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Original Article

LOCALIZATION OF MMP-2 AND MMP-9 IN CANINE MAMMARY GLAND DURING PREGNANCY

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Summary

The mammary gland is unique in its development because most of its branching occurs in adolescent rather than in prenatal development. During early pregnancy extensive ductal side branching occurs while during the second half, secretory lobuloalveolar units are formed within the mammary gland. As modulators of the extracellular matrix, matrix metalloproteinases (MMPs) are the major enzymes taking part in the development of the gland. The activity of MMP-2 and MMP-9 has mostly been associated with tumor progression, while their participation in the physiological development of the mammary gland is not well characterized. In the present study the cell-specific localization of MMP-2 and MMP-9 in the developing dog mammary gland during pregnancy was investigated. In the early stages, both gelatinases were present, being located mostly in the epithelium of the ducts and less so in the surrounding stroma. After the formation of alveoli, MMP-2 was still present but MMP-9 was absent from the glandular epithelium and the stroma, being present only in the epithelium of the larger ducts. The results show that most likely, both gelatinases take part in ductal branching during early pregnancy, but only MMP-2 is associated with the differentiated stage of lactation.

Key words: MMP-2, MMP-9, mammary gland, pregnancy

Introduction

The mammary gland is unique in its development as the organ anlage is formed during the embryonic period, but its definitive functional maturity is not achieved until the first pregnancy. Unlike other branched organs (e.g. salivary glands, kidneys, lungs etc.) most of its branching occurs in adolescent rather than in prenatal development. During the embryonic development of the mammary gland only a rudimentary ductal tree is formed. After birth the gland enters a period of relative quiescence during which it grows just enough to keep up with body growth. This period lasts until puberty when the ovarian hormones stimulate mammary gland proliferation. In response, the "primary" ducts start lengthening and branching and terminal end buds

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(TEBs) form at the end of the ducts. These TEBs continue to branch forming new primary ducts. At the same time "secondary" branches grow laterally from the primary ducts until the entire fat pad is filled by a network of branched ducts, but still there is room for alveoli to develop when pregnancy is established [1].

During early pregnancy extensive ductal side branching occurs while during the second half, secretory lobuloalveolar units are formed within the mammary gland in preparation for lactation. Ultrastructural investigations on the beagle mammary gland show that on day 20 of pregnancy the glandular ducts are lined by a stratified epithelium consisting of two or three cell layers. From day 40 of gestation the glandular parenchyma shows alveolar arrangement with clear distinction between secretory and myoepithelial cells [2]. After mid-pregnancy the gland enters the secretory initiation phase, so near parturition it is ready for active milk secretion post partum. The formation of the secretory units is associated with massive tissue remodeling triggered by an increase in the level of serum prolactin and progesterone. These hormones activate the alveolar switch, a genetic program that coordinates changes in the mammary epithelial cell proliferation, migration, differentiation and deletion [3]. During lactation the secreting phenotype is maintained but following weaning the mammary gland undergoes involution.

The extracellular matrix plays an essential role throughout mammary gland development. Epithelial cell proliferation, differentiation and survival depend on the integrity of the underlying basement membrane. An intact basement membrane is necessary for the expression of differentiation-related genes and maintenance of the differentiated state. On the contrary, alteration and degradation of the basement membrane is believed to be critical for the involution, characterized by programmed cell death of the epithelial cells and extensive remodeling of the extracellular matrix [4].

According to recent data the processes of mammary gland development and remodeling are mediated by a series of enzymes. MMPs, as modulators of extracellular matrix (ECM) and basement membrane composition and structure, play a central role in the mammary gland development [5]. They are widely studied because of their participation not only in normal physiological processes but in diseases too, such as arthritis, cancer, atherosclerosis, aneurisms, nephritis, tissue ulcers and fibrosis. Most MMPs are secreted in the ECM in an inactive form (proMMPs) and as a result of already activated MMPs and other enzymes. Control over MMPs' activity can be realized at the following levels: transcriptional, translational and through the natural inhibitors of MMPs – TIMPs (tissue inhibitors of metalloproteinases).

