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EXPRESSION, ACTIVATION, AND REGULATION OF MATRIX METALLOPROTEINASE 2 (MMP-2) IN THE BOVINE CORPUS LUTEUM

BY

BO ZHANG

B. S., Henan Normal University, 1994
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DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements of the Degree of

Doctor of Philosophy

in

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May, 2002
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This dissertation is dedicated to

my wife Hongying Zhang

and

my son Nickolas J. Zhang
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It is my sincerest wish to acknowledge several people without whom this work would have been impossible.

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ABSTRACT

EXPRESSION, ACTIVATION, AND REGULATION OF MATRIX
METALLOPROTEINASE 2 (MMP-2) IN THE BOVINE CORPUS LUTEUM

by

Bo Zhang

University of New Hampshire, May, 2002

Matrix metalloproteinases (MMPs) have been postulated to be important for angiogenesis and for tissue remodeling events associated with corpus luteum (CL) development. The MMPs are mainly regulated at three levels, transcription, activation, and inhibition by endogenous tissue inhibitors of metalloproteinases (TIMPs). Unlike most MMPs, pro-MMP-2 activation is accomplished on the cell surface rather than extracellularly. The objectives of the present study were to clone bovine cDNAs for MMP-2 and its activator, membrane type 1- (MT1-)MMP, to investigate the expression and localization of MMP-2, MT1-MMP, TIMP-1, and TIMP-2 in three ages of CL, and to explore the regulation of MMP-2 expression in luteal and endothelial cells by cytokines. Molecular cloning and sequence analysis demonstrated that the bovine MMP-2 and MT1-MMP genes are highly similar to their homologs in other species. Although the levels of MMP-2 and MT1-MMP transcripts were constant in the early (day 4), mid (day 10), and late (day 16) stage CL, active MMP-2 and MT1-MMP proteins were
significantly increased (p<0.05) from the early to the mid and late stages. In addition, the patterns of TIMP-2 mRNA and protein expression were similar to MMP-2 and MT1-MMP, being expressed at higher (p<0.05) levels in the mid and late stages than the early. Immunohistochemistry demonstrated that MMP-2, MT1-MMP, and TIMP-2 were localized in endothelial and large luteal cells. In contrast, TIMP-1 mRNA and protein were highly expressed in the early and mid cycle CL, but decreased in the late stage. TIMP-1 was detected in vascular smooth muscle cells and large luteal cells. In a luteal cell culture system, TNFα stimulated MMP-2 expression in a dose and time dependent manner. In luteal-derived endothelial cells, TNFα increased while IFNγ inhibited MMP-2 expression. In the presence of both cytokines, IFNγ attenuated the stimulatory effects of TNFα alone, bringing MMP-2 expression down to control levels. In conclusion, the coordinate expression of TIMP-2, and active MMP-2 and MT1-MMP suggests that the MT1-MMP/TIMP-2/pro-MMP-2 system may be spatially and temporally available for pro-MMP-2 activation in the bovine CL. Furthermore, cytokines regulate MMP-2 expression in luteal and endothelial cells.
I. Introduction

The ovary, a primary female reproductive organ, has two principal functions. Ovaries release mature egg(s) or oocyte(s) at appropriate intervals for fertilization and successful propagation of the species (Findlay, 1991). In addition, they produce steroid hormones to regulate reproductive cyclicity, to prepare the adult female for fertilization and implantation of the zygote, and to support pregnancy. The follicle and corpus luteum, two structures found in the ovary, are largely responsible for these functions.

Follicles consist of an inner avascular granulosa compartment harboring the oocyte, an external vascularized thecal layer, and a basement membrane in between to separate these two compartments. The development of follicles from primordial to primary, then to secondary, and
eventually the final Graafian stages involves not only the proliferation of granulosa and theca cells, but also the breakdown and resynthesis of extracellular matrix to accommodate the enlargement and maturation of the follicle (Rodgers et al., 1999; Rodgers et al., 2000). When stimulated by the preovulatory surge of gonadotropins, the dominant Graafian follicle ruptures and releases the mature oocyte into the oviduct. This event requires the breakdown of tissue barriers, such as the basement membrane where granulosa cells rest, the theca externa, collagen fibers, tunica albuginea, and a second basement membrane beneath the surface epithelium, before the ovarian surface is breached to release the egg (Espey, 1994a; Espey, 1994b). Following ovulation, fibroblasts and endothelial cells of the thecal layer invade the granulosa layer. The gonadotropin surge also initiates luteinization, a process by which the remaining ruptured follicle is transformed into a new endocrine gland called the corpus luteum, which secretes progesterone to support pregnancy (Niswender and Nett, 1994). If fertilization does not occur, the CL will undergo a degenerative process, referred to as luteolysis or luteal regression, enabling a new reproductive cycle to commence.

All these physiological events that occur during the course of a normal reproductive cycle necessitate remodeling of the extracellular matrix (ECM), which require the participation of four classes of proteolytic enzymes: serine proteases, cysteine proteases, aspartic proteases, and metalloproteases. Of these, a wealth of evidence suggests that matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), are known to play important roles, not only in ECM remodeling in pathological situations such as rheumatoid arthritis, wound healing (Ravanti and Kahari, 2000) and tumor invasion and metastasis (Kahari and Saarialho-Kere, 1999; Stamenkovic, 2000), but in physiological processes such as embryo development, bone formation, implantation, and the ovarian events described above (Smith et al., 1999; Vu and Werb, 2000). The present review focuses on the molecular and biochemical characteristics of MMPs and TIMPs, and their roles in follicular development, ovulation, corpus luteum development and regression, and angiogenesis in the ovary.
II. Characteristics and Properties of Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs)

The matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent proteolytic enzymes that share a similar protein structure and collectively are able to digest all known ECM macromolecules (Matrisian, 1990; Birkedal-Hansen et al., 1993). However, individual MMP members have distinct protein structures and different preferences for a particular group of matrix proteins (Massova et al., 1998). The MMP family members are products of different genes. So far, more than 25 members have been identified in this enzyme family (see Table 1-1). The characteristic domains of MMPs include (see Figure 1-1): (1) the signal peptide domain, which directs the enzyme into the rough endoplasmic reticulum during synthesis; (2) the propeptide domain, which maintains enzyme latency until it is removed or disrupted; (3) the catalytic domain, which contains the highly conserved Zn\(^{2+}\) binding region (HExGxxGxxHS/T) and dictates enzyme activity; (4) the hemopexin domain, which is responsible for the substrate specificity of MMPs; and (5) a small hinge region, which acts as a "hub", enabling the hemopexin region to present substrate to the active core of the catalytic domain. For the subfamily of membrane-type MMPs, there is also a transmembrane domain, which has a membrane-spanning segment of about 20 hydrophobic amino acids and an intracellular domain of approximately 24 amino acids.

1. MMP family

Based on these structural and functional features, MMP family members can be classified into different subfamilies (see Table 1-2): (1) collagenases, including MMP-1, MMP-8, and MMP-13; (2) gelatinases, including MMP-2 and MMP-9; (3) stromelysins, including stromelysin-1 and -2; (4) membrane-type metalloproteinases (MT-MMPs), including MT1-6 MMP; and (5) other MMPs, which require further characterization. This last group includes

- 3 -

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MMP-19, MMP-18, and MMP-7. There are now more than twenty-five members in the MMP family.

(1) Collagenases. Collagenases are the first enzymes to be characterized in the MMP family (Gross and Lapiere, 1962). Thus far, four members have been identified in this collagenase subfamily. They are: collagenase 1 (MMP-1), collagenase 2 (MMP-8), collagenase 3 (MMP-13), and Xenopus collagenase 4 (MMP-18). These collagenases cleave the triple helix of type I, II, and III collagen exclusively at the Gly\textsuperscript{775}-Leu/Ileu\textsuperscript{776} peptide bond, which is located very close to three-quarters of the distance from the amino terminus of the substrate molecule.

