the upregulation of cytokines during mammogenesis. It is known that macrophages and eosinophils mediate the growth of the mammary gland during development (Gouon-Evans et al. 2002). In mouse, the formation and outgrowth of terminal end buds (i.e. end of the growing duct) closely parallels recruitment to the postnatal mammary gland of macrophages. Mice depleted of circulating leukocytes presented a dramatic curtailment of mammary ductal development (Gouon-Evans et al. 2000). Overall these data indicate functions for macrophages in ductal outgrowth and differentiation, possibly acting through their well-documented roles in supplying trophic factors for epithelial cell growth, angiogenesis or their phagocytic activity but also through matrix remodeling (Welgus et al. 1992; Polverini 1997). Cytokines regulate these processes through the recruitment and regulation of macrophages. Moreover, TNF-α-deficient mice displayed fewer secondary and tertiary branches of the mammary epithelium during puberty. TNF-α was shown to stimulate growth as well as to induce extensive branching and alveolar morphogenesis of isolated rat mammary epithelial cells in primary culture (Ip et al. 1992; Varela & Ip 1996). Thus, cytokines could play by themselves a critical role for the good progression of mammary morphogenesis.

This study is based on the examination of transcript expressions in order to evaluate their modulation. The advantages of using real-time PCR over conventional PCR have been thoughtfully described (Leutenegger et al. 2000). However, we often encountered large variations in the folds of induction of the target genes. We observed apparent regulation in the expression of the transcript of cytokines without that statistical test has detected any significant difference between the different periods of development or function of the gland as it is the case for GM-CSF. There was large variation among animals which explains why some comparisons were not statistically significant. The variation may be attributed to several factors such as the phenotype and the physiology which are specific to each animal and the high sensitivity of real-time PCR probably further augmented this variation.

5 CONCLUSION

Here, the common patterns of gene expression of cytokines and matrix proteases have been outlined across the mammary developmental time course. These patterns have been subsequently correlated with biological function of the gland. The induction of matrix proteases coincides with the upregulation of cytokines. A range of mammary cell types can be involved in the synthesis of these factors. The possibility for MEC to produce cytokines has been confirmed by our study while the production of proteases has been delimited to very few cell types.

One common link between cytokines and matrix proteases is the previously defined role for leukocytes. Cytokines signaling induces leukocyte homing and activation. When activated and present in large number in the mammary gland, leukocytes could elicit an important production of proteases. However this possible role played by leukocytes has not been investigated yet in bovine mammary gland. The proposed mechanism with which they could contribute to the critical structural changes occurring during mammary development clearly needs further investigation.

The novelty value of the present studies lies in being one of the first to examine matrix proteases and cytokines during all the development of bovine mammary gland.

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APPENDIX

Appendix 1. Rabot A., Sinowatz F., Berisha B., Meyer H.D.D., Schams D. Expression of extracellular matrix-degrading proteinases and their inhibitors in the bovine mammary gland during development, function and involution. *Journal of Dairy Science* 90:740-748.

Appendix 2. Rabot A., Wellnitz O., Meyer H.D.D., Bruckmaier R. 2007. Use and relevance of a bovine mammary gland explant model to study infection responses in bovine mammary tissue. *Journal of Dairy Research* 74:93-99.

Appendix 3. Rabot A., Berisha B., Meyer H.D.D., Schams D. Potential Role of Granulocyte Monocyte-Colony-Stimulating Factors in Bovine Coliform Mastitis. To be submitted to *Journal of Dairy Science*.

Expression and Localization of Extracellular Matrix-Degrading Proteinases and Their Inhibitors in the Bovine Mammary Gland During Development, Function, and Involution

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ABSTRACT

In degrading the extracellular matrix, matrix metalloproteinases (MMP) and the plasminogen activator (PA) system may play a critical role in extensive remodeling that occurs in the bovine mammary gland during development, lactation, and involution. Therefore, the aim of our study was to investigate the mRNA expression of MMP-1, MMP-2, MMP-14, MMP-19, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, urokinase-type PA, tissue-type PA, urokinase-type PA receptor, and PA inhibitor-1 by quantitative PCR and to localize with immunohistochemistry MMP-1, MMP-2, MMP-14, and TIMP-2 proteins in the bovine mammary gland during pubertal mammogenesis, lactogenesis, galactopoiesis, and involution. Expression of mRNA for each of the studied factors was relatively lower during galactopoiesis and early involution but was markedly increased during mammogenesis and late involution, 2 stages in which tissue remodeling is especially pronounced. The localization of proteins for MMP-1, MMP-14, and TIMP-2 showed a similar trend with strong staining intensity in cytoplasm of mammary duct and alveolar epithelial cells during pubertal mammogenesis and late involution. Interestingly, MMP-2 protein was localized only in the cytoplasm of endothelial cells during late involution. Our study demonstrated clearly that expression of extracellular matrix-degrading proteinases coincides with a concomitant expression of their inhibitors. High expression levels of MMP, TIMP, and PA family members seem to be a typical feature of the nonlactating mammary gland.
Key words: matrix metalloproteinases, plasminogen activator, mammary gland, bovine

INTRODUCTION

The mammary gland undergoes substantial morphological changes during development, lactogenesis, galactopoiesis, and involution. At puberty, the extent of mammary gland development accelerates with ductal elongation and branching, followed by lobulo-alveolar development and maturation during pregnancy. This results in fully functional differentiation and production of milk by the secretory epithelium during lactation. Degenerative events then take place during involution of the gland following cessation of lactation after weaning or the end of milking. These different stages of normal mammary development require a finely controlled degradation and remodeling of the extracellular matrix. Several proteinases are implicated in the turnover of the extracellular matrix. The matrix metalloproteinases (**MMP**) are considered the key enzymes in this process and can be divided into 8 groups according to their structure. Metalloproteinases are zinc-dependent endopeptidases that are usually secreted as soluble latent proenzymes, 6 of which are membrane bound. They are then activated in the extracellular environment by a variety of factors, including members of the plasminogen activator (**PA**) system. This system belongs to the serine proteinase family, the second main family of matrix-degrading proteinases. These proteins are involved in direct degradation of extracellular matrix substrates (Sternlicht and Werb, 2001) and the activation of MMP precursors at the cell surface through the urokinase-type PA (**uPA**), uPA receptor (**uPAR**)/plasminogen cascade (Murphy et al., 1999). Plasmin is produced from its inactive zymogen precursor, plasminogen, through 2 PA, uPA and tissue-type PA (**tPA**). Plasminogen activation is limited by the action of PA inhibitor (**PAI**).

In addition to their classical role, the matrix-degrading proteinases also function to release growth factors and cytokines. This expands the repertoire of MMP actions to include modulation of cell growth (Sternlicht and Werb, 2001; Green and Lund, 2005).

Regulation of most of the MMP family members is tightly controlled and is 3-fold: at the level of gene transcription, enzyme activation, and the balance between MMP and their natural inhibitors, the tissue inhibitors of metalloproteinases (**TIMP**). Activation of the proenzymes and the function of the mature MMP can be inhibited by the binding of TIMP.

Because extracellular matrix proteinases have been associated with tumor growth and metastasis (Egeblad and Werb, 2002), a considerable body of research exists concerning the expression and role of the MMP and inhibitors in the mammary gland in murine models (Benaud et al., 1998; Rudolph-Owen and Matrisian, 1998; Green and Lund, 2005). However, very few data concerning the bovine mammary gland are available, although events occurring during development of the mammary gland are critical to the success of the first lactation, and those occurring during involution are likely to influence the following lactating period. In the mouse, mammogenesis and early involution are clearly associated with an up-regulation of the expression and activity of ECM-degrading proteinases and a down-regulation of their inhibitors. This study aimed to verify whether these observations were also true for the bovine. To test this hypothesis, we established the expression pattern of some MMP and inhibitors in the bovine mammary gland in an attempt to further elucidate their role and possible importance in mammary development and mammary function. Profiles of mRNA expression of MMP-1, MMP-2, MMP-14, and MMP-19; TIMP-1 and TIMP-2; and members of the PA system, uPA, tPA, uPAR, and PAI-1 were determined for the bovine mammary gland at well-defined stages of development (lactogenesis, galactopoiesis, and involution) by real-time PCR. In addition, we analyzed the localization patterns of MMP-1, MMP-2, MMP-14, and TIMP-2 to evaluate the correlation with gene expression data and to determine which cell types were involved. By selecting different proteinases and their inhibitors, we tried to cover a wide range of substrates that could be degraded during development and remodeling associated with involution.

MATERIALS AND METHODS

Animals

The mammary glands from nonpregnant German Fleckvieh and Holstein Friesian cows (38 in total) were removed within 20 min of slaughter during defined stages. The classification of the animals was established as follows: 1) **M**: pubertal mammogenesis (18-mo-old heifers, n = 4); 2) **L**: lactogenesis (onset of secretion during d 4 to 8 postpartum, n = 5); galactopoiesis: 3) **G1**: peak lactation (2 to 8 wk postpartum, n = 5); 4) **G2**: mid lactation (4 to 5 mo, n = 4); 5) **G3**: late lactation (8 to 12 mo, n = 4); involution (after dry off): 6) **I1**: 24 to 48 h (n = 5); 7) **I2**: 96 to 108 h (n = 3); 8); **I3**: 14 to 28 d (n = 8). Small pieces (1 to 2 g) of mammary tissue were frozen in liquid nitrogen and stored at –80°C for RNA extraction or were fixed for immunohistochemistry study.

Total RNA Extraction and Reverse Transcription

Total RNA was isolated from tissues using an adapted guanidinium thiocyanate/phenol method as described previously (Plath et al., 1997). To quantify the amount of total RNA extracted, the optical density (**OD**) was determined with a spectrophotometer (Eppendorf, Hamburg, Germany) at 3 different dilutions of the final RNA preparations at 260 nm, corrected by the 320-nm background absorption. The integrity of RNA was verified electrophoretically by ethidium bromide staining and by an $OD_{260}:OD_{280}$ nm absorption ratio of >1.7.

Synthesis of the first strand cDNA was performed in a volume of 40 μL containing 1 μg of RNA and 2,000 U of M-MLV reverse transcriptase (Promega, Mannheim, Germany) according to the manufacturer's instructions. A reaction without the reverse transcriptase enzyme was performed to detect residual DNA contamination.

Real-Time PCR

Primers for housekeeping and target genes were designed by use of a software program (HUSAR program; DKFZ, Heidelberg, Germany) according to published bovine sequences (Table 1). Quantitative real-time PCR was performed with a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia). Polymerase chain reactions were carried out using a LightCycler DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) with 1 μL of each cDNA in a 10-μL reaction mixture (3 mM $MgCl_2$, 0.4 μM of each forward and reverse primer, 1× LightCycler DNA Master SYBR Green I). After initial incubation at 95°C for 10 min to activate the *Taq* DNA polymerase, templates of all specific transcripts were generated with a 3-segment amplification and quantification program (95°C for 10 s, 60°C for 10 s, 72°C for 15 s with a single fluorescence acquisition point) repeated for 40 cycles.