According to their substrate specificity, sequence similarity and domain organization, MMPs can be divided into the following major groups: collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs. MMP-2 and MMP-9 belong to the gelatinases subfamily and their substrates include denatured collagen, gelatin and type IV collagen present in basement membranes [6]. Their activity has been associated with tumor progression but the expression during the physiological development of the mammary gland is not well characterized.

During the embryonic development, formation of branching ducts requires a tightly regulated ratio of TIMPs and MMPs. It is believed that expression of MMPs induces formation and growth of straight ducts, while increased TIMP expression favors collagen bundles deposition which results in branch point formation [6]. Inappropriate expression of MMPs or TIMPs can lead to aberrant mammary gland phenotype [7].

During the postembryonic development an initial proliferative phase occurs during puberty followed by a second phase of proliferation and differentiation when pregnancy is established. Under physiological conditions in the virgin or early pregnant gland tightly controlled expression and activation of MMPs are necessary for the development of the gland. According to recent data, MMP-2 is produced by the periductal fibroblasts while MMP-9 is found predominantly in immune cells [8, 9]. During branching morphogenesis MMP-2 is expressed in the stromal compartment [10]. It has been shown that MMP-2 regulates ductal invasion and represses lateral branching but does not participate in the pre-pubertal development of the gland. During early puberty MMP-2 promotes the invasion of the TEBs into the fat pad by supporting epithelial cell survival and protecting them against apoptosis and that later in puberty it represses lateral branching [9].

The role of MMP-9 in the development of the gland is not completely revealed. It has been

reported that MMP-9 is a major determinant of TNF-induced branching morphogenesis in mouse epithelial cells [11] but other authors suggest that MMP-9 has no obvious role in mammary gland branching morphogenesis as they found no difference in the mammary gland during puberty in MMP-9 deficient mice [9].

The data concerning MMP expression during lactation is scarce and controversial. Some studies do not detect MMP-3 in the lactating gland, while others report low levels of MMP-3 and MMP-2. The extracellular proteases are generally more abundant during mammary involution as this involves restructuring of the mammary gland, whereas their expression is inhibited during lactation [12].

Materials and Methods

Tissue samples

Three groups of animals were included in the current investigation. Group A included 5 pregnant bitches with no alveoli present in their mammary glands, group B consisted of 3 bitches with completed branching of the glands that had resulted in alveoli formation and Group C comprised 3 sexually mature nonpregnant bitches used as a reference. All of the animals were stray dogs (mongrels) that were either victims of car incidents or had been spayed. Samples from the mammary glands of each dog were taken for tissue sections.

Immunohistochemistry

Tissue samples were fixed in 10% buffered formalin for 24 h, and then dehydrated and placed in cedar oil until they became translucent. Then the samples were rinsed in xylene and embedded in paraffin. Paraffin sections (5 μm) were mounted on Hydrophilic Plus Slides (Bio SB Inc., USA). Sections were deparaffinized, rehydrated and washed in a washing buffer (Bio SB Inc., USA). Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min at room temperature and after that the sections were rinsed in a washing buffer. Rabbit anti-human antibodies to MMP-2 and MMP-9 (Santa Cruz Biotechnology, Inc., USA) (diluted 1:100 in antibody diluent, ScyTek, Inc., USA) were added and sections were incubated overnight at 4°C in a humidified chamber. After incubation, the sections were washed and immunoreactivity of MMP-2 and MMP-9 was visualized with UltraTek HRP Anti-Polyvalent (DAB) Staining System (ScyTek, Inc., USA). Sections were counterstained with Mayer's haematoxylin, dehydrated in graded series of ethanol, cleared in xylene and cover-slipped with Canada balsam. For controls, the primary antibody was replaced with isotype-matched anti-rabbit IgG.

The average score was taken from slides of three animals on each experimental group and photomicrographs taken from one of three slides is presented here. Photomicrographs of representative fields of immunohistochemistry were evaluated using Olympus BX 51 microscope fitted with an Olympus C5050Z digital camera (Olympus Optical Co, Ltd). Digital images were captured using Adobe Photoshop v.7.0 (Adobe Systems Inc.).