The N-terminal ⅔ and C-terminal ⅓-length fragments that are generated then undergo spontaneous denaturation and become susceptible to further cleavage by other MMPs such as gelatinases (Miller et al., 1976; Hasty et al., 1987; Dioszegi et al., 1995; Mitchell et al., 1996; Reboul et al., 1996). However, each of these collagenases has various preferences for different collagen types.

Although the first vertebrate collagenase activity was originally described by Gross et al. (1962) in amphibian tissues, it took about one decade before human collagenase was purified in skin (Bauer et al., 1970) and fibroblasts (Stricklin et al., 1977). The cDNA of human collagenase was also cloned from fibroblasts (Goldberg et al., 1986). This enzyme, bearing the designation matrix metalloproteinase-1 (MMP-1), has served as the prototype for all other interstitial collagenases. MMP-1 is secreted from cells as two latent forms, one with molecular weight of 52 kDa and the other 57 kDa, which depend on the status of the Asn-linked glycosylation (Dioszegi et al., 1995). After activation, the enzyme is processed to a 42 kDa form (Table 1-2). MMP-1 preferentially degrades type III collagen. It also cleaves type I, II, and X collagens, type I gelatin, the antiprotease β\textsubscript{1}-antitrypsin, and it is likely that other substrates exist for this enzyme as well (Welgus et al., 1981a; Welgus et al., 1981b). A variety of cell types, including tumor cells, endothelial cells, fibroblasts, keratinocytes, chondrocytes, osteoblasts, and hepatocytes, have the
ability to produce MMP-1, indicating the importance of this MMP in both pathological and physiological conditions (Birkedal-Hansen et al., 1993; Birkedal-Hansen, 1995).

Collagenase-2 (MMP-8) is also called neutrophil collagenase due to its original identification in human neutrophils as a distinct protein (Macartney and Tschesche, 1983; Hasty et al., 1984) and gene product (Hasty et al., 1990) from MMP-1. However, the collagenase activity in human neutrophil was first described in the late 1960s (Lazarus et al., 1968). One feature unique to MMP-8 is that this enzyme is stored in specific granules in neutrophil leukocytes (Murphy et al., 1977) and released upon phagocytic stimulation (Oronsky et al., 1973). In addition, although the function is still unclear, there is a high level of glycosylation in MMP-8 (Birkedal-Hansen et al., 1993). The reported molecular weight of MMP-8 varies depending on the investigators. This may be due to method of determination (purified protein vs. deduced from cDNA). In the purified protein, the calculated molecular weight may be affected by the level of glycosylation on the enzyme. In general, it is believed that the latent form of MMP-8 is approximately 75 kDa, but it is usually processed during extraction protocols to about 58 kDa (this form still lacks catalytic activity). The active form of MMP-8 has a molecular mass of around 40-42 kDa. In addition to its expression in polymorphonuclear leukocytes during their maturation (Hasty et al., 1987), MMP-8 is also produced in melanoma cells (Giambernardi et al., 1998), rheumatoid synovial fibroblasts (Hanemaaijer et al., 1997), endothelial cells, and chondrocytes (Cole et al., 1996). Furthermore, its high expression in rat postpartum uterus indicates that it may play a potential role during involution of this reproductive organ (Balbin et al., 1998). This enzyme displays a substrate specificity profile different from that of MMP-1 (Hasty et al., 1987). The preferred substrate for MMP-8 is type I collagen, not type III collagen, which is digested at a higher rate by MMP-1 (Hasty et al., 1987). Intriguingly, MMP-8 also degrades collagen type II at a much greater (~ 150 times) rate than MMP-1 (Knauper et al., 1997b; Jeffery, 1998), indicating that it may be involved in diseases such as rheumatoid arthritis and osteoarthritis, where type II collagen degradation plays a major role. Moreover, MMP-8 is
capable of cleaving aggrecan (Fosang et al., 1996a; Arner et al., 1997), but at sites distinct from the major enzyme called cartilage aggrecanase (Arner et al., 1997).

Collagenases 3, designated as MMP-13, was first sequenced from human breast tumor (Freije et al., 1994). The latent and active forms of MMP-13 possess a molecular mass of about 52 and 42 kDa, respectively. Unlike MMP-1, which has a low activity on type II collagen, MMP-13 cleaves type II collagen at a higher rate than types I and III. Similar to MMP-8, this efficient catalytic action of MMP-13 on type II collagen signifies its importance in the physiology and pathology of cartilage, where type II collagen is the predominant ECM protein. Additionally, MMP-13 displays considerably greater gelatinolytic activity than collagenolytic activity (Welgus et al., 1985a; Mitchell et al., 1996). Of particular interest, MMP-13 has broad substrate specificity. MMP-13 also catalyzes type IV, IX, X and XIV collagens, laminin, fibrillin-1, fibronectin, tenascin, aggrecan, and inhibitors of serine proteinases (Fosang et al., 1996a; Knauper et al., 1996a; Knauper et al., 1997a; Ashworth et al., 1999). Thus, in addition to its original localization in breast carcinomas (Freije et al., 1994; Heppner et al., 1996; Uria et al., 1997), MMP-13 is expressed in a variety of tumor types, including chondrosarcomas (Uria et al., 1998), melanomas (Airola et al., 1999), and squamous cell carcinomas (Johansson et al., 1997; Cazorla et al., 1998). It is also associated with a number of diseases, where excessive collagen degradation has been postulated to be causative, such as periodontitis (Uitto et al., 1998), rheumatoid synovium (Stahle-Backdahl et al., 1997), osteoarthritic cartilage (Mitchell et al., 1996; Shlopov et al., 1997), and atherosclerosis (Sukhova et al., 1999). Interestingly, this enzyme is also highly expressed in the uterus (Welgus et al., 1985b), suggesting its potential roles during tissue remodeling of this reproductive organ.

Collagenase 4 (MMP-18) was recently identified in the African clawed toad, *Xenopus laevis* (Stolow et al., 1996). This is also the first sequenced MMP from an amphibian. The predicted molecular masses of the latent and active forms of MMP-18 are 51 and 42 kDa, respectively (Stolow et al., 1996). Collagenase 4 has the ability to degrade type I collagen (Stolow et al.,
1996), rat type II and human type III collagens, α1-antitrypsin, and α2-macroglobulin (Shi and Sang, 1998). MMP-18 is transiently expressed in the region of branchial arches, thorax, and the dorsal anterior axis during the late stages of Xenopus embryogenesis (Damjanovski et al., 1999; Damjanovski et al., 2000; Damjanovski et al., 2001). The embryo lethality during the late stages of embryogenesis caused by over-expression of MMP-18 further indicates the restricted temporal and spatial expression of this enzyme (Damjanovski et al., 2001). The homologs of collagenase 4 in other species have not been identified.

(2) Gelatinases There are two members in this group: gelatinase A and gelatinase B. Although a gelatinase activity, which was retrospectively determined to be gelatinase B, was detected from rheumatoid synovial fluid in 1972 (Harris and Krane, 1972), it was about six years later that gelatinase activity was first separated from collagenase and stromelysin (Sellers et al., 1978). Whereas collagenase was the first described MMP to degrade native collagen into fragments (Gross and Lapiere, 1962), gelatinases have activity against denatured collagens (or collagen fragments) (Sopata et al., 1974). Based on their sizes and substrate specificities, these two identified gelatinases are named 72-kDa gelatinase (gelatinase A, MMP-2) and 92-kDa gelatinase (gelatinase B, MMP-9), respectively. The early experiments demonstrated that both gelatinases were also able to digest soluble type IV collagen, producing characteristic ¼ N-terminal and ¾ C-terminal fragments (Liotta et al., 1979; Mainardi et al., 1980; Fessler et al., 1984; Murphy et al., 1989). Therefore, gelatinase A and B are also called 72-kDa and 92-kDa type IV collagenase, respectively. However, it has been questioned whether MMP-2 and MMP-9 are true type IV collagenases in vivo since the full-length type IV collagen is not susceptible to cleavage by either enzyme (Mackay et al., 1990; Moll et al., 1990). Structurally, gelatinases are characterized by three repeats of type II fibronectin-like gelatin binding regions, which enable gelatinases to bind to denatured collagens (O'Farrell and Pourmotabbed, 1998).