Confirmation of PCR product identity and specificity was obtained through melting curve analysis (Rotor-Gene 3000 software, version 5.0; Corbett Research) and subsequent gel electrophoresis separation, in which PCR products showed a single band at the expected length.

The cycle threshold (**CT**) for the target gene and the CT for an endogenous control, the housekeeping gene β-actin, were determined for each sample (Rotor-Gene 3000 software, version 5.0; Corbett Research). Values were then normalized to the endogenous control according to the ΔCT equation, where $\Delta CT = CT_{target} - CT_{\beta\text{-actin}}$ (Leutenegger et al., 2000; Livak and Schmittgen, 2001).

Table 1. Sequences of primers used in PCR amplification reactions

Gene[1]	Sequence (5′ to 3′)	Product size, bp	EMBL[2] accession number
β-Actin	For AACTCCATCATGAAGTGTGACG	234	U39357
	Rev GATCCACATCTGCTGGAAGG		
MMP-1	For CAAACCAGGTGCAGGTATCG	232	NM174112
	Rev AAGGTCCGTAGATGGCCTG		
MMP-2	For CCCAGACAGTGGATGATGC	248	NM174745
	Rev TTGTCCTTCTCCCAGGGTC		
MMP-14	For ACTTGGAAGGGGGACACC	236	AF144758
	Rev AGGGGGCATCTTAGTGGG		
MMP-19	For TTTCAAGGGGGACTATGTGTG	240	X92521
	Rev CAATAGAGAGCTGCATCCAGG		
TIMP-1	For CATCTACACCCCTGCCATG	231	AF144763
	Rev CAGGGGATGGATGAGCAG		
TIMP-2	For GGGTCTCGCTGGACATTG	256	AF144764
	Rev TTGATGTTCTTCTCCGTGACC		
tPA	For GGGGAAGCACAACCACTG	263	X85800
	Rev AGCTGATCAGGATCCCCC		
uPA	For TGCAGCCATCTACAGGAGG	240	X85801
	Rev TGGTGAGCAAGGCTCTCC		
uPAR	For TGTTTCCAGAAACCGCTACC	234	S70635
	Rev AAGTGGAAGGTGTGGTTGTTG		
PAI-1	For CAGCGACTTACTTGGTGAAGG	231	X52906
	Rev TCCAGGATGTCGTAGTAACGG		

[1]MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinases; tPA = tissue-type plasminogen activator; uPA = urokinase-type plasminogen activator; uPAR = uPA receptor; PAI = plasminogen activator-inhibitor.

[2]EMBL = European Bioinformatics Institute (Cambridge, UK).

Statistical Analyses

The statistical significance of differences in the ΔCT values of examined factors was assessed by ANOVA, followed by the Holm-Sidak test. Differences were considered significant if $P < 0.05$. A high cycle number indicated a low level of gene expression and vice versa. We expressed our experimental data as 20 minus the mean of the normalized cycle threshold $(20 - \Delta CT) \pm$ standard error of the means (SEM). Consequently, the more template present at the beginning of the reaction, the larger the $20 - \Delta CT$ value.

Immunohistochemistry

For histology and immunohistochemistry, tissue samples (approximately 5 mm thick) were fixed in Bouin's solution for 48 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 gelatine/chrom alum-coated glass slides.

Following deparaffinization, the presence of the proteinases was demonstrated immunohistochemically by the streptavidin-biotin horseradish peroxidase complex (ABC) technique (Hsu et al., 1981). To expose antigenic sites for proteinases, dewaxed sections were heated 4 times to 95°C in a 600-W microwave oven, maintained for 5 min, and allowed to cool for 20 min. Endogenous peroxidase activity was then eliminated by incubation with a 0.5% (vol/vol) hydrogen peroxide solution in absolute methanol for 10 min at 20°C. Nonspecific protein binding was eliminated by incubation with Protein Block Serum-Free (Dako, Hamburg, Germany) for 10 min at room temperature. Sections were then incubated overnight at 4°C with first antibodies, dilution 1:100: polyclonal anti-MMP-1, monoclonal anti-MMP-2 (clone Ab-4); polyclonal anti-TIMP-2; polyclonal anti-MMP-14 (clone Ab-1; NeoMarkers, Fremont, CA). Incubation for 30 min with biotinylated porcine antirabbit IgG at a 1:100 or 1:300 dilution and with biotinylated rabbit antimouse IgG, 1:300 (Dako) followed. The sections were then reacted with ABC reagent from a commercial kit (Dako). The bound complex was made visible by reaction with 0.05% 3,3′-diaminobenzidine hydrochloride and 0.0006% hydrogen peroxide in 0.1 M PBS. Between each step, sections were washed 3 times in PBS. All incubations were carried out in humidified chambers to prevent evaporation. Sections were counterstained in Mayer's hematoxylin, dehydrated, cleared, and mounted.

Controls were performed by 1) replacing the primary antibody with nonimmune serum; 2) substituting the

primary antibody with buffer; 3) replacing the secondary antibody with buffer; and 4) incubating with diaminobenzidine reagent alone to exclude the possibility of nonsuppressed endogenous peroxidase activity. Lack of detectable staining of tissue elements in the controls demonstrated the specificity of the reactions. Positive controls were performed with bovine placental tissue to test the primary antibodies.

RESULTS

Expression and Tissue Distribution of MMP Family Members

The mRNA expression data for MMP and TIMP are shown in Figure 1, and the localization and staining patterns of the proteins are shown in Figure 2. The level of expression of MMP-1 transcripts did not change significantly across the stages examined (Figure 1A). By immunohistochemistry, during pubertal mammogenesis (heifers), the mammary duct epithelial cells were strongly immunoreactive (Figure 2, panel 2), stained weakly during lactation (Figure 2, panel 3), and strongly stained again during involution (Figure 2, panel 4). The protein was distinctly detected in the cytoplasm of the epithelial cells. The negative control of heifer tissue (replacement of the primary antibody with nonimmune serum) showed no staining at all (Figure 2, panel 1).

Expression of MMP-2 mRNA was highest during pubertal mammogenesis and lactogenesis, significantly decreased during galactopoiesis, and further decreased during early involution (I2), followed by a significant increase (I3; Figure 1B). The only positive staining for MMP-2 detected in the mammary gland was during involution and was restricted to endothelial cells of blood vessels (Figure 2, panel 5).

Matrix metalloproteinase-14 and TIMP-2 presented the same profile of mRNA expression. Their mRNA levels decreased constantly from mammogenesis to galactopoiesis, were stable until the latest stage of involution, and tended to recover to a level comparable to that in mammogenesis (Figure 1C and 1D). Mammary duct epithelial cells of heifers were distinctly stained with antibody to MMP-14 (Figure 2, panel 6) but alveolar epithelial cells were poorly stained at all other stages examined (data not shown). The insert in Figure 2, panel 6, shows a positive control for MMP-14 (giant cells of bovine placenta). In contrast, mammary duct epithelial cells in heifers (Figure 2, panel 7) and alveolar cells (data not shown) of all other stages stained only weakly to TIMP-2. In Figure 2, panel 8, a negative control is presented in heifer tissue as well as a positive control in the insert (bovine placenta with stained giant cells). Table 2 summarizes the immunochemistry data

indicating the relative intensity of staining and the cell types concerned.

Expression of MMP-19 transcripts was high during mammogenesis but was strongly down-regulated during lactation until the last stage of involution, when the transcripts recovered their initial level of expression (Figure 1E). Tissue inhibitor of metalloproteinase-1 mRNA was also expressed with a significantly higher value during pubertal mammogenesis, followed by down-regulation during lactation until early involution, with an increase again in late involution (Figure 1F).
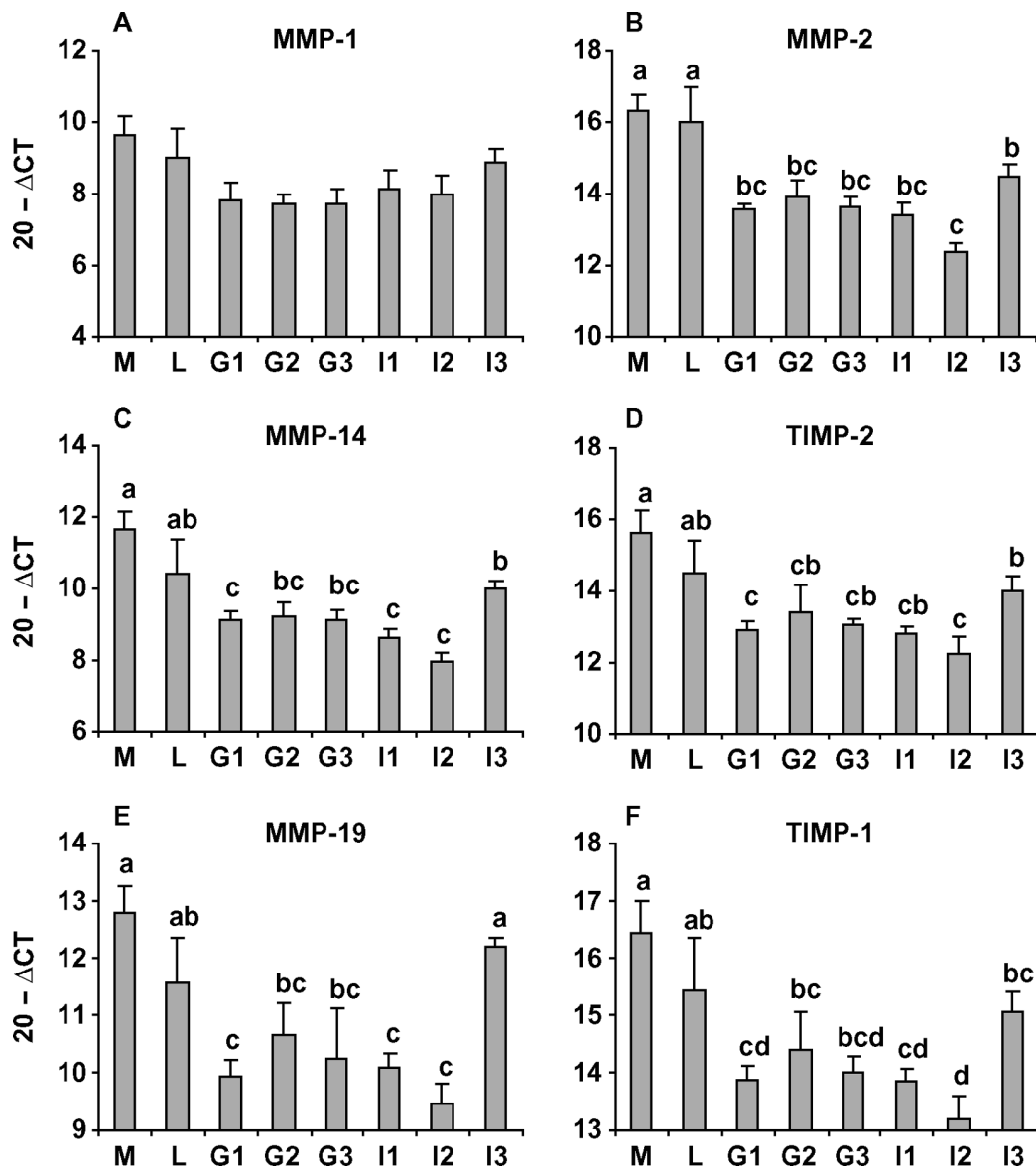
Expression of PA Family Members

The mRNA expressions for PA, PAI-1, and uPAR are shown in Figure 3. The expression of uPA mRNA was consistently high during pubertal mammogenesis and lactogenesis but was significantly decreased during late lactation and early involution, followed by a significant increase in late involution (Figure 3A). In contrast, tPA expression remained constant across stages (Figure 3C). Expression of mRNA of the uPAR was up-regulated during mammogenesis and late involution and exhibited relatively low expression during other stages (Figure 3B). Similarly, PAI-1 mRNA was significantly higher during mammogenesis and lactogenesis but also tended to be higher during late involution (Figure 3D).