Results

Group A included 5 pregnant bitches with no alveoli present in their mammary gland, which corresponds to the early stages of pregnancy when ductal branching takes place. In the investigated tissue sections, both MMP-2 and MMP-9 were present. The localization of the gelatinases was predominantly in the ductal epithelium and less so in the stroma. (Figure 1a,b). Group B included 3 pregnant bitches with completed branching of the glands in which alveoli were present. The reaction for MMP-2 was still present (mainly in the epithelium of the ducts and alveoli) (Figure 1d), although it was weaker as compared to the reaction during early pregnancy, while for MMP-9 it was absent in the glandular epithelium and the stroma but still present in the epithelium of larger ducts (Figure 1e).

Group C consisted of 3 sexually mature nonpregnant bitches used as a reference. The immunohistochemistry results in nonpregnant dogs were similar to those obtained during early pregnancy, where MMP-2 and MMP-9 were both present in the ductal epithelium. A difference was found in the intensity of stroma staining which was stronger in nonpregnant dogs. (Figure 1g, h). The epithelium of the larger ducts showed a stronger reaction than that of the smaller ones.

Discussion

The development of the mammary gland differs from that of other branched organs, because most of its branching occurs not during the embryonic

period but after birth. In puberty there is rapid development of the duct system, and functional maturity is achieved with the formation of alveoli when pregnancy is established. In early pregnancy there is rapid development of the duct system of the mammary gland and secondary and tertiary branching occurs. This involves ECM matrix remodeling, because the branching of the ducts requires cell proliferation and matrix degradation. The same processes are responsible for tumor progression and invasion and the participation of both gelatinases in them has been widely studied and well documented [6, 13, 14], but there are no studies demonstrating the presence of MMP-2 and MMP-9 in the mammary gland during pregnancy. It is well-known that MMP expression and activity during normal development is strictly regulated, while in tumor progression matrix degradation is guided by the local microenviroment created by tumor cells. The stronger reaction for both gelatinases in early pregnancy (Figure 1a,b) is most likely associated with the intensive branching of the duct system but in advanced pregnancy, when the formation of alveoli and initiation of lactation take place, obviously matrix degradation is decreased, suggested by the weaker reaction in immunohistochemical staining (Figure 1d,e). Presumably, during the highly differentiated state of lactation, MMP expression and activity should both be repressed because of the presence of an intact basement membrane supporting the differentiated epithelial cells [15], but the presence of MMP-2 during this stage of the development of the gland implies other roles that the enzyme may have in lactation. It could be assumed that the role of MMP-2 is different during branching morphogenesis and during lactation because of the changing microenvironment. As the antibody used is polyclonal, no distinction between the active and inactive forms of the enzymes can be made, so