The 72-kDa gelatinase A (MMP-2) is processed to an approximately 62 kDa active enzyme via a membrane-dependent mechanism, while most other MMPs are activated extracellularly.
(Brown et al., 1990; Strongin et al., 1993). In addition to gelatin and type IV collagen, MMP-2 possesses proteolytic actions on type I (Aimes and Quigley, 1995), type V (Okada et al., 1990), type VII (Seltzer et al., 1989), and type X (Gadher et al., 1989; Welgus et al., 1990) collagens, and other ECM components such as elastin (Senior et al., 1991), laminin (Giannelli et al., 1997), vitronectin (Imai et al., 1995a), and aggrecan (Fosang et al., 1992; Fosang et al., 1996b).

Surprisingly, MMP-2 also cleaves the non-ECM component amyloid protein precursor with a β-secretase-like activity (LePage et al., 1995), indicating its involvement in Alzheimer's disease. It is because of this wide range of substrate specificities that enables MMP-2 to play important roles in a number of cellular processes, such as cell proliferation, differentiation, adhesion, and migration. Most importantly, MMP-2 also has a significant role in tumor invasion and angiogenesis. It binds integrin α2β1, facilitating the pericellular proteolysis during tumor invasion and angiogenesis (Brooks et al., 1996).

In addition to the cysteine-rich repeats of type II fibronectin-like inserts in the catalytic domain, MMP-9 also has a unique proline-rich type V collagen-like insert at the end of its hinge region; the significance of which is still unclear (Wilhelm et al., 1989). The predicted molecular weight of MMP-9 from its cDNA is about 76 kDa. However, the molecular weight determined by biochemical analyses is about 92 kDa. This difference is due to N- and O-linked glycosylation on this enzyme (Wilhelm et al., 1989; Murphy and Crabbe, 1995). The active form of MMP-9 is approximately 82 kDa, but progressive proteolytic and/or autolytic cleavage on the N- and C-termini of the enzyme may process it into 65 kDa or even smaller forms (Fridman et al., 1995; Murphy and Crabbe, 1995; Sang et al., 1995). MMP-9 shares a similar range of substrates with MMP-2 (Table 1-3). Distinct from MMP-2, MMP-9 is not able to hydrolyze type I collagen. However, it has been reported that MMP-9 cleaves the N-terminal telopeptide of type I collagen in an acidic environment (Okada et al., 1995bb). Although MMP-9 degrades aggrecan, and link protein, which stabilizes the interaction between aggrecans and hyaluronate in proteoglycan
aggregates, its efficiency is much lower than MMP-2 (Fosang et al., 1992; Nguyen et al., 1993). MMP-9 is also localized on the cell surface, where CD-44 serves as a docking protein to promote tumor invasion and angiogenesis (Yu and Stamenkovic, 1999; Yu and Stamenkovic, 2000). Accumulating evidence indicates that MMP-9 is a major MMP in a variety of normal physiological processes such as implantation (Librach et al., 1991; Behrendtsen et al., 1992), and bone development (Reponen et al., 1994; Blavier and Delaisse, 1995; Vu and Werb, 2000). Not surprisingly, high levels of MMP-9 expression are also observed in pathological conditions, including tissue injury (Partridge et al., 1993; La Fleur et al., 1996; Pender et al., 1997), inflammation (Weeks et al., 1993; Leppert et al., 1995a; Leppert et al., 1995b; Opdenakker et al., 2001), arthritis (Stein-Picarella et al., 1994), and wound healing (Salo et al., 1994; Buisson et al., 1996), and tumor angiogenesis (Bergers et al., 2000). Additionally, its expression has been demonstrated in tumor cells from diverse sites of the body including colorectum (Pyke et al., 1993; Nielsen et al., 1996), bone (Vu et al., 1998), bone marrow (Ries et al., 1994), brain (Rao et al., 1993), breast (Farias et al., 2000), liver (Ashida et al., 1996), lungs (Canete-Soler et al., 1994; Farias et al., 2000), skin (Pyke et al., 1992), and prostate (Dong et al., 2001).

(3) Stromelysins The stromelysins include stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10). These two enzymes are classified into their own subgroup because they lack the ability to cleave the triple helical regions of collagen, even though they share a similar domain structure with the collagenases (Stemlicht and Werb, 2001). Furthermore, they are distinguished from other MMPs by their amino acid sequences being noticeably similar to each other, but not to other MMPs (Muller et al., 1988). Lastly, they possess a 9-amino acid residue insert in the hinge region, which is absent from other MMPs.

The first neutral non-collagenolytic proteinase activity was noticed in human cartilage (Sapolsky et al., 1974) and in the conditioned medium from rabbit synovial fibroblasts (Werb and Reynolds, 1974) at the same time by two independent research groups. The enzyme was later named “stromelysin” because it was produced by stromal cells (Chin et al., 1985). Historically,
stromelysin was also known as “collagenase activator protein” (Treadwell et al., 1986) and “transin” (Matrisian et al., 1986). cDNA cloning ultimately determined that the three proteins were the same metalloproteinase, MMP-3 (stromelysin 1) (Matrisian et al., 1985; Whitham et al., 1986; Fini et al., 1987; Wilhelm et al., 1987; Saus et al., 1988). The latent form of MMP-3 has a molecular mass of 56 kDa, while the active form is about 45 kDa (Nagase, 1995; Nagase, 1998). Asn-linked glycosylation is also observed in MMP-3 (Wilhelm et al., 1987). However, not all the secreted MMP-3 is glycosylated, for example, 80% of MMP-3 from fibroblasts is unglycosylated, while the remaining ones are produced as a glycoprotein (Nagase, 1995). MMP-3 hydrolyzes numerous ECM components including aggrecan (Wilhelm et al., 1993), cartilage link protein (Nguyen et al., 1989), gelatin (Murphy et al., 1992a), vitronectin (Imai et al., 1995a), tenascin (Imai et al., 1994), fibronectin (Muir and Manthorpe, 1992), versican (Perides et al., 1995), elastin (Murphy et al., 1992a), laminin (Bejarano et al., 1988), perlecan (Whitelock et al., 1996), proteoglycan (Fosang et al., 1991), and type II, IX, X, and XI collagens (Wu et al., 1991). However, it is unable to hydrolyze triple-helixal collagen peptide (Lauer-Fields et al., 2000).

Stromelysin 2 (MMP-10) was the first MMP to be identified directly from rat cDNA clones (Breathnach et al., 1987). The deduced protein was also designated as transin 2, which has 71% identity to MMP-3 (transin 1) at the amino acid level. The human MMP-10 was subsequently cloned from human tumors (Muller et al., 1988). MMP-10 is capable of activating pro-MMP-8 (Knauper et al., 1994). There is limited information about the substrate specificity of MMP-10, except its ability to effectively degrade cartilage link protein (Nguyen et al., 1993) and gelatin (Sanchez-Lopez et al., 1993). In human endometrium, MMP-10 mRNA expression increases during premenstrual and menstrual phases (Hampton and Salamonsen, 1994; Osteen et al., 1994). The function of MMP-10 during tumor progression is still not clear.

(4) Membrane-type metalloproteinases (MT-MMPs) At present, there are six membrane-type metalloproteinases: MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), MT5-MMP (MMP-24), and MT6-MMP (MMP-26). MT-
MMPs contain a cleavage site for furin proteinases between the propeptide and catalytic domains, providing the basis for furin enzyme-like dependent activation of latent MT-MMPs prior to secretion.