DISCUSSION

Prelactational Development

Mammogenesis occurs in 2 steps: the first one, early mammogenesis, consists of the construction of a branched ductal network throughout the mammary fat pad. The final developmental fate of the udder is fulfilled only when pregnancy occurs, under the impulse of reproductive hormones. As expected, our study demonstrates that matrix-degrading proteinases are expressed and up-regulated in the glands of virgin cows. Matrix metalloproteinase-2 expression and activity were also detected in developing mouse mammary tissue (Witty et al., 1995; Fata et al., 1999). Despite evidence in our study that mRNA expression of MMP-2 was up-regulated during mammogenesis and lactogenesis, the protein was not detected at this stage. The failure to detect MMP-2 protein is puzzling and unexplained. However, because MMP-14 is an activator of MMP-2 activity (Rudolph-Owen and Matrisian, 1998; Egeblad and Werb, 2002), it is logical to detect a synchronized expression of this factor.

Up-regulation of MMP-19 expression in nonlactating mammary tissue (i.e., that from virgin cows and postinvolution glands) corresponds with the study by Djonov et al. (2001) on human mammary tissue. High uPA and

Figure 1. Messenger RNA expression of matrix metalloproteinase (MMP) family members in bovine mammary gland tissue. M: pubertal mammogenesis (18-mo-old heifers, n = 4); L: lactogenesis (onset of secretion during d 4 to 8 postpartum, n = 5); G1: galactopoiesis peak lactation (2 to 8 wk postpartum, n = 5); G2: galactopoiesis midlactation (4 to 5 mo, n = 4); G3: galactopoiesis late lactation (8 to 12 mo, n = 4); I1: early involution (24 to 48 h after the end of milking, n = 5); I2: involution (96 to 108 h, n = 3), I3: late involution (14 to 28 d, n = 8). Results represent 20 minus means of ΔCT (cycle threshold) $\pm$ SEM from n = 3 to 8/group. Different letters indicate different means ($P < 0.05$). TIMP = Tissue inhibitor of metalloproteinases.

tPA levels in the developing bovine mammary gland are consistent with the fact that plasminogen plays an important role in branching morphogenesis (Busso et al., 1989; Delannoy-Courdent et al., 1996; Simian et al., 2001). Given that the PA system was also proposed to participate in activating MMP (Simian et al., 2001; Curry and Osteen 2003), it is not surprising to observe an elevation of the expression of its members when MMP are active.

Interestingly, TIMP are up-regulated during mammogenesis, although they have an inhibitory effect on morphogenesis (Fata et al., 1999). This most likely is to facilitate normal udder growth rather than uncontrolled proliferation. The up-regulation of TIMP-2 expression is expected because this factor, along with MMP-14, is required for the activation of MMP-2 (Wang et al., 2000). However, the TIMP-2 protein seems to be present at a constant level throughout the development
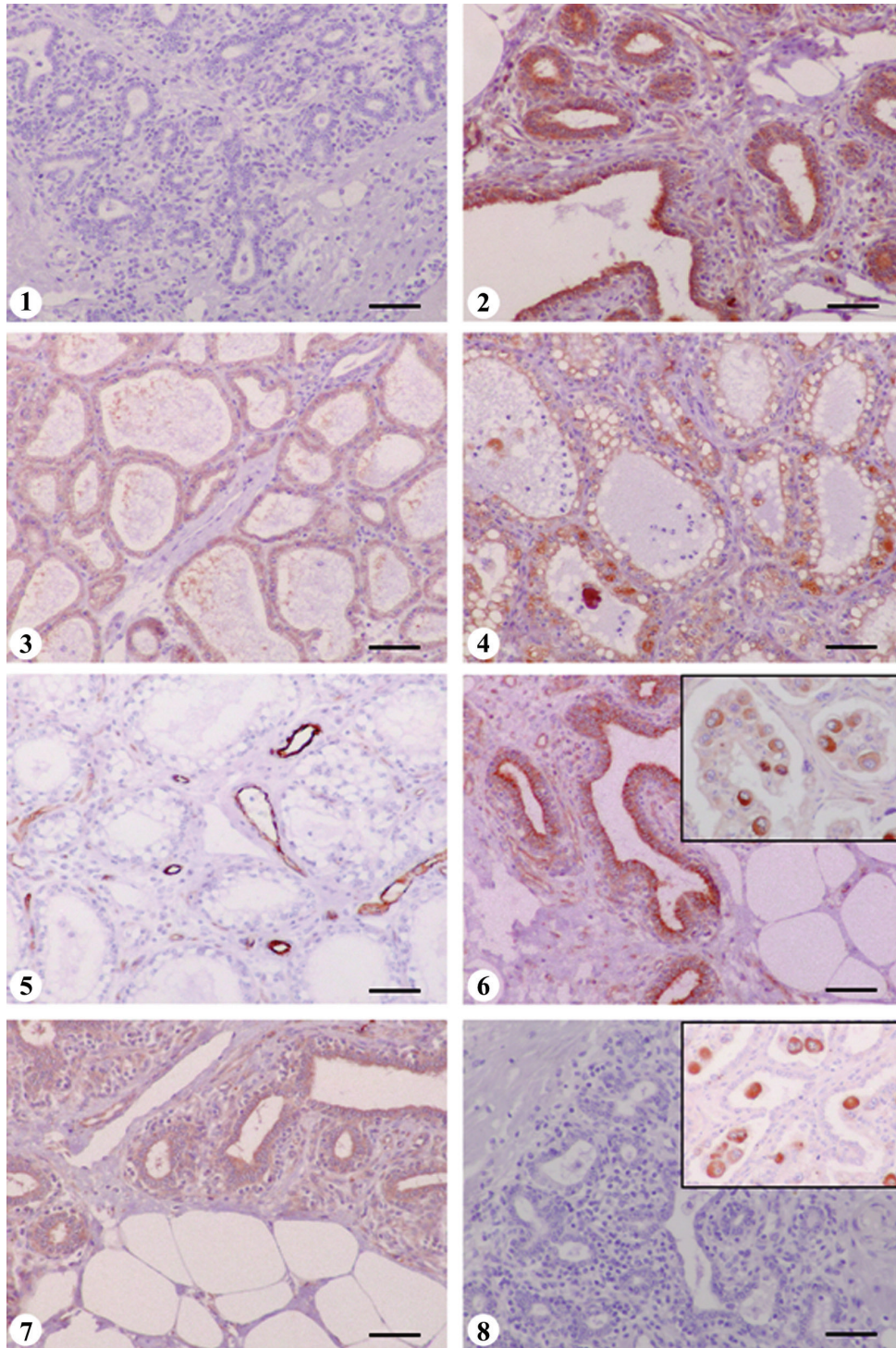
Figure 2. Immunohistochemical localization of matrix metalloproteinase (MMP)-1, MMP-2, and tissue inhibitor of metalloproteinase (TIMP)-2. Matrix metalloproteinase-1: Pubertal mammogenesis (heifers). No signal is visible on the negative control where MMP-1 primary antibody has been replaced with nonimmune serum (panel 1; bar: 100 μm). The ductular epithelial cells are strongly stained for MMP-1 at pubertal mammogenesis (panel 2; bar: 50 μm), appear moderately stained at galactopoiesis, 2 to 8 wk postpartum (panel 3; bar: 50 μm), and show a strong signal again in late involution, 28 d (panel 4; bar: 35 μm). Matrix metalloproteinase-2: Only vascular endothelial cells display distinct immunostaining at late involution (panel 5; bar: 50 μm). Matrix metalloproteinase-14: Duct epithelial cells are distinctly stained during pubertal mammogenesis. The insert shows giant cells of bovine placenta as the positive control (panel 6; bar: 100 μm). Tissue inhibitor of metalloproteinase-2: Epithelial cells from the mammary duct show a weak staining during pubertal mammogenesis (panel 7; bar: 100 μm). Negative and positive (insert, giant cells of bovine placenta) controls for TIMP-2 antibody (panel 8, bar: 100 μm).

Table 2. Summary of the intensity of the immunostaining for each antibody, tissue area, and stage of development in bovine mammary gland tissue[1]

| Antibody[2]	Tissue area	Mammogenesis	Lactation	Involution
MMP-1	Ductal epithelial cells	+++	+	+++
MMP-2	Endothelial cells	−	−	+++
MMP-14	Ductal epithelial cells	++	−	−
	Alveolar cells	+	+	+
TIMP-2	Ductal epithelial cells	+	+	+
	Alveolar cells	+	+	+

[1]From − (no staining) to +++ (very strong intensity).

[2]MMP = Matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase.

period. This suggests that the formation of branching ducts requires a tightly regulated ratio of MMP and inhibitors.

The case of MMP-1 is quite interesting. Sorrell et al. (2005) demonstrated in the mouse that expression levels of most MMP genes are actually low and, moreover, that they stay constant throughout development. Therefore, the absence of significant variation of MMP-1 expression in the cow would not be unexpected if the protein were not remarkably up-regulated at late involution. We cannot reach a conclusion regarding regulation at the zymogen level because the anti-MMP-1 recognized both the zymogen and the active form of the protein. Because mRNA expression and the presence of the protein were not correlated for MMP-2 and MMP-1, we assume that regulation at the transcript level is probably not the most important in the mechanisms controlling the activity of the MMP.
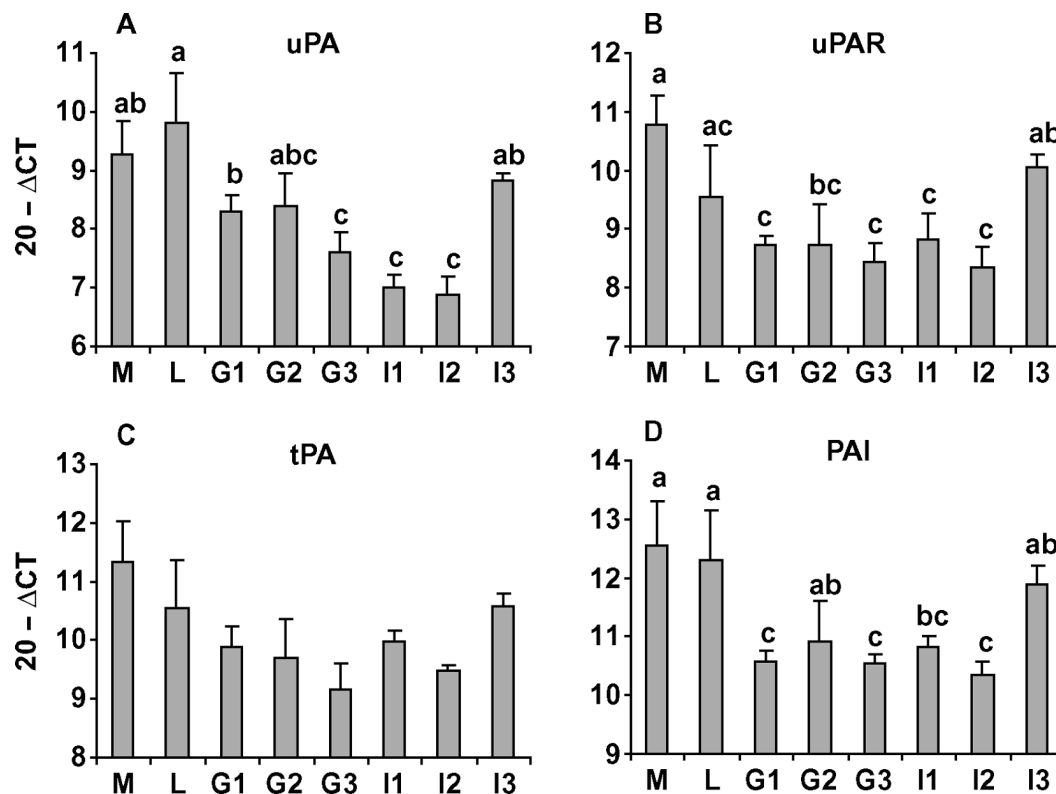
Figure 3. Messenger RNA expression of plasminogen activator (PA) system members in bovine mammary gland tissue. M: pubertal mammogenesis (18-mo-old heifers, n = 4); L: lactogenesis (onset of secretion during d 4 to 8 postpartum, n = 5); G1: galactopoiesis peak lactation (2 to 8 wk postpartum, n = 5); G2: galactopoiesis midlactation (4 to 5 mo, n = 4); G3: galactopoiesis late lactation (8 to 12 mo, n = 4); I1: early involution (24 to 48 h after the end of milking, n = 5); I2: involution (96 to 108 h, n = 3), I3: late involution (14 to 28 d, n = 8). Results represent 20 minus the means of ΔCT (cycle threshold) ± SEM from n = 3 to 8/group. Different letters indicate different means ($P < 0.05$). uPA = Urokinase-type PA; uPAR = urokinase-type PA receptor; tPA = tissue-type PA; PAI = PA inhibitor.

Lactation

Most of the matrix-degrading proteinase transcripts studied were down-regulated during different stages of lactation in the bovine mammary gland. Our data are in agreement with studies showing low levels or no detection at all of MMP in the lactating murine mammary gland (Strange et al., 1992; Li et al., 1994; Lund et al., 1996). This is consistent with the presence of a well-defined, intact basement membrane supporting the differentiated, milk-secreting epithelial cells. The need for an intact basement membrane in the maintenance of a functional, differentiated phenotype in mammary epithelial cells is known (Sympson et al., 1994). During all stages of lactation, expression of MMP inhibitors, as well as members of the PA-plasmin system, is also repressed except for uPA and tPA, which are expressed at a stable level. This last observation is in contradiction with the study of Busso et al. (1989), in which both uPA and tPA expression decreased in lactating murine glands, but the data are in accordance with the increased concentrations of plasmin and plasminogen during lactation in cows detected by Politis et al. (1989).

Involution

After cessation of lactation by removing suckling young or by suspending milking (dry period), the mammary gland undergoes involution. In rodents, involution has been characterized as a 2-stage process. The first stage is characterized by induction of apoptosis of mammary epithelial cells without visible degradation of the extracellular matrix, and the second phase is characterized by activation of proteinases that destroy the lobular-alveolar structure of the gland and trigger remodeling of the extracellular matrix (Lund et al., 1996). Epithelial cells lose their adhesion to a basement membrane, which is destroyed by the increased proteinase activity and, as a result, the cells lose survival signals generated by the extracellular matrix (Wiseman and Werb, 2002). These events result in a return to the fully regressed, nonfunctional stage of the mammary gland in preparation for a new reproductive cycle. Several matrix-degrading proteinases have been implicated in the degradation and remodeling of mouse mammary stroma during involution, including MMP-2 (Dickson and Warburton, 1992; Talhouk et al., 1992) and uPA (Busso et al., 1989). Indeed, PA play an important role during involution because postlactational involution has been demonstrated to be profoundly compromised in the absence of plasmin (Lund et al., 2000). Mammary involution proceeds more slowly in dairy animals than in rodents, and morphological changes that occur during the dry period are less pronounced. There is no significant tissue regression during the dry period prior to parturition in dairy cows, and even without the protective effect of a concurrent pregnancy, the alveolar structure is partially maintained for several weeks after cessation of milking (Capuco and Akers, 1999). Our data suggest that involution in the bovine occurs in 2 phases, consistent with those noted in mice: an initial one corresponding to the stages of I1 and I2 (1 to 5 d of involution), when expression of MMP and their inhibitors are strongly down-regulated, and another one corresponding to the later stage, I3 (14 to 28 d of involution), with an up-regulation of proteinases and members of the PA system. This is also reflected in immunohistochemistry data in which MMP-1 and MMP-2 proteins are more abundant during involution, in the epithelium for the first one and, interestingly, in the endothelial cells for the second one. This suggests different activities for these 2 proteinases. Matrix metalloproteinase-2 has also been detected in stroma, epithelial, and myoepithelial cells in the murine model (Dickson and Warburton, 1992; Wiseman et al., 2003).

Despite a difference in timing between rodents and ruminants, involution involves similar physiological events in both species. Our data suggest that high expression of MMP and inhibitors is a characteristic feature of the "resting" mammary gland. Because this expression also corresponds with an elevation of morphogenic growth factor expression as transforming growth factor α, fibroblast growth factor, and IGF (Plath et al., 1997, 1998; Plath-Gabler et al., 2001), one can assume that matrix-degrading proteinases are working synergistically with such factors. Studies to determine the nature of this relation and the influence that lactogenic hormones might have on their expression are warranted.

In general, the fluctuation in expression of the different transcripts measured were much less dramatic than in rodents. This can be explained by the fact that development and lactation periods of the bovine mammary gland are much longer than in the murine mammary gland (weeks for the mouse to a month for the cow). Consequently, in the cow, the action of matrix-degrading proteinases does not need to be as acute and intense as it is in the mouse.

CONCLUSIONS

This characterization study shows that all of the tested matrix-degrading proteinases and their inhibitors are up-regulated in stages in which the mammary gland undergoes extensive morphological modeling. Their localization is not confined to the alveolar epithelial cells but can also be observed in mammary duct epithelial cells and vascular endothelial cells. This indi-

cates that matrix-degrading proteases might play a cell-specific role that highlights another level of control of their action. Matrix-degrading proteinases are under complex regulation to prevent inappropriate degradation as well as inappropriate proliferation of mammary tissue. Both growth factors and reproductive hormones influence this regulation, but by largely unknown mechanisms.

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Use and relevance of a bovine mammary gland explant model to study infection responses in bovine mammary tissue

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Our aim was to develop an explant model to define more precisely the early response of bovine mammary epithelial cells to infection. Therefore we investigated the mRNA expression encoding for some soluble immunological factors in lipopolysaccharide (LPS)-treated bovine mammary gland explants. Explants were taken out from the mammary gland of eight lactating cows after slaughter then incubated with LPS (10 μg/ml) for 6 h. The mRNA expression of α-lactalbumin (α-la), various cytokines, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, and two immunoglobulin receptors, the neonatal Fc receptor (FcRn) and polymeric immunoglobulin receptor (pIGR), were assessed with qPCR before and after 3 h and 6 h of LPS challenge. Both immunoglobulin receptors and α-la increased at 3 h then recovered their initial level at 6 h whereas IL-1β, IL-6 and IL-8 increased only after 6 h ($P < 0.05$). Surprisingly, TNF-α transcripts did not show any regulation in response to the LPS treatment. We nevertheless concluded that our model was valid to examine the short-term response of mammary epithelial cell challenged with LPS.

Keywords: Cytokine, immunoglobulin receptor, LPS, bovine, explant.

Mammary gland immunity, represented as protection and resistance to infectious diseases, is facilitated through a variety of factors. Cytokines are part of the important line of defence represented by both specific and innate soluble factors of defence mechanisms. Numerous reports show immunomodulatory capabilities of cytokines (Sordillo & Streicher, 2002; Alluwaimi, 2004). They are described as cell-free soluble factors that function as communicator molecules between leucocytes and also between leucocytes and tissue. Pro-inflammatory cytokines are thought to initiate the inflammatory reactions in mammary tissues and to induce migration of leucocytes into the udder in response to invading microorganisms or their components such as lipopolysaccharide (LPS) (Riollet et al. 2000a). Indeed, the rapid influx of neutrophils at the site of infection is capital, their ability to phagocytose and kill bacteria being the key to recovery from infection. Interleukin-1 beta (IL-1β), IL-6, IL-8, and tumour necrosis factor-alpha (TNF-α) are some of the cytokines known to play an important role in this process (Okada et al. 1997; Boudjellab et al. 2000; Watanabe et al. 2000).

The inflammatory response is thought to be initiated by leucocytes via the production and release of cytokines but also epithelial cells were recently shown to have the capacity to mount an innate immune response, and they were also demonstrated to express inflammatory mediators (Boudjellab et al. 1998). However, though several studies suggest a non-negligible role of mammary epithelial cell (MEC) in cytokine expression, the extent of this capability remains unclear; precise information about the importance of the part played by MEC would be very useful. Greater understanding of the initial host response to infection may lead to more accurate selection of resistant animals or to novel prophylactic or therapeutic intervention strategies.

To address this concern, studies *in vivo* are not suitable since several types of cells in the mammary gland can produce cytokines, including resident macrophages, endothelial cells, and leucocytes that migrate into the gland in response to the infection. Consequently, several approaches to study bovine MEC are based on cell culture techniques. A homogeneous population of cells is exposed to the same defined infection pressure in the absence of environmental influences. Culture of bovine MEC is considered by many as an established *in-vitro* model to study

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different functions in the mammary gland. However, several aspects of epithelial cell culture show that this model provides only limited information about regulatory mechanisms. Transformed cell lines such as the MAC-T, BME-UV or L1 cells developed to study hormonal influences appeared to be phenotypically unstable and to express milk proteins erratically (Huynh et al. 1991; Zavizion et al. 1996; German & Barash, 2002). To eliminate limits due to a single genotype, cell culture models have been attempted with non-transformed cells, primary cell culture. But still, most investigations with cultured cells were performed on plastic surfaces, which led to drastic alterations of morphology and function from parent tissue. Epithelial cells cultured on plastic dishes lost their mammary specific functions: loss of polarization of organelles, changes in metabolite patterns, alteration of synthesis and secretion of most milk proteins within a couple of days. In most systems the level of milk protein synthesis is either low or non-existent and, if it does occur, it is independent of some or all of the factors (hormones or extra cellular matrix) known to regulate lactogenesis *in vivo* (Rose et al. 2002). Therefore, cell culture is not entirely appropriate for investigations of lactating MEC function.

Incubations of slices of mammary tissue have shown that tissue-specific functions can be maintained for at least 4 d, though this method has never been used to study the inflammation response of MEC (Baumrucker & Stemberger, 1989). Here we report the development of an explant culture model, which allows study of the response of epithelial cells in 'in vivo-like' configuration while guaranteeing a homogeneous inflammatory pressure. Our particular interest was to determine the role of epithelial cells in the early host responses and their ability to respond rapidly to infection; we therefore focused on the short-term changes.

LPS-induced changes in the transcriptional activity kinetics of pro-inflammatory cytokines such as TNF-α, IL-1B, IL-6, IL-8 were monitored by qRT-PCR until 6 h after challenge. In addition, the transcripts of two immunoglobulin receptors, the neonatal Fc receptor (FcRn) and the polymeric immunoglobulin receptor (pIgR) were also assessed. FcRn and pIgR are expressed on the basolateral surface of mammary alveolar cells to transport immunoglobulins (Ig) from plasma into milk. Transcripts of α-lactalbumin (α-la) were also studied as a control of the lactating properties of the alveolar cells of the explants.

Materials and Methods

Source of tissues

Eight lactating dairy cows were used. Mammary glands were removed from the animals at slaughter and an incision was made aseptically at the proximal part of the gland, through the connective tissue, to expose the secretory tissue. For each of the eight cows, one quarter of the mammary gland was chosen for withdrawal of three samples. All samplings were carried out through the incision, using a Bard magnum Biopsy Instrument (BARD, Covington GA, USA) and a Core Tissue Biopsy Needle (BARD) so that the tissue was removed no later than 20 min after slaughter. Samples were immediately incubated at room temperature in microfuge tubes containing 2 ml of media supplemented or not with LPS until distribution to 12-well dishes under a sterile hood.

Using the biopsy tool allowed a calibration of the samples. Owing to the unchangeable dimensions of the needle (1·6 cm, 2 mm), samples were similar in shape and weight. These very slim and long samples have the advantage of offering a large surface of contact and absorption to the medium and its components and do not require further manipulation.

Explant culture

Explants were incubated in 12-well dishes, one explant per well, in 1 ml of medium, at 37 °C and 5% CO_2. From each cow, explants for all three treatment times of LPS treatment and control were used. The basal medium was DMEM-F12 medium supplemented with insulin (10 µg/ml), hydrocortisone (0·5 µg/ml), penicillin G (100 µg/ml) streptomycin (100 µg/ml), amphotericin B (2·5 µg/ml). Final concentrations of 10 µg/ml of LPS from *Escherichia coli* (0111:B4; cat. No. L4391) were used in the explant cultures for 3 h and 6 h and each stimulation was performed on explants from the eight cows. All chemicals were purchased from Sigma Chemical Co. Explants were processed for RNA extraction either upon arrival at the laboratory for the controls or immediately after being challenged for 3 h and 6 h.

Total RNA extraction and reverse transcription

Tissue homogenization was performed with the homogenizer FastPrep120 centrifuge (Q.Biogene). Each explant was mixed with 1 g Matrix Green and 1 ml TriFast (TriFast, Peqlab, Erlangen, Germany) in Green-Caps and centrifuged twice with the FastPrep120 for 40 s at speed 4·5. Between homogenizations, the samples were stored on ice. Then, total RNA of mammary explants was isolated according to the manufacturer's recommendations (TriFast, Peqlab, Erlangen, Germany).

To quantify the amount of total RNA extracted, the optical density at 260 nm was determined with a photometer (Eppendorf, Hamburg, Germany). RNA integrity was verified by the OD_{260}/OD_{280} nm absorption ratio between 1·7 and 2·0. Total RNA was reverse transcribed to cDNA with reverse transcriptase (MMLV-RT, Promega, Madison WI, USA) and random hexamer primers (Gibco BRL, Grand Island NY, USA) according to the manufacturer's instructions. Final concentration of cDNA was 25 ng/µl.

**Table 1.** Sequences of PCR primers of the factors investigated in the experiment

Target†	Sequence (5′-3′)		Product size, bp	EMBL accession number
β-actin	For	AACTCCATCATGAAGTGTGACG	234	U39357
	Rev	GATCCACATCTGCTGGAAGG		
GAPDH	For	GTCTTCACTACCATGGAGAAGG	197	U85042
	Rev	TCATGGATGACCTTGGCCAG		
αla	For	ACCAGTGGTTATGACACACAAGC	233	M18780
	Rev	AGTGCTTTATGGGCCAACCAGT		
IL1-β	For	TTCTCTCCAGCCAACCTTCATT	214	M37211
	Rev	ATCTGCAGCTGGATGTTTCCAT		
TNF-α	For	TAACAAGCCGGTAGCCCACG	277	AF011926
	Rev	GCAAGGGCTCTTGATGGCAGA		
IL-6	For	GCTGAATCTTCCAAAAATGGAGG	188	NM173923
	Rev	GCTTCAGGATCTGGATCAGTG		
IL-8	For	ATGACTTCCAAGCTGGCTGTTG	200	BC103310
	Rev	TTGATAAATTTGGGGTGGAAAG		
FcRn	For	TGAACGGCGAGGAGTTCATG	288	AJ313190
	Rev	GCTCAGGTGGGTAGAAGGAGA		
PigR	For	ATGTGAGCCTGGAGGTCAGCCA	353	AJ313189
	Rev	CTCCAGCACCTGGAGGTCAA		

† See text for abreviations of targets

Quantification by real-time PCR

Quantitative real-time PCR was performed with the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). PCR reactions were carried out using a LightCycler DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) with 1 µl of cDNA (25 ng) in a 10-µl reaction mixture (3 mM-$MgCl_2$, 0·4 µmol/l of each primer, 1× LightCycler DNA Master SYBR Green I). Mixtures underwent the following real time PCR protocol: a denaturation program (95 °C for 30 s) and a three segment amplification and quantification program (95 °C for 10 s, 60 °C for 10 s, 72 °C for 15 s with a single fluorescence acquisition point) repeated for 40 cycles. Primer sequences used for housekeeping gene and target gene amplification are shown in Table 1. The specificity and identity of each PCR product was determined by melting curve analysis (Rotor-Gene 3000 software, version 5.03) and subsequent gel electrophoresis separation. Crossing point (CP) values were acquired by using the comparative quantification method of the Rotor Gene software version 5. CP is defined as the point at which the fluorescence rises appreciably above background.

Relative mRNA expression levels are given by the arithmetic formula $2^{-\Delta\Delta CP}$ where the CP of the target gene is normalized to the mean of housekeeping genes GAPDH and β-actin relative to total RNA (ΔCP) and then compared with the control group collected before LPS stimulation (ΔΔCP): quantity of a transcript in treated explants relative to that in the control groups was expressed as fold change in expression (Livak & Schmittgen, 2001). The mRNA expression of both housekeeping genes resulted in constant expression levels in all samples.
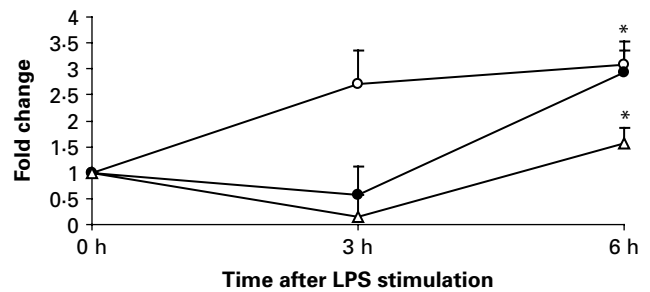
Fig. 1. Relative mRNA expression of Il-1β (○), IL-6 (●) and IL-8 (△) in mammary explants incubated with LPS (10 µg/ml) at 0, 3, and 6 h. Values are the mean ± SEM fold increase of eight cows at each time point. Means with * are significantly different ($P < 0·05$).

Statistical analyses

Statistical significance of differences in mRNA expression between control and infection were tested for normal distribution then assessed by a paired *t* test performed with Sigma Stat 3.0 (SPSS Inc., Chicago IL, USA). $P < 0·05$ was considered significant.

Results

Cytokines

IL-1β, IL-6, IL-8 mRNA expression increased significantly ($P < 0·05$) after 6 h of LPS challenge, about 3-fold higher for Il-1β and Il-6 and 1·5-fold higher for IL8 than in the control (Fig. 1). TNF-α mRNA expression did not change
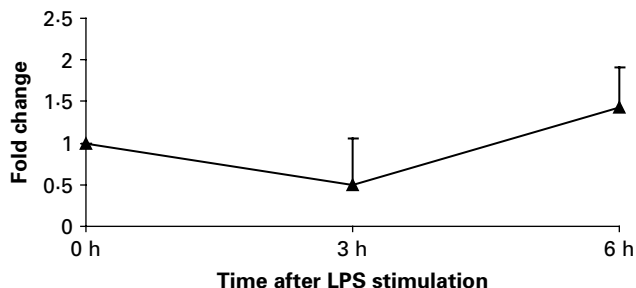
Fig. 2. Relative mRNA expression of TNF-α in mammary explants incubated with LPS (10 μg/ml). Values are means±SEM of eight cows at each time point. No significant difference was detected.
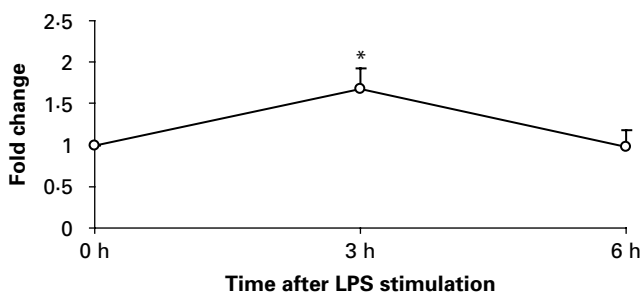
Fig. 3. Relative mRNA expression of α-la in mammary explants incubated with LPS (10 μg/ml). Values are means±SEM of eight cows at each time point. Means with * are significantly different ($P<0.05$).

Fig. 4. Relative mRNA expression of immunoglobulin receptors, FcRn (receptor of IgG; ▲) and pIgR (receptor of IgA and M; △), in mammary explants incubated with LPS (10 μg/ml). Values are means±SEM of eight cows at each time point. Means with * are significantly different ($P<0.05$).

significantly although a small rise seemed to take place at 6 h (Fig. 2). IL-6, IL-8 and TNF-α tended to be down-regulated 3 h after the stimulation whereas IL-1β seemed to be already up-regulated.
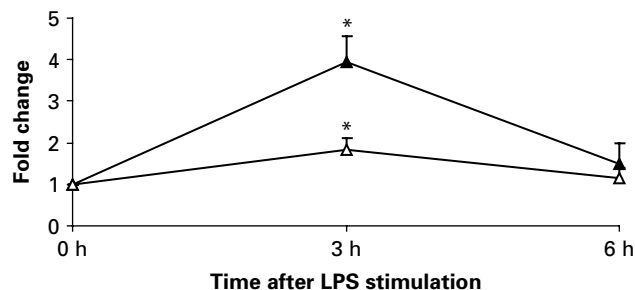
α-Lactalbumin and immunoglobulin receptors

A transient increase in mRNA expression was observed for α-la (Fig. 3) and both Ig receptors, FcRn and pIgR (Fig. 4), at time 3 h ($P<0.05$). They recovered their basal level of expression after 6 h of stimulation.

Discussion

To our knowledge, mammary gland explants have not been used to measure innate immune response under inflammation conditions. In the current study, we determined whether this model would be relevant to examine the ability of MEC to produce cytokines when stimulated with LPS.

To get information that would be as close as possible to conditions *in vivo*, the first step was to verify that the alveolar cells of the explants were fully functional and kept their lactating phenotype. α-Lactalbumin as one major milk protein was a good candidate. The transient increase of α-la transcripts due to the LPS treatment

observed in the explants attested to the capability of the epithelial cells to express milk proteins and validated the functionality of the model. However, these results differed from a previous report of α-la mRNA being down-regulated in bovine mammary gland challenged with LPS (Schmitz et al. 2004). One possible explanation is that a reaction to the release of milk from the explants could explain this increase of α-la mRNA whose expression would have been repressed in the mammary glands engorged with milk before slaughter.

Among the cytokines playing key roles in mediating acute inflammatory reactions, IL-1β and TNF-α are extremely potent inflammatory molecules. They recruit and activate neutrophils, induce fever and the production of acute phase reactant proteins by the liver (Feghali & Wright, 1997). They also enhance the expression of vascular endothelial adhesion molecule expression, thereby promoting neutrophil transendothelial migration to the site of infection (Gray et al. 1982; Craven, 1986).

TNF-α seems to be constitutively expressed by cultured bovine MEC but natural coliform mastitis, experimental infection, or LPS exposure increase considerably and rapidly TNF-α protein and mRNA expression (Persson Waller et al. 2003; McClenahan et al. 2005; Strandberg et al. 2005). Surprisingly, LPS stimulation had no significant effect on the production of TNF-α mRNA by explants under our experimental conditions. This could be attributed to marked variability in individual response, as production of TNF-α mRNA was very heterogeneous among the eight mammary glands.

Production of IL-1 by normal MEC *in vitro* has been reported (Okada et al. 1997) but like TNF-α, it experiences a sharp elevation after infection. In *Esch. coli* infection, IL-1β concentration increases in milk (Riollet et al. 2000b) but this increase is detectable only at 40 h post infection (pi) (Bannerman et al. 2004) whereas in cultured and LPS-stimulated ovine MEC, the greatest concentration of IL-1β is reached sooner and peaks at 24 h post challenge (pc) (Okada et al. 1999). As this sharp elevation is also associated with the influx of neutrophils, it is difficult

to determine whether cytokines in milk originated from immune cells or from mammary cells *in vivo*. However, IL-1β gene expression is markedly up-regulaed by more than 500-fold in bovine MEC. That strongly suggests an active role of MEC in the production of these pro-inflammatory cytokines. The increase in MAC-T was only greater by 4·26-fold after 24 h pc (Strandberg et al. 2005). In our study we were able to detect a significant 3-fold increase of IL-1β mRNA within 6 h.

IL-8 is a powerful chemotactic factor that attracts neutrophils and T lymphocytes and is also involved in neutrophil activation (Harada et al. 1994). If the transcriptional level shows no significant variation at different stages of lactation in the healthy bovine mammary gland (Alluwaimi et al. 2003), it is elevated early in experimental coliform mastitis. The time required for significant elevation of the level of the protein to be detectable *in vivo* varies considerably according to the study. The protein is detected in milk as early as 4 h pi (Persson Waller et al. 2003), 14–16 h pi (Bannerman et al. 2004) or 24 h pi (Shuster et al. 1997). In our study, a significant increase in IL-8 mRNA was detectable after 6 h of exposure to LPS. Many studies indicate that MEC is a major source of IL-8. MEC lines stimulated with LPS or *Esch. coli* copiously produce IL-8. Both bovine MEC and MAC-T cell lines showed a significantly increased production of IL-8 after 24 h of LPS stimulation although the increase was more marked in bovine MEC. Thus, after 24 h IL-8 transcripts are increased by 137-fold in bovine MEC against 22 in MAC-T. IL-8 mRNA peaks within 2 h then remains constant for the following 22 h and within 1 h in the study of (McClenahan et al. 2005). In addition, the protein level assessed with ELISA also showed an up-regulation. To investigate further the main source of production, levels of IL-8 were measured in milk and lymph, revealing a higher amount in milk, indicating that the mammary epithelium rather than the sub-epithelial tissue is the major source of IL-8 (Persson Waller et al. 2003).

IL-6 is one of the proinflammatory cytokines incriminated in the development of signs of acute septic shock in coliform mastitis. It has been postulated that IL-6 facilitates the transition of the inflammatory process from influx of neutrophils to monocytes (Kaplanski et al. 2003). IL-6 mRNA is expressed by normal MEC; a IL6-like activity is shown as well (Okada et al. 1997) and both are up-regulated in the same cell type but stimulated by LPS (Okada et al. 1999). Bovine MEC cell line responds to LPS stimulation in producing IL-6 in a dose-dependent manner. Standberg et al. (2005) showed IL-6 to be up-regulated to a lesser extent than the other cytokines: about 3·32–3·70-fold in bovine MEC and MAC-T. In mammary gland infected with *Esch. coli*, IL-6 expression was detected as early as 14 h pi (Shuster et al. 1997). We demonstrated a significant increase of IL-6 mRNA expression after 6 h of LPS stimulation.

To our knowledge, the present study is the first to examine the regulation of the expression of immunoglobulin receptors in mammary gland under inflammation conditions. FcRn is detected in epithelial cells of mammary gland of many species: mouse (Cianga et al. 1999) ruminant (Kacskovics et al. 2000; Mayer et al. 2002) and human (Cianga et al. 2003). In bovine mammary gland, FcRn is uniformly distributed in epithelial cells before parturition then only on their apical side after calving (Mayer et al. 2005). After LPS challenge, FcRn mRNA was up-regulated at 3 h then recovered its prechallenge level. Transcripts of pIgR showed a similar pattern. Little is known about the distribution of this receptor in mammary gland, the only study reported on ruminant being in sheep. pIgR mRNA is detectable from the third part of pregnancy, accumulates until established lactation when it reaches its highest level (Rincheval-Arnold et al. 2002). These results are important in suggesting a possible role for epithelial cells in the early stage of inflammation. Immunoglobulins are part of the soluble defences that elicit effective protective responses to invading pathogens. IgG and IgM present in serum and milk can opsonize bacteria to enhance phagocytosis (Rainard & Riollet, 2006). IgA contribute to agglutination of bacteria to prevent colonization and neutralize bacterial toxin (Sordillo et al. 1997; Sordillo & Streicher, 2002). Epithelial cells would have the capacity to enhance their production of Ig receptor in order to concentrate Ig at the place of infection to thwart the bacterial invasion. Thus mammary epithelial cells might provide a rapid immunological response to infection.

The response of the explant to LPS stimulation is somehow encompassed between those of the *in-vitro* and *in-vivo* models and corresponded to what would have been expected from a model having the characteristics of its two precedents. However, the degree of change does not exceed a 3-fold increase and is much less than expected on the basis of cell culture studied. Although the medium surrounded the explants and the MEC could be stimulated from their luminal as well as their basal side, we can assume that the number of cells in the explant that could come into contact with LPS and could respond to its stimulation was less than in a cell culture model. Another limit to the model might be trauma caused by the biopsy procedure. The metabolism of the cells injured by the tool could be perturbed and this might explain the unexpected initial trends observed for α-la, IL-6 and IL-8. This trauma might also have been caused by the culture procedure since the explants had not been previously cultivated in the medium before the LPS stimulation.

The underlying assumption made in these analyses is that the enhanced mRNA expression observed has a direct relationship with the quantities of the corresponding proteins. It was not possible to verify whether it was the case in our experimental conditions but several studies report this direct correlation. Several studies report increased IL-8 protein levels secreted into the cell culture medium after IL-8 mRNA up-regulation caused by LPS (Boudjellab et al. 2000; Wellnitz & Kerr, 2004; Strandberg et al. 2005). However, these data concern only one cytokine

and it must be kept in mind that mRNA expression is not necessarily associated with protein expression.

A tissue explant as an intact and functional piece of the original mammary gland might contain resident macrophages, endothelial cells and cell types other than epithelial cells that could produce cytokines. Thus, the possibility that cytokines may also be produced by other cell types present in the explant cannot be formally ruled out but the cytokine expression by leucocytes that migrate at the place of the infection can be excluded. The clear majority of epithelial cells in the explant leads to the assumption that their contribution is a relevant one. The sheer number of epithelial cells in the explants gives them the potential to be a major contributor to the cytokine secretion.

The current study demonstrates that explant models can be used to study the immune response of MEC. The model developed responded to challenge with LPS by up-regulation of mRNA expression for a range of cytokines and immunoglobulin receptors.

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Potential Role of Granulocyte Monocyte-Colony-Stimulating Factors in Bovine Coliform Mastitis.

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ABSTRACT

The critical role played by cytokines in bovine mastitis has been long demonstrated. However, the functioning for many of these cytokines is still not described in details and it is especially the case for GM-CSF. To determine whether GM-CSF is a key factor or not in the mammary immune system. we compared the strength of GM-CSF expression during the inflammatory response in the blood system and in the mammary gland in parallel. The real-time RT-PCR technology was used to investigate the transcriptional activity of GM-CSF and its receptor in healthy bovine mammary gland at different stages of development and function and in LPS-challenged leucocytes. We could detect GM-CSF mRNA at all stages of development and function studied. Although no significant change between the different stages could be detected, GM-CSF transcript tended to be up regulated at mammogenesis and involution. Interestingly the two genes coding for each chain of this receptor are expressed and regulated differentially regarding the development stage of the gland. The experiment with bovine WBC culture clearly showed that stimulated leucocytes answer with a sharp increase of GM-CSF mRNA synthesis as soon as 1h after challenge. Then the expression peaked at 2h with a 3.4-fold expression and remained up regulated until the end of the experiment. These data let presume that GM-CSF is not an acute phase cytokine.

INTRODUCTION

Bovine mastitis is one of the most costly diseases to the dairy industry. Chemotherapy being usually not recommended during the lactating period, various treatment methods that are not

dependent on antibiotics have been tried in studies on the prevention and treatment of mastitis. One of these non-antibiotic methods of treating mastitis is a therapy using cytokines. Cytokines are immunoregulatory mediators that play a central role in the regulation of immune responses against different infections and their capability to mobilize the innate and specific immunity of the bovine mammary gland is well documented (Sordillo et al. 1997).

The colony-stimulating factors (**CSF**) are a group of cytokines central in the regulation of hematopoiesis and inflammation. Initially discovered for its role in the differentiation of progenitor cells into granulocytes and macrophages, the granulocyte monocyte-CSF (**GM-CSF**) has pleiotropic and widespread effects on hematopoietic cells. It stimulates proliferation, growth, differentiation of various cell lineages to a mature phenotype and enhance the effector activities of immune cells. It has also been shown proinflammatory effects on granulocytes, monocytes activation and T cell function and is required for normal function of some populations of dentritic cells and contributes to inhibition of granulocyte apoptosis at the site of inflammation (Coxon et al. 1999; Boutet et al. 2004). All of the biological effects of GM-CSF are mediated via the GM-CSF receptor (**GM-CSFR**), composed of two distinct chains, α and β. The α chain is the primary binding chain that associates with GM-CSF with low affinity while the β chain does not display intrinsic binding affinity for GM-CSF alone but is necessary for signal transduction. Interaction of GM-CSF with the α-chain of the GM-CSFR leads to a high affinity association through the formation of the α-/β-subunit complex followed by the transduction of intracellular signals.

Many studies suggest the prevalent role that GM-CSF could play in udder health. Recombinant bovine GM-CSF (**rboGM-CSF**) has been demonstrated to enhance superoxide anion production and phagocytosis, to activate the bactericidal activities of polymorphonuclear cells in vitro (Sordillo et al., 1992) and in vivo (Reddy et al. 1990; Kehrli et al. 1991; Daley et al. 1993; Hirai et al. 1999) suggesting that GM-CSF could be a good candidate as a therapeutic agent in cattle. GM-CSF has also been shown to induce the influx of neutrophils into the mammary gland (Wedlock et al. 2004). At last, milk bacteria count decrease drastically by GM-CSF injection during *S. aureus* mastitis (Takahashi et al. 2004).

Although these compelling evidences that GM-CSF could play an important role, some authors still stipulates that its participation in the udder immune system during infection is rather limited (Alluwaimi 2004). Bovine mammary gland cells constitutively produce GM-CSF mRNA (Okada et al., 1997; Leutenegger et al., 2000) but it is uncertain whether this production is markedly increased in response to infection. To answer this question, we compared the strength of GM-CSF expression during the inflammatory response in the blood

system and in the mammary gland in parallel. The real-time RT-PCR technology was used to investigate the transcriptional activity of GM-CSF and its receptor in healthy bovine mammary gland at different stages of development and function and in LPS-challenged leucocytes.

MATERIALS AND METHODS

Normal Mammary Gland Study. The mammary glands from non-pregnant German Fleckvieh and Holstein Frisian cows (38 in total) were removed within 20 min of slaughter during defined stages. The classification of the animals was established as follows. (i) M: Pubertal mammogenesis (18-month-old heifers, n=4); (ii) L: lactogenesis (onset of secretion during days 4-8 post partum, n=5); galactopoiesis: (iii) G.1: peak lactation (2-8 weeks post partum, n=5); (iv) G.2: mid lactation (4-5 months, n=4); (v) G.3: late lactation (8-12 months, n=4); involution (after dry off): (vi) I.1: 24-48h (n=5); (vii) I.2: 96-108 h (n=3), (viii) I.3: 14-28 days (n=8). Small pieces (1-2g) of mammary tissue were frozen in liquid nitrogen and stored at -80°C for RNA extraction.

White Blood Cell Samples

The white blood cell sampling has been described earlier (Prgomet et al., 2005). Briefly, three Brown Swiss dairy cows were selected based on somatic cell counts (SCC). SCC ranged from $4x10^5$ to $1x10^6$ cells/ml milk. A 25 ml blood was taken from a jugular vein, diluted 1:1 (v/v) with lysis buffer (830 mg NH4Cl; 3.7 mg Na-EDTA, 100 mg KCl in 100 ml H2O pH 7.4) and centrifuged for 10 min at 220 g. The bovine WBC suspension was adjusted to $2x10^7$ cell/ml in PBS/0.1% bovine serum albumin (BSA).

Cell Culture and LPS Challenge

The white blood cells (**WBC**) were incubated as duplicates in RPMI1640 medium supplemented with 10% FCS (Gibco-BRL,Life Technologies, Gaithersburg, MD, USA) and 0.1% gentamycin (Selectavet, Weyarn-Holzolling, Germany) at a cell density of $4x10^6$/ml in 96-well plates. After 24 h culture at 37°C in an atmosphere of 5% CO2, the cells were stimulated in duplicates by addition of 10 µg/ml LPS (E. coli O26:B6; Sigma Chemicals, Seelze, Germany) and allowed to incubate for (0, 1, 2, 3, 4 or 8 h). After LPS stimulation total RNA was extracted by using the Macherey-Nagel NucleoSpin RNAII kit (Macherey-Nagel, Düren, Germany). RNA pellets were resuspended in 50 µl of diethyl pyrocarbonate treated

H2O. Purity of the total RNA extracted was determined by optical density measurement at 260 nm and 280 nm. The 260:280 nm ratio reached values between 1.6 and 1.8.

Total RNA Extraction and Reverse Transcription

Total RNA of tissue and cells was isolated using Trifast (Peqlab) according to the manufacturer's recommendations. To quantify the amount of total RNA extracted, the optical density was determined with a spectrophotometer (Eppendorf, Hamburg, Germany) at three different dilutions of the final RNA preparations at 260nm, corrected by the 320nm background absorption. RNA integrity was electrophoretically verified by ethidium bromide staining and by OD_{260}/OD_{280}nm absorption ratio >1.7.

Synthesis of the first strand cDNA was performed in a volume of 40μl containing 1μg RNA and 2000 U of M-MLV reverse transcription (Promega, Mannheim, Germany) according to the manufacturer's instructions. A reaction without RT-enzyme was performed to detect residual DNA-contamination.

Quantitative PCR

Primers for housekeeping and target genes were designed by use of a software program (HUSAR program, DKFZ, Heidelberg, Germany) according to published bovine sequences (Table 1).

Quantitative real-time PCR was performed with a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia). PCR reactions for the mammary gland samples were carried out using a LightCycler DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) with 1μl of each cDNA in 10μl reaction mixture (3m*M* MgCl$_2$, 0.4μ*M* of each forward and reverse primer, 1x LightCycler DNA Master SYBR Green I). After initial incubation at 95°c for 10 min to activate Taq DNA polymerase, templates of all specific transcripts were generated with a three-segment amplification and quantification program (95°C for 10s, 60°C for 10s, 72°C for 15s with a single fluorescence acquisition point) repeated for 40 cycles.

For the white blood samples, a one-step RT-PCR method was chosen. Real-time RT-PCR was performed using QuantiTect SYBR Green RT-PCR (Qiagen, Hilden, Germany) by a standard protocol recommended by the manufacturer with 5 ng total RNA. The master-mix was prepared as follows: 5 μl 2*QuantiTect SYBR Green RT-PCR master-mix, 0.5 μl forward primer (1 μm), 0.5 μl reverse primer (1 μm) and 0.1μl QuantiTect RT Mix, used at a volume

of 6.1 µl. As PCR template, 3.9 µl of total RNA was added. A four-step run protocol was used: (i) reverse transcription (20 min at 50°C); (ii) denaturation program (15 min at 95°C); (iii) amplification and quantification program repeated 35 times (15 s at 94°C; 30 s at 60°C; 20 s at 72°C with a single fluorescence measurement).

Confirmation of PCR product identity and specificity was obtained through melting curve analysis (Rotor-Gene 3000 software, version 5.0) and subsequent gel electrophoresis separation where PCR products showed a single band at the expected length.

The crossing point (CP) for the target gene and the CP for an endogenous control, the housekeeping gene GAPDH, were determined for each sample (Rotor-Gene 3000 software, version 5.0). Then, values were normalized to the endogenous control according to the ΔCP equation where $\Delta CP = CP_{target} - CP_{GAPDH.}$ (Leutenegger et al. 2000; Livak & Schmittgen 2001). In LPS experiments, mRNA expressions are presented as ΔΔCP increase calculated in relation to time point zero.

Statistical Analyses

For the normal mammary gland samples, the statistical significance of differences in the ΔCT values of examined factors was assessed by ANOVA, followed by the Holm-Sidak test. For the LPS-challenged samples, two-way ANOVA with length of stimulation as the first factor and treatment (LPS treated versus control) was applied on normalized mRNA expression results (ΔΔCT). Differences were considered significant if *P< 0.05*. All statistical evaluations were performed by using Sigma Stat 3.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

GM-CSF and GM-CSF Receptors Gene Expression in Normal Mammary Gland Tissue
The amplification of GM-CSF transcripts did not succeed in some of the tested cows. Consequently, the number of animals per group changed from the ones described in Material and methods and their composition is detailed in Fig.1. The expression of GM-CSF mRNA did not change significantly among the different stages of development and function although an up regulation seem to occur during mammogenesis and late involution.

Unlike the ligand, both receptors, α and β chains, are expressed and regulated regarding the stage of development or function of the mammary gland (Fig.2). GM-CSFRα is up regulated during mammogenesis and late involution while GM-CSFRβ is strongly down regulated

during mammogenesis and significantly increased during lactogenesis, peaked at galactopoesis then recovered the level it had during lactogenesis.

GM-CSF mRNA Expression in LPS-treated WBC

The kinetic of GM-CSF mRNA expression during culture of LPS-stimulated WBC is shown in Fig. 4. The gene expression for GM-CSF mRNA increased rapidly and the maximum was obtained after 2 h. At this time the incubation of bovine white blood cells with 10μg/ml LPS caused a 3.4-fold increase in mRNA content of GM-CSF compared with value at time 0h. GM-CSF mRNA continued to be expressed to the end of the culture period although a progressive decrease.

DISCUSSION

The critical role played by cytokines in bovine mastitis has been long demonstrated. However, the functioning for many of these cytokines is still not described in details and it is especially the case for GM-CSF. In the udder of ewes infected with E. coli, GM-CSF was hardly detectable (Waller & Colditz 1999) but it has not been addressed extensively in bovine coliform mastitis although several studies demonstrated it is likely to play an important role in the outcome of the infection. The present study was designed to bring further information about GM-CSF role in the mammary immune system and to determine whether GM-CSF is a key factor or not. We therefore investigated and compared its expression in healthy mammary gland and LPS-challenged immune cells.

GM-CSF seems to be constitutively expressed in the bovine mammary gland under normal condition. We could detect its transcript at all stages of development and function studied even if some cows did not display any expression at all. Although no significant change between the different stages could be detected, GM-CSF mRNA tended to be up regulated at mammogenesis and involution. Origin of this production is unclear: primary culture of mammary epithelial cells has also been shown to express GM-CSF mRNA (Okada et al. 1997) whereas GM-CSF mRNA has also been monitored in milk somatic cells (**SC**) extracted from healthy cows at mid- and late lactation (Leutenegger et al., 2000; Alluwaimi & Cullor 2002). In this late study, a significant elevation in GM-CSF transcriptional activity in late lactation has been detected. As cytokines are supposed to be released transiently, the physiological role of such constitutive expression is still unknown. High mRNA transcript of GM-CSF at specific stages such as involution may account for the basic immunological needs

of the mammary gland. It might also be part of an alert system that contributes to assure the quickest inflammatory response possible in case of infection.

To our knowledge this is the first time the GM-CSF receptor is investigated in bovine mammary gland. Interestingly the two genes coding for each chain of this receptor are expressed and regulated differentially regarding the development stage of the gland. To resume, one is up regulated when the other is down regulated and inversely. The interpretation of such results is unsure since the full functionality of the receptor is acquired only when its both chains are unified. One possible explanation may be another mechanism to prevent over stimulation of the immune system by GM-CSF when there is no infection. Indeed, besides the regulation at the transcription level of the ligand itself, GM-CSF activity can also be controlled through ligand-receptor complex formation, resulting in a unresponsiveness of the target cell.

Following bacteria entry into the gland, neutrophils are the first cells recruited into the focus of infection. The effectiveness of neutrophils in preventing or eliminating intramammary infections depends on the promptness and magnitude of the migratory response and the subsequent bactericidal capabilities. GM-CSF may participate actively to this recruitment since administration of recombinant GM-CSF has the ability to augment this important host defense mechanism. Dose-dependent increases in circulating leucocytes as well as enhanced phagocytic and bactericidal capabilities of neutrophils has been demonstrated in vitro with rboGM-CSF (Reddy et al. 1990; Sordillo et al. 1992). Similar observation and a dose-dependent increase in circulating leucocytes after infusion were confirmed in vivo (Daley et al. 1993). However, expression and secretion of GM-CSF in case of natural, induced mastitis or LPS stimulation have gone largely unstudied in bovine. The only data available about GM-CSF expression during bovine coliform mastitis are from Lee et al. (2006). They observed a transient expression of the transcript in milk SC within the first 24 hour of infection of cows with E. coli without that statistical test has detected any significant difference over control quarter though. There was large variation among animals and as a consequence most tested comparisons were not statistically significant. The differences existing between the answer of the different animals may be an explanation. However our experiment with bovine WBC culture clearly showed that stimulated leucocyte answer with a sharp increase of GM-CSF mRNA synthesis. Expression is up regulated as soon as 1h after challenge, peaked at 2h with a 3.4-fold expression and remained up regulated until the end of the experiment. TNF-α, IL1-β and IL6 exhibited comparable pattern of expression in these cells under the same conditions of culture (Prgomet et al. 2005). This similarity between proinflammatory cytokines and GM-

CSF presumes a comparable importance in the infection response. The variety of inflammatory response between animals together with the great sensitivity of the investigation methods used make difficult the detection of a difference by statistical test. Despite a difficulty to define the GM-CSF expression profile under inflammatory conditions, its possible importance of this factor in the inflammatory response can not be excluded.

In conclusion, if an up regulation trend for GM-CSF mRNA during infection has been demonstrated for the bovine mammary gland, one should keep in mind that increased expression of a given mRNA transcript does not necessarily correspond with up regulated expression of a protein. Moreover, due to the migration of a great number of leucocyte toward the foci of infection and the ability of epithelial cells to express cytokines, the origin of the expression remain to be deciphered. Further studies conducted at the protein level and on both cell types would improve our knowledge on GM-CSF ability to mediate disease resistance.

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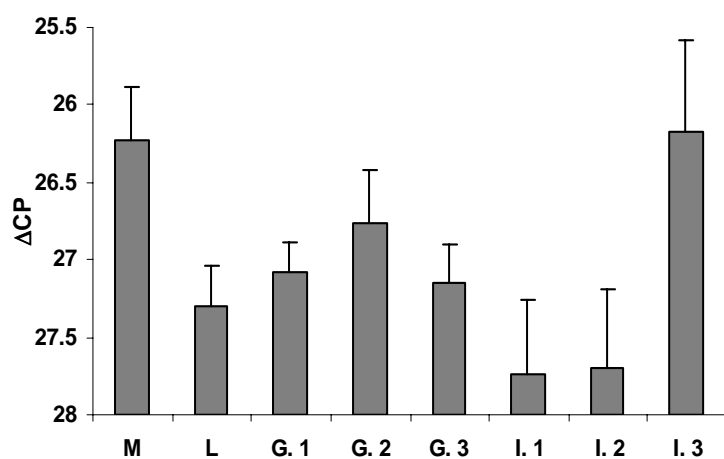
Figure 1: GM-CSF mRNA expression in bovine mammary gland tissue. M: pubertal mammogenesis (18-month-old heifers, n = 4); L: lactogenesis (onset of secretion during days 4-8 post partum, n = 5); G.1: galactopoiesis peak lactation (2-8 weeks post partum, n = 4); G.2: galactopoiesis mid lactation (4-5 months, n = 3); G.3: galactopoiesis late lactation (8-12 months, n = 4); I.1: early involution (24-48 h after end of milking, n=5); I.2: involution, 96-108 h (n = 3); I.3: late involution (14-28 days, n = 6). Results represent $\Delta CT \pm SEM$ from n = 3-6/group. No statistically significant difference was detected ($P = 0.090$).

Figure 2: Relative mRNA expression of the two chains of the GM-CSF receptor, GM-CSFRα (A) and GM-CSFRβ (B) in bovine mammary gland tissue. M: pubertal mammogenesis (18-month-old heifers, n = 4); L: lactogenesis (onset of secretion during days 4-8 post partum, n = 5); G.1: galactopoiesis peak lactation (2-8 weeks post partum, n = 5); G.2: galactopoiesis mid lactation (4-5 months, n = 4); G.3: galactopoiesis late lactation (8-12 months, n = 4); I.1: early involution (24-48 h after end of milking, n=5); I.2: involution, 96-108 h (n = 3); I.3: late involution (14-28 days, n = 8). Results represent $\Delta CT \pm SEM$ from n = 3-8/group. Different superscript letters indicate significant different means ($P < 0.05$).

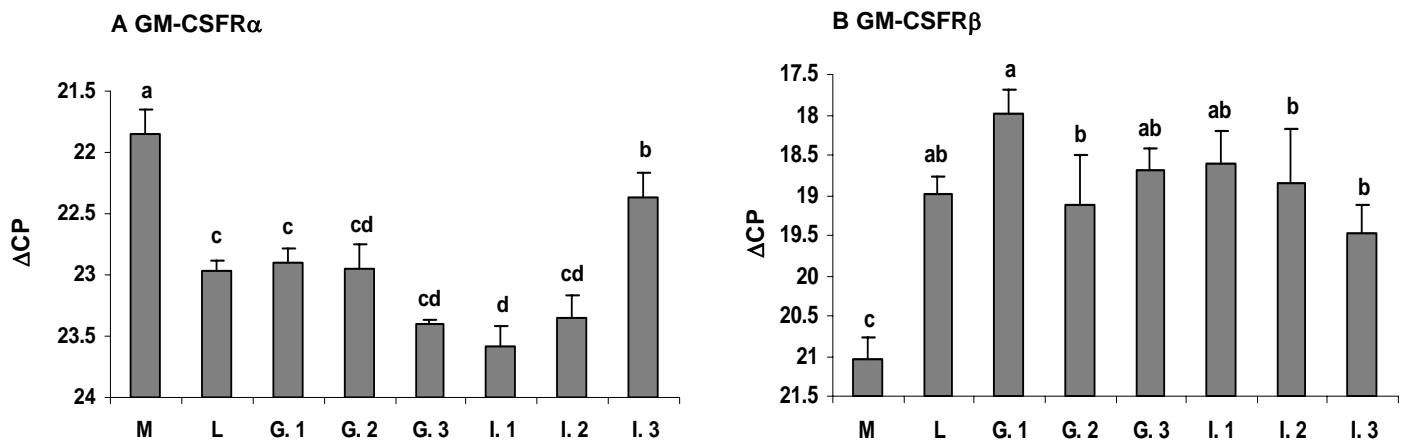Figure 3: Kinetics of GM-CSF transcripts in bovine LPS (10 µg/ml) stimulated WBC. The GM-CSF mRNA expression was assessed by real time one-step RT-PCR using GAPDH as house keeping gene. Results are presented as $\Delta\Delta CP \pm SEM$ of three dairy cows conducted in duplicates (n = 6). Significant changes between time 0 h and other time points are shown by * (*$P < 0.01$, **$P < 0.001$).

Table 1. : Sequences of primers used in PCR amplification reactions

Genes		Sequence (5'-3')	Product size (bp)	EMBL accession number
GAPDH	For	gtc ttc act acc atg gag aag g	197	U85042
	Rev	tca tgg atg acc ttg gcc ag		
GM-CSF	For	atg tgg ctg cag aac ctg ctt c	316	U22385
	Rev	ctg gtt ccc agc agt caa agg g		
GM-CSF Rα	For	cca aat gcc agc cta aat gt	227	XM 589264
	Rev	ttc tga aag ccg tct ctg gt		
GM-CSF Rβ	For	tct cca gag ctg aat cac ga	212	XM 606956
	Rev	ctg cgg tag agg gtc aca tt		

Figure 1.

Figure 2.

A GM-CSFRα

B GM-CSFRβ

Figure 3.