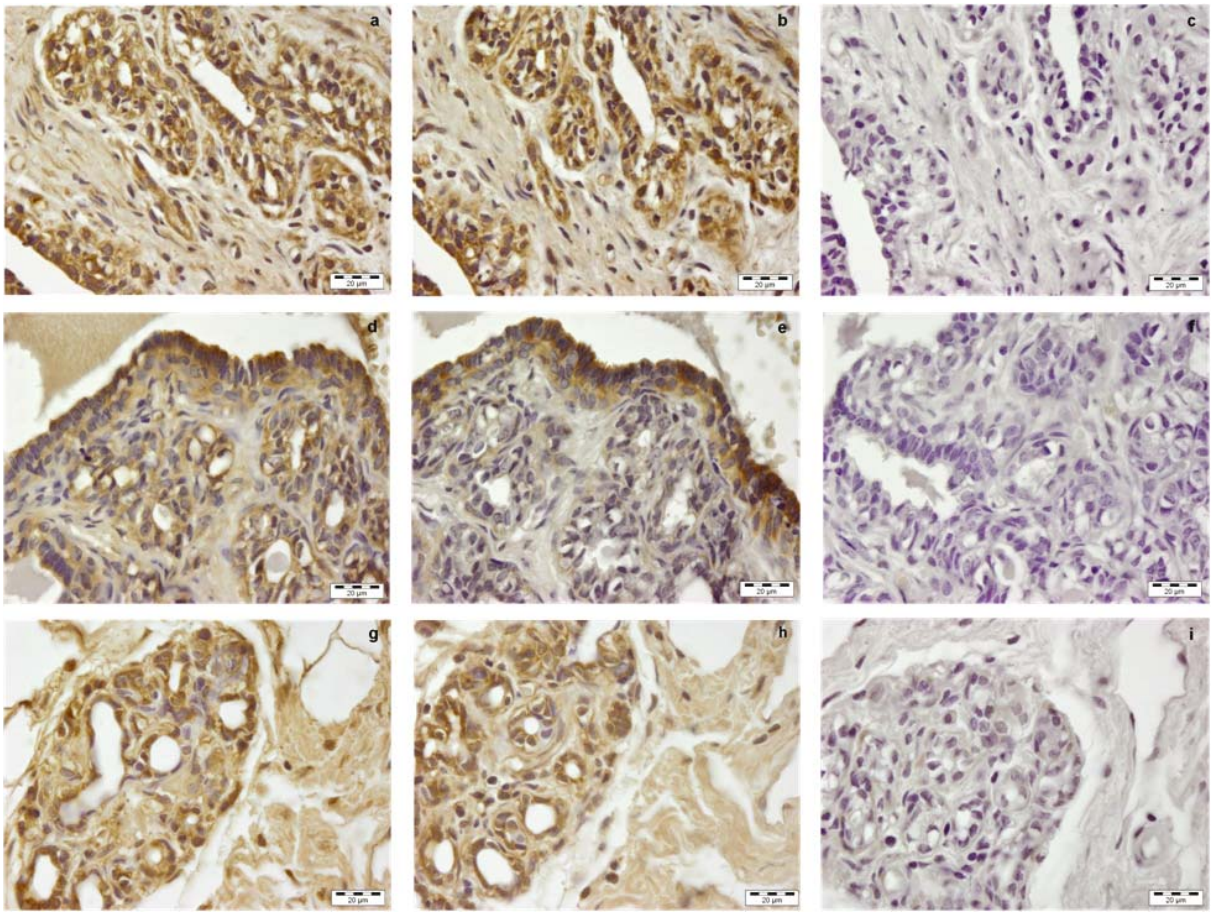

Figure 1. Immunohistochemical staining for MMP-2: 1a, d and g (Group A, Group B and Group C, respectively), and MMP-9: 1b, e and h (Group A, Group B and Group C, respectively); 1c, f, and i – controls, without primary antibody

further investigations are necessary to assess the activity of the enzymes.

The reaction for both gelatinases in nonpregnant dogs (Figure 1g,h) is not surprising as even in the lack of pregnancy the gland is not "resting". During each estrous cycle it is subjected to physiological remodeling that is associated with proliferation of the duct epithelium and alveoli formation, followed by extensive apoptosis [16]. It is necessary for the gland to undergo intensive remodeling during each estrous cycle as this is associated with the preparation of the gland for lactation if pregnancy occurs. It has been reported that MMP-2, -3, -7, -9 and - 13 were all expressed in the mouse mammary gland at each stage of the estrous cycle [16]. Though there are no records on the MMP profile changes in the dog mammary gland, with respect to the stages of the estrous cycle, it can be assumed that the two gelatinases take part in the remodeling of the canine mammary gland during the stages of the estrous cycle. Further investigations are necessary to relate the possible changes in their expression to the stages of the estrous cycle.

Conclusions

The results from this study show that MMP-2 and MMP-9 are both present in the mammary gland during early pregnancy, but after the formation of alveoli and the onset of secretion, only MMP-2 is found in the glandular epithelium. This raises further considerations about the role of MMP-2 and MMP-9 in the developmental processes of the mammary gland during pregnancy as, up to date; there is no evidence of their participation in the development of the pregnant mammary gland.

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