The notion that pro-MMP-2 is activated on the cell membrane rather than in the extracellular environment led to the discovery of MT-MMPs. MT1-MMP, the first member of the membrane-type MMP subfamily, was cloned from a human placenta cDNA library (Sato et al., 1994). Distinct from all other MMPs that have been identified, this enzyme has a hydrophobic transmembrane segment at the C-terminus following the hemopexin domain. Indeed, MT1-MMP activates pro-MMP-2 (Sato et al., 1994). Subsequent studies suggest that this activation process also involves TIMP-2, which binds to MT1-MMP to form a MT1-MMP/TIMP-2 “co-receptor” for pro-MMP-2. The latter is then presented to an adjacent, active MT1-MMP, which cleaves the propeptide domain of pro-MMP-2, initiating the activation process (Deryugina et al., 1997; Butler et al., 1998; Cao et al., 1999; Murphy et al., 1999b). In addition, MT1-MMP converts latent MMP-13 (collagenase-3) to the active form (Knauper et al., 1996c). Moreover, MT1-MMP also functions as a proteolytic enzyme, capable of cleaving various ECM components, including type I, II, and III collagen, gelatin, fibronectin, laminin-1, vitronectin, cartilage proteoglycans, and fibrillin-1 (Imai et al., 1997; Pei and Weiss, 1996; d’Ortho et al., 1997; Ohuchi et al., 1997; Aznavoorian et al., 2001).

MT2-MMP was identified from a human lung cDNA library (Will and Hinzmann, 1995). MT2-MMP is predominantly expressed in liver, placenta, intestine, colon, and testis (Will and Hinzmann, 1995). MT2-MMP also activates pro-MMP-2. Different from MT1-MMP, however, MT2-MMP activates pro-MMP-2 via a pathway independent of TIMP-2 (Morrison et al., 2001). MT2-MMP also degrades laminin, fibronectin, and tenascin (d’Ortho et al., 1997).

Having been identified at the same time as MT2-MMP, MT3-MMP was originally designated as MT-MMP-2 (Takino et al., 1995). MT3-MMP is primarily expressed in the brain. MT3-MMP activates pro-MMP-2 and also hydrolyzes gelatin, casein, type III collagen, and...
fibronectin (Shimada et al., 1999; Kang et al., 2000), and can be detected in membrane-bound and soluble forms (Matsumoto et al., 1997; Shofuda et al., 1997).

In addition to its original identification in breast carcinoma, MT4-MMP is also expressed in normal tissues including brain, colon, ovary, and testis (Puente et al., 1996). MT4-MMP is structurally different from other MT-MMPs. Instead of having a 20-amino acid tail after the transmembrane domain (as observed in other MT-MMPs), MT4-MMP has a very short or no cytoplasmic tail following the putative transmembrane domain (Itoh et al., 1999). Further sequence and biochemical analyses suggest that the C-terminus of MT4-MMP acts as a glycosylphosphatidylinositol (GPI) anchoring signal, rather than as a transmembrane domain (Itoh et al., 1999). MT4-MMP is the first GPI-anchored proteinase in the MMP family. This property enables it to be shed from plasma membranes by endogenous enzymes. MT4-MMP converts membrane-bound pro-TNFα to its mature form (Wang et al., 1999b; English et al., 2000). There are contradictory reports regarding its capability to activate pro-MMP-2 (Kolkenbrock et al., 1999; Wang et al., 1999b; English et al., 2000). MT4-MMP is unable to hydrolyze collagen types I, II, III, IV, and V, fibronectin, laminin or decorin, but degrades gelatin and some synthetic MMP substrates (Kolkenbrock et al., 1999; Wang et al., 1999b).

MT5-MMP, originally identified from a human brain cDNA library, activates latent MMP-2 and is predominantly expressed in brain, kidney, pancreas, and lung (Llano et al., 1999; Pei, 1999a). It is highly expressed in brain tumors (Llano et al., 1999). Interestingly, MT5-MMP is also shed from the cell membrane by a furin type convertase, suggesting that it may function both as a membrane-bound and soluble proteinase (Pei, 1999a; Wang and Pei, 2001). Proteoglycans are the preferred substrates for MT5-MMP (Wang et al., 1999a).

MT6-MMP was cloned from leukocytes (Pei, 1999b). Thus, it is also called leukolysin (Pei, 1999b). High levels of MT6-MMP expression was also observed in brain tumors (Velasco et al., 2000). MT6-MMP is the second GPI-anchored enzyme in the MMP family, showing a similar C-terminal sequence as MT4-MMP (Kojima et al., 2000). MT6-MMP activates pro-MMP-2
and cleaves type-IV collagen, gelatin, fibronectin and fibrin, but not laminin-1 (Pei, 1999b; English et al., 2001; Kang et al., 2001). Cytokines may play an important role in processing this enzyme from secretory vesicles and plasma membrane to the extracellular milieu as a soluble, active protein (Kang et al., 2001).

5) Matrilysins  Currently, two members have been identified in this subfamily, matrilysin 1 (MMP-7) and matrilysin 2 (MMP-26). They are the smallest MMPs since they lack the hemopexin domain.

MMP-7 was first purified from the postpartum involuting rat uterus (Sellers and Woessner, 1980; Woessner and Taplin, 1988). Its cDNA was cloned and sequenced from a human tumor library (Quantin et al., 1989). This enzyme was also previously named as PUMP-1 (putative metalloproteinase-1). Due to its simple domain organization, MMP-7 is small in size, with a 28 kDa latent and 19 kDa active forms (Wilson and Matrisian, 1998). However, the minimal domain structure does not affect its broad substrate specificities at all. MMP-7 hydrolyzes type IV collagen (Murphy et al., 1991), gelatins (Quantin et al., 1989), laminin (Imai et al., 1995a), fibronectin (Imai et al., 1995b), elastin (Imai et al., 1995b), vitronectin (Imai et al., 1995a), aggrecan (Fosang et al., 1992), cartilage link protein (Nguyen et al., 1993), proteoglycan (Imai et al., 1995b; Halpert et al., 1996), fibulin (Sasaki et al., 1996), and entactin (Sires et al., 1993).

MMP-7 is involved in many normal tissue remodeling events by being expressed in the uterus (Sellers and Woessner, 1980), hair follicles and sweat glands, small intestinal crypts, skin, and airway epithelium (Giambemardi et al., 1998; Wilson and Matrisian, 1998). Distinct from other MMPs, expression of MMP-7 is restricted to glandular epithelium, and is secreted apically. MMP-7 is also expressed by malignant epithelial cells in tumors of the breast (Heppner et al., 1996), prostate (Klein et al., 1997), colon (Yoshimoto et al., 1993), lung (Bolon et al., 1997), and gastrointestinal tract (Adachi et al., 2001). This is unlike other MMPs, whose source is the stromal cells that surround epithelial tumors, rather than the tumor cells themselves (Wilson and Matrisian, 1996; Wilson et al., 1997).
A novel metalloproteinase (MMP-26) has been recently identified independently by several
groups (de Coignac et al., 2000; Park et al., 2000; Uria and Lopez-Otin, 2000; Marchenko et al.,
2001). Even though this enzyme has a high homology (45% identity) with MMP-12 (macrophage
metalloproteinase), its protein structure is more related to MMP-7 (matrilysin) by having the
minimal domain organization, which consists of only the signal peptide, propeptide and catalytic
domains (de Coignac et al., 2000; Marchenko et al., 2001). Furthermore, MMP-26 also contains a
Thr(207) residue upstream of the zinc binding motif, which is a specific feature of all known
matrilysins (Abramson et al., 1995; Uria and Lopez-Otin, 2000; Marchenko et al., 2001).
Accordingly, the name matrilysin-2 has been suggested for this novel MMP (Uria and Lopez-
Otin, 2000; Marchenko et al., 2001). The name “endometase” has also been used for this enzyme
as it is derived from a human endometrial tumor cDNA library (Park et al., 2000). Distinct from
other MMPs, MMP-26 has a unique “cysteine switch” motif sequence PHCGVPD, which
replaces the highly conserved motif PRCGXXD, indicating its activation may be sensitive to pH
(Marchenko et al., 2001). Of note, MMP-26 may not be a classical matrilysin in that there is low
sequence similarity between MMP-26 and MMP-7. Also, they are located on different
chromosomes and they have dissimilar predicted surface modeling. MMP-26 has a diverse array
of substrates, including gelatins, fibronectin, fibrogen, vitronectin. However, MMP-26 is unable
to degrade types I-IV collagen, tenascin C, and laminin V (Uria and Lopez-Otin, 2000;
Marchenko et al., 2001). MMP-26 is highly expressed in the placenta (de Coignac et al., 2000;
Park et al., 2000), the uterus (Uria and Lopez-Otin, 2000) and a number of developing fetal
tissues (Marchenko et al., 2001). Expression of MMP-26 in carcinomas of the endometrium, lung,
and prostate suggests its unique role in the progression of epithelial tumors (Uria and Lopez-Otin,
2000).

(6) Other MMPs Based on structure and substrate specificity, several MMPs do not fit into
any of the subgroups describe so far, although some investigators argue that matrilysin and
macrophage metalloelastase should be included in the group of stromelysins.
Stromelysin-3 (MMP-11) was first cloned from a breast carcinoma cDNA library. Its expression is restricted to the stromal cells surrounding the tumor cells (Basset et al., 1990). While stromelysins 1 and 2 share a 76% identity to each other in amino acid sequence, MMP-11 only shows 39% and 36% sequence identity to other stromelysins and other MMPs, respectively (Sang and Douglas, 1996). Structurally, MMP-11 is also different from stromelysins 1 and 2. MMP-11 contains a 10-amino acid residue insert (RXK/RR) at the end of the propeptide domain, which resembles a furin-like recognition site (Pei and Weiss, 1995). Indeed, intracellular activation of pro-MMP-11 by Golgi-associated furin enzyme, which is different from the extracellular activation of other MMPs, was observed (Pei and Weiss, 1995). The just described characteristics distinguish MMP-11 (stromelysin-3) from the subfamily of stromelysins.

Although MMP-11 cleaves some ECM proteins such as type IV collagen, laminin, fibronectin, and aggrecan, its efficiency is much lower than stromelysin-1 (Murphy et al., 1993b). However, MMP-11 is critical for tumor progression, since MMP-11-deficient mice are resistant to chemically-induced tumors (Noel et al., 1996; Basset et al., 1997). It has also been postulated that expression of MMP-11 in adjacent cells is pivotal for the epithelial-to-mesenchymal conversion of tumor cells (Ahmad et al., 1998). This tumor promoting capability of MMP-11 may result from its ability to release mature or active growth factors from the corresponding latent forms sequestered in the extracellular matrix (Basset et al., 1997).

Macrophage metalloelastase (MMP-12) was first purified from the mouse as a 22 kDa enzyme (Banda and Werb, 1981), which is processed from its 54 kDa latent form by conventional propeptide removal and atypical processing at the C-terminus (Shapiro et al., 1992). Cloning and sequence analysis of mouse and human MMP-12 revealed that this enzyme has a domain structure typical of collagenases, but it only shares 33-48% homology with other MMPs at the amino acid level (Shapiro et al., 1992; Shapiro et al., 1993). Although MMP-12 is unable to hydrolyze interstitial collagens, it cleaves a wide array of ECM components including elastin, type IV collagen, type I gelatin, vitronectin, fibronectin, proteoglycans, laminin-1, and myelin.
basic protein (Chandler et al., 1996; Gronski et al., 1997). MMP-12 has been related to a number of macrophage-involved diseases such as pulmonary emphysema (Hautamaki et al., 1997) and Lewis lung carcinoma (Dong et al., 1997).

Human MMP-19 was the first MMP identified by using the EST (expressed sequence tags) database search, although its 5′ end was determined by 5′-RACE (Cossins et al., 1996). These investigators called their enzyme MMP-18, without being aware that the then newly identified Xenopus MMP was already given this designation. Two identical cDNAs, which were designated as MMP-19, were later cloned from a human liver cDNA library and a library constructed from rheumatoid arthritis synovium (Kolb et al., 1997; Pendas et al., 1997b). Since the latter research was done on rheumatoid arthritis synovium inflammation, the enzyme was also called RASI-1 (Kolb et al., 1997). MMP-19 lacks all the specific structural features of collagenases, gelatinases, and membrane-type metalloproteinases. For example, MMP-19 has a 16-acidic residue insert in the hinge region rather than the 9-residue insertion rich in hydrophobic amino acids that is specific for stromelysins (Pendas et al., 1997b). MMP-19 is a potent basement membrane enzyme capable of hydrolyzing type IV collagen, laminin, fibronectin, type I gelatin, nidogen, tenascin, aggrecan, and cartilage oligomeric matrix protein (COMP) (Stracke et al., 2000a; Stracke et al., 2000b). MMP-19 is highly expressed in intestine, ovary, spleen, heart, pancreas, lung, thymus, testis, prostate, and placenta, indicating that it may play a role in remodeling events in these tissues (Cossins et al., 1996; Pendas et al., 1997b). Furthermore, the expression of MMP-19 in capillary endothelial cells of acutely but not chronically inflamed synovium, demonstrates that it may be involved in angiogenesis (Kolb et al., 1999). Interestingly, MMP-19 expression is observed in tumor and endothelial cells of benign tumors, while it disappears as the tumor progresses towards the invasive and neoplastic phenotypes (Djonov et al., 2001). This suggests that MMP-19 may be important for tumor survival but not progression.

Enamelysin (MMP-20) was first cloned from a cDNA library of porcine enamel organ (Bartlett et al., 1996). Its human homolog was subsequently cloned from an odontoblastic cell.
cDNA library (Llano et al., 1997). The deduced MMP-20 protein consists of 483 amino acid residues, bearing a 54 kDa molecular mass. Although MMP-20 has the characteristic domain organization of MMPs, the specific structural features of collagenases, gelatinases, membrane-type MMPs, and stromelysins are absent in MMP-20. MMP-20 degrades amelogenin, casein, aggrecan, and cartilage oligomeric matrix protein (COMP), but has weak action against gelatin (Llano et al., 1997; Stracke et al., 2000a). The restricted expression of MMP-20 signifies its importance in the processing of enamel matrix during tooth enamel formation (Grant et al., 1999). Moreover, MMP-20 expression is also detected in odontogenic tumors and tongue carcinomas (Takata et al., 2000; Vaananen et al., 2001). Intriguingly, MMP-20 is expressed in granulosa cells, and is highly elevated by bradykinin treatment, indicating its specialized role during ovulation (Kimura et al., 2001).

MMP-21 (XMMP) was first cloned from *Xenopus laevis* (Yang et al., 1997). Different from other MMPs, MMP-21 has a 37-amino acid-residue insert at the c-terminal end of the propeptide domain. This insert, resembling a vitronectin segment, is followed by an RRKR motif, a furin-like recognition site that is present in stromelysin-3 and MT-MMPs. In addition, MMP-21 lacks the hinge region, which is typically present in other MMPs for connecting the catalytic and hemopexin domains. Another specific feature of MMP-21 is that its C-terminal domain consists of four repeats of vitronectin-like segments (Yang et al., 1997). The expression of MMP-21 is transiently induced at the gastrula stage, maintained at the neurula stage, but down-regulated in the pre-tailbud embryo, suggesting its unique role during early embryo development of *Xenopus laevis*. The human homolog of MMP-21, which shows 73% homology to XMMP at the amino acid level, has been recently identified (Marchenko et al., 2001). However, its substrate specificity, and involvement in physiological and pathological processes require further investigation.

MMP-22 (CMMP) was characterized in chicken embryo fibroblasts (Yang and Kurkinen, 1998). In the catalytic domain, MMP-22 has a unique cysteine residue in the sequence motif that
interacts with the structural (noncatalytic) zinc ion. A homologous cysteine residue is also present in XMMP (Yang et al., 1997) and MMP-19 (Cossins et al., 1996; Pendas et al., 1997b). The recombinant MMP-22 enzyme digests casein and gelatin (Yang and Kurkinen, 1998), suggesting that it possesses collagenolytic and gelatinolytic activities. The presence of MMP-22 in mammalian species is not known at this time.

MMP-23 was first cloned from human uterus and testis cDNA libraries, but it was named MMP21/22 by the investigators (Gururajan et al., 1998a; Gururajan et al., 1998b). The identical sequence was re-discovered from a human ovary cDNA library, bearing the name MMP-23 (Velasco et al., 1999). The mouse and rat homologs were also reported at the same time (Pei, 1999c; rat homolog with GenBank accession number AB010960 by Ohnishi et al., 1999). In all of these species, the domain organization of MMP-23 is strikingly different from all other MMPs in that it lacks not only a recognizable signal peptide at the N-terminus and the cysteine switch consensus PRCGVPD in the propeptide domain, but also the hemopexin-like sequence at the C-terminal end, which is replaced by an Ig-like C2 type (or IL1R-like) domain (Pei, 1999c; Velasco et al., 1999). Although the cysteine switch motif is absent in MMP-23, the enzyme contains a conserved furin recognition sequence (RRRR), which is also present in MMP-11 (stromelysin-3) and MT-MMPs, suggesting it has an activation mechanism different from other MMPs (Pei, 1999c; Velasco et al., 1999). Surprisingly, a cysteine array motif, which is located downstream of the catalytic domain, is noted in mouse MMP-23 (Pei, 1999c), while it is not mentioned in the human homolog (Velasco et al., 1999). Thus, this enzyme is also named cysteine array matrix metalloproteinase or CA-MMP (Pei, 1999c). Even though the typical signal peptide is absent, MMP-23 contains a hydrophobic segment at the N-terminal end (Pei, 1999c). Studies demonstrate that this hydrophobic region serves as a signal anchor to localize MMP-23, enabling it to act as a type II transmembrane MMP [type I transmembrane has either a transmembrane segment (MT1, 2, 3, 5-MMPs) or a glycosylphosphatidylinositol region (MT4, 6-MMPs) at the C-terminus], which couples enzyme secretion and activation by a single proteolytic cleavage (Pei...
et al., 2000). This novel mechanism may establish a new pathway for regulating MMP trafficking. The recombinant MMP-23 degrades a common synthetic MMP substrate and gelatin (Pei, 1999c; Velasco et al., 1999). In addition to its expression in adult tissues such as heart and lung, MMP-23 is predominantly expressed in reproductive tissues including prostate, ovary, and testis. However, its expression in placenta is relatively low or negative (Pei, 1999c; Velasco et al., 1999). The expression of MMP-23 in the ovary will be discussed in a later section.

MMP-27 is a newly identified member in the MMP family, and has a domain organization characteristic of collagenases. However, although the sequence information has been reported in the NCBI (GenBank accession number NM_022122 by de Coignac), there are no reports regarding the biochemical and molecular properties, or tissue expression and cellular localization of this enzyme.

MMP-28 (Epilysin) is a novel MMP member identified independently by two research laboratories (Lohi et al., 2001; Marchenko and Strongin, 2001). Since this novel endopeptidase is prominently expressed in the epidermis, it is appropriately designated as “epilysin”. MMP-28 contains the characteristic signal peptide, propeptide, catalytic, hinge, and hemopexin domains, characteristic of most MMPs. It also has several peptide sequences typical of MMPs, such as the conserved cysteine switch sequence PRCGVTD and the catalytic domain sequence HEIGH (Lohi et al., 2001; Marchenko and Strongin, 2001). Unlike other MMPs, however, a unique feature of its catalytic domain is that it has threonine residues (HEIGHTLGLTH). Structurally, MMP-28 is similar to MMP-11 (stromelysin-3) in that it has a furin recognition sequence (RRKRR) following the cysteine switch sequence. But phylogenetic analysis on the catalytic domains of MMP-28 and other MMPs demonstrates that this enzyme is most closely related to MMP-19, which shows 46% identity to MMP-28 (Lohi et al., 2001). Therefore, it has been postulated that MMP-28 is the first member of the MMP-19 subgroup (Marchenko et al., 2001). The recombinant MMP-28 protein has caseinolytic activity, which can be completely inhibited by EDTA and a synthetic MMP inhibitor, batimastat (Lohi et al., 2001). In addition to its strong
expression in keratinocytes, MMP-28 expression is also observed in a broad range of fetal tissues (lung, brain, skeletal muscle, and kidney), adult tissues (kidney and pancreas), and a number of carcinomas including colon adenocarcinoma, ovarian carcinoma, and pancreatic adenocarcinoma (Marchenko et al., 2001). This suggests that this novel MMP may have a significant role in normal tissue remodeling and tumor progression.

2. Regulation of MMP activity

MMP activities are pivotal for normal physiological processes. While over-expression of these enzymes typically results in a variety of diseases and pathological conditions. Therefore, the temporal expression and spatial localization of MMPs need to be closely coordinated with activity. This requires strict regulation of MMPs, which occurs at different levels.

1) Transcriptional regulation

As a general rule, MMPs are not constitutively expressed in most tissue types. Instead, the transcriptional expression of MMPs can be induced by a variety of growth factors and cytokines, while their expression can also be suppressed, for example, by transforming growth factor beta (TGFβ) and glucocorticoids (Matrisian, 1990). Other extracellular signals regulating MMP expression also include extracellular matrix proteins, integrin-derived signals, and cell stress factors.

The regulation by the above extracellular signals is ultimately accomplished by acting on the promoters of MMP genes (Borden and Heller, 1997). The most common promoter found in MMP genes is the AP-1 cis-element, which is usually localized at 65 to 79 base pairs upstream of the transcriptional start site. MMP-1, -3, -7, -9, -10, -12, -13, and -19 are under the regulation of the AP-1 promoter (Gack et al., 1994; Quinones et al., 1994; Korzus et al., 1997; Pendas et al., 1997a; Chapman et al., 1999; Mueller et al., 2000; Campbell et al., 2001; Wu et al., 2001). Other inducible cis-regulatory elements, such as Ets, SP1, SP3, and NF-κB, are also present in the promoter region of most MMPs (Borden and Heller, 1997). Thus, transcriptional regulation of
MMPs is complex, since there are numerous signal transduction pathways for extracellular signals, and one promoter may be affected by various signaling components. For example, the AP-1 cis-element is switched on when an AP-1 transcription factor complex, which consists of members of Jun and Fos proteins, is attached (Curran and Franza, 1988). c-Jun and c-Fos components are under the regulation of at least three distinct signaling pathways, including mitogen-activated protein kinases (MAPKs), stress-activated protein kinase/Jun N-terminal kinases (SAPK/JNKs), and p38 (Heldin, 1995). Interactions between multiple signaling components may, therefore, influence the expression pattern of MMPs (Korzus et al., 1997; Simon et al., 2001).

Different from most other MMPs, MMP-2 is often constitutively expressed at the transcriptional level. Gene structure analysis found that the MMP-2 promoter has high similarity with the other two molecules that are involved in its activation process: TIMP-2 and MT1-MMP. In vivo studies further demonstrated that MMP-2, MT1-MMP, and TIMP-2 are coordinately expressed in a number of tissue types (Apte et al., 1997; Chen and Wang, 1999; Kanwar et al., 1999; Kurschat et al., 1999; Longin et al., 2001), suggesting that not all members of this protein family are regulated at the same levels.

(2) Activation of latent MMPs As potent proteolytic enzymes on various ECM components, MMPs are also tightly regulated at the activational level. MMPs are produced as latent forms, and most are subsequently activated by extracellular proteolytic cleavage. The latency of MMPs is maintained by the cysteine switch consensus sequence in the propeptide domain. The conserved cysteine residue covalently interacts with the catalytic zinc ion in the active core of the enzyme. The latter is thus prevented from interacting with a water molecule (Van Wart and Birkedal-Hansen, 1990). Either proteolytic cleavage on the propeptide by proteinases, or chemical modification on the SH group of cysteine residues, can bring about disruption of the Zn$^{2+}$-Cys interaction, allowing the catalytic zinc ion to interact with H$_2$O (Nagase, 1997). The activity of MMPs is attained by this intramolecular reaction. The final step
may also involve autocatalytic removal of the propeptide. Chemical modification is achieved by reagents such as 4-aminophenylmercuric acetate (APMA), HOCL, and denaturants. Also, the propeptide of most MMPs is susceptible to cleavage by extracellular proteinases such as trypsin, plasmin, chymotrypsin, and MMPs themselves (Nagase, 1997; Murphy et al., 1999b; Nagase and Woessner, 1999). Therefore, these proteinases may serve as endogenous activators of MMPs.

Unlike other MMPs, MMP-11 (stromelysin-3) and all MT-MMPs have an additional 10-amino acid residue furin-like enzyme recognition motif (RxK/RR) at the C-terminal end of the propeptide domain (Pei and Weiss, 1995). The same insert is also observed in a novel member of the MMP family, MMP-23, which does not possess the typical cysteine switch consensus (PPCG[V/N]PD) in the propeptide domain (Pei, 1999c). This structural feature indicates that these MMPs can possibly be activated intracellularly rather than extracellularly, by furin, a Golgi-associated subtilisin-like proteinase. Indeed, it has been demonstrated that furin is able to process latent MMP-11 to its active form (Pei and Weiss, 1995). Furthermore, this processing can be stopped by synthetic furin inhibitors (Santavicca et al., 1996). The transmembrane-deleted MT-MMP1 can also be activated intracellularly (Pei and Weiss, 1996). Intracellular processing of MMP-23 is also observed (Pei et al., 2000; Ohnishi et al., 2001), even though furin is unable to enhance MMP-23 processing in a transfection model (Ohnishi et al., 2001).

Notably, although MMP-2 harbors the characteristic cysteine switch motif in its propeptide domain, it is resistant to cleavage by many proteinases that are able to activate most other MMPs. Instead, it is activated by membrane-type metalloproteinases (MT-MMPs) (Sato et al., 1994; Sato and Seiki, 1996; Murphy et al., 1999a; Seiki, 1999). Among them, MT1-MMP is the most studied. Efficient activation of pro-MMP-2 by MT1-MMP requires TIMP-2, which forms a trimeric complex by binding to MT1-MMP to form a "co-receptor" for pro-MMP-2. On the cell surface, an adjacent active MT1-MMP then cleaves the propeptide of pro-MMP-2 to initiate the activation process (Cao et al., 1998; Zucker et al., 1998; Deryugina et al., 2001). However,
TIMP-2 may not be necessary for activation by other MT-MMPs. For example, MT2-MMP activates pro-MMP-2 in a TIMP-2 independent manner (Morrison et al., 2001).

(3) Inhibition by endogenous inhibitors Once activated, the activity of MMPs in the extracellular milieu is controlled by a group of endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), which belongs to a multigene family (Woessner and Nagase, 2000). Other proteinase inhibitors, such as α-macroglobulins, also inhibit MMP activity (Sottrup-Jensen, 1989). There are two significant aspects regarding the roles of TIMPs and α2-macroglobulin. On the one hand, α2-macroglobulin, which is abundantly present in all tissue fluids and acts nonspecifically against MMPs, may serve as a major inhibitor throughout the entire body, whereas TIMPs may regulate MMP activity locally by their tissue or cell specific expression. On the other hand, the inhibitory action of α2-macroglobulin on MMPs is irreversible until the α2-macroglobulin/MMP complexes are eliminated via scavenger receptor-mediated endocytosis, while the inhibition of MMPs by TIMPs is reversible (Sternlicht and Werb, 2001).

In addition to α2-macroglobulin and TIMPs, another group of inhibitors is emerging. Recent studies suggest that ECM proteins may play dual roles during tissue remodeling. Besides their functions as structural components for supporting cells and tissues and as targets of MMPs for ECM turnover, they may also regulate MMP activities via their proteolytic products. For example, the noncollagenous NC1 domain of type IV collagen possesses not only sequence similarity with TIMPs but also inhibitory activity against MMPs (Netzer et al., 1998), being capable of inhibiting tumor progression and angiogenesis (Petitclerc et al., 2000). The C-terminal domain of procollagen C-terminal proteinase enhancer (CT-PCPE) also shows MMP inhibitory activity by reverse zymography (Mott et al., 2000). N-terminal sequencing and structural comparison further demonstrate that this fragment harbors six cysteine residues, which are conserved in the N-terminal domain of TIMPs (Murphy and Willenbrock, 1995). Nevertheless, although the inhibitory effects of the C- and N-terminal fragments of CT-PCPE on MMP-2 are

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remarkably lower than TIMPs (Netzer et al., 1998; Mott et al., 2000), they may present a new
group of inhibitors whereby they are released upon proteolytic cleavage of the full length
molecule.

3. TIMPs

TIMPs specifically and reversibly inhibit the activity of MMPs. So far, four members of the
tissue inhibitors of metalloproteinases (TIMPs) gene family are known: TIMP-1, -2, -3, and -4.
Table 1-4 summarizes some of the basic molecular and biochemical features of these inhibitors.
All TIMPs have 12 conserved cysteine residues, which form six disulfide bonds and 6-looped, 2-
domain structures. Loops 1-3, which are localized at the N-terminal domain, are highly
conserved. The importance of these loops is established by the fact that they are necessary and
sufficient for the inhibitory ability of TIMPs against MMPs (Murphy and Willenbrock, 1995;
Woessner and Nagase, 2000). The three loops at the C-terminus domain (loops 4-6) may be
responsible for determination of preferred MMP targets of individual TIMPs (Willenbrock and
Murphy, 1994). Overall, the four TIMPs show 37-51% sequence identity (Woessner and Nagase,
2000). The ability of TIMPs to block the autocatalytic activation of latent MMPs and to limit the
degradative actions of activated MMPs is attained by their capability to bind both latent and
active MMPs with a 1:1 stoichiometric ratio (Bode and Maskos, 2001). The relatively high
variability in the C-terminal domains of TIMPs may contribute to determine their preferred MMP
targets (Brew et al., 2000; Bode and Maskos, 2001). In addition, the differential regulation and
wide tissue localization of each individual TIMP are associated with their corresponding distinct
biological functions.

TIMP-1 is a 28.5 kDa N-linked glycoprotein (Gasson et al., 1985). Although it has been
generally recognized that TIMP-1 inhibits the activities of all known MMPs (Edwards, 2001), it
has no or very low inhibitory actions on MT-MMPs (Will et al., 1996). TIMP-1 preferentially

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binds to MMP-9 by interacting strongly with the hemopexin domain of MMP-9 with its C-terminal domain (Goldberg et al., 1989; Murphy and Willenbrock, 1995).

TIMP-2 is an unglycosylated protein with a molecular mass of about 21 kDa (De Clerck et al., 1989; Stetler-Stevenson et al., 1989). Unlike other TIMPs, the C-terminal sequence of TIMP-2 is extended and negatively charged (Murphy and Willenbrock, 1995). TIMP-2 readily forms complexes with MMP-2 through its C-terminal domain (Goldberg et al., 1989; Kolkenbrock et al., 1994).

TIMP-3 is an N-glycosylated protein (Apte et al., 1995). Its molecular mass ranges from 24 to 27 kDa depending on the degree of glycosylation. It preferentially inhibits the activity of MMP-1, -2, -3, -9, and -13 (Apte et al., 1996; Knauper et al., 1997a; Butler et al., 1999). TIMP-3 is the only TIMP that is strongly associated with the ECM (Leco et al., 1994; Yu et al., 2000). The biological importance of TIMP-3 is highlighted by the fact that mutation in this molecule results in Sorsby’s Fundus Dystrophy, an autosomal dominant disorder that is manifested by early degeneration of the retina (Apte et al., 1995).

TIMP-4 has a molecular mass of about 24 kDa (Greene et al., 1996; Liu et al., 1997). It is not known whether this protein is glycosylated. TIMP-4 is the only member of the TIMP family that was identified by using the expressed sequence tag approach (Greene et al., 1996). The full-length cDNA was cloned from a human heart cDNA library (Greene et al., 1996). The gene structure of TIMP-4 was determined from genomic DNA (Olson et al., 1998). Homology analysis revealed that TIMP-4 is closer to TIMP-2 and TIMP-3 (51%) than to TIMP-1 (37%). In addition to the original observation of its abundant presence in human heart tissues (Greene et al., 1996), TIMP-4 is also highly expressed in a broad range of rodent tissues, including brain, heart, ovary, and skeletal muscles ((Leco et al., 1997; Wu and Moses, 1998), suggesting its role as an important tissue-specific regulator of ECM turnover. TIMP-4 significantly inhibits tumor growth and metastasis of human breast cells probably via its potent inhibitory action on a number of MMPs, including MMP-1, -2, -3, -7, -8, -9, -12, -13, and -14 (Bigg et al., 1997; Liu et al., 1997;
Wang et al., 1997; Stratmann et al., 2001a; Stratmann et al., 2001b). This is achieved primarily through TIMP-4’s reliance on residue 2 (Ser2) in its inhibitory domain (Stratmann et al., 2001a). TIMP-4 binds to MMP-2 with high efficiency similar to TIMP-2. However, unlike TIMP-2, which promotes activation of pro-MMP-2 by coupling MMP-2 and MT1-MMP to form a trimeric complex on the cell membrane, TIMP-4 inhibits pro-MMP2 activation (Bigg et al., 2001; Hernandez-Barrantes et al., 2001). Therefore, the balance between TIMP-2 and TIMP-4 may be part of a mechanism that dictates the local regulation of MMP-2 activity.

4. Non-conventional functions of MMPs and TIMPs

Accumulating evidence suggests that MMPs are multifunctional proteins. In addition to facilitating ECM turnover, MMPs have broad substrate specificities on non-ECM proteins (Table 1-3).

One notable example of the novel functions of MMPs is their ability to regulate the activity of enzymes, including themselves. MT1-MMP can activate pro-MMP-2 on the cell surface (Sato et al., 1994), while MMP-3 initiates the activation of pro-MMP-1, -3, -7, -8, -9 and -13 (Nagase, 1995; Nagase, 1998). In addition, proteolytic enzyme activity is mainly regulated by endogenous inhibitors. For example, serine proteinase inhibitors (serpins) inhibit plasmin, which belongs to a class of proteases that has a serine amino acid residue in their active site. These inhibitors, in turn, are cleaved by MMP-9 (Liu et al., 2000) and MMP-11 (Pei et al., 1994), resulting in their inactivation, which alters the balance between enzymes and their inhibitors.

Cleavage of ECM proteins by MMPs can regulate the availability of growth factors and cytokines, which are associated with matrix protein. For example, fibroblast growth factor (FGF) is released when perlecan is cleaved by MMP-1 and MMP-3 (Whitelock et al., 1996), while transforming growth factor β (TGFβ) becomes available when decorin is degraded by MMP-2, -3, and -7 (Imai et al., 1997). Furthermore, there are a number of growth factors and cytokines that are synthesized as pro-forms, which are biologically inactive. Direct cleavage of these precursors...
by MMPs processes them into their active forms. For example, MMP activity is required for release of TNFα from its cell membrane bound form (Gearing et al., 1994; McGeehan et al., 1994; Lombard et al., 1998), while removal of the N-terminal sequence of interleukin 1β (IL1β) by MMP-2, -3, and -9 converts it to its biologically functional form (Schonbeck et al., 1998). On the other hand, the biological activity of these cytokines can also be down-regulated by MMPs. N-terminal proteolytic processing of monocyte chemoattractant protein 3 (MCP-3) by MMP-2 results in a MCP-3 antagonist, which binds to MCP receptors, but does not induce signal transduction (McQuibban et al., 2000). Furthermore, MMPs act on growth factor receptors. For example, cleavage of FGF receptor type I by MMP-2 releases its ectodomain from the cell surface. This soluble FGF receptor then alters the availability of FGF to its cell membrane receptors (Levi et al., 1996).

Cell-cell and cell-matrix interactions are important for cell migration and invasion. MMPs influence these processes by directly cleaving cell adhesion molecules, such as integrin β4 and E-cadherin (Lochter et al., 1997; von Bredow et al., 1997). Proteolytic cleavage of E-cadherin by MMP-3 and MMP-7 releases an 80 kDa fragment, which stimulates cell migration and invasion (Noe et al., 2001). Keratinocyte migration, on the other hand, is induced by MMP-2 dependent cleavage of laminin-5 (Giannelli et al., 1997). Lastly, hydrolysis of cell adhesion molecule CD-44 and cell surface tissue transglutaminase (tTG) by MT1-MMP alters tumor cell adhesion and migration (Belkin et al., 2001; Kajita et al., 2001).

Besides MMPs, the TIMPs are also a multifunctional group of proteins. In addition to inhibiting MMP activity, TIMP-1 and -2 exhibit growth promoting ability on several different cell lines (Docherty et al., 1985; Gasson et al., 1985; Stricklin and Welgus, 1986; Hayakawa et al., 1992; Hayakawa et al., 1994; Nemeth et al., 1996). Also, TIMP-1 is associated with steroidogenesis in rat gonadal cells (Boujrad et al., 1995). Furthermore, the nuclear localization of TIMP-1 shows a cell cycle dependent pattern (Zhao et al., 1998), indicating that this protein may...
regulate the cell cycle. In addition to its inhibitory activity, TIMP-2 facilitates pro-MMP-2 activation by assembling an MT1-MMP/TIMP-2/pro-MMP-2 tri-molecular complex on the cell surface (Butler et al., 1998). Lastly, a death domain is found in the N-terminus of the TIMP-3 protein (Bond et al., 2000). Indeed, TIMP-3 over-expression induces apoptosis in smooth muscle cells (Baker et al., 1998). This may be due to its ability to induce the type II apoptotic pathway (Bond et al., 2002).

Taken together, MMPs regulate cell proliferation, survival, adhesion, migration, and cell-cell and cell-matrix interactions by their proteolytic actions on non-ECM proteins that include the precursors and receptors of growth factors and cytokines, these hormones themselves, cell adhesion molecules, and enzymes. As a group of proteins with small molecular masses, TIMPs also affect cell growth and survival. Because they possess multiple functions, the roles of MMPs and TIMPs in physiological and pathological processes are even more complex (Chambers and Matrisian, 1997; Blavier et al., 1999; McCawley and Matrisian, 2000).

III. MMPs/TIMPs in Follicular Development and Atresia

In all mammalian species, the follicle is a morphological and functional unit which sustains oocyte growth and maturation. Follicular growth begins with primordial follicles which are located in the inner part of the ovarian cortex. Entry of primordial follicles into the growth phase is characterized by conversion of the non-growing, flattened (squamous) pre-granulosa cells surrounding the oocyte into a single layer of cuboidal granulosa cells. At this stage, it is termed a primary follicle, which is also distinguished by the formation of a zona pellucida around the oocyte (Cortvrindt and Smitz, 2001). Granulosa cells continue to proliferate resulting in the oocyte being surrounded by two or three layers of these cells. At this stage, they are termed secondary follicles. As secondