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

A. Rabot,* F. Sinowatz,† B. Berisha,* H. H. D. Meyer,* and D. Schams*[1]
*Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, D-85350 Freising, Germany
†Institute of Veterinary Anatomy, University of Munich, D-80539 Munich, Germany

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.

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[1]Corresponding author: physio@wzw.tum.de

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 m*M* $MgCl_2$, 0.4 μ*M* 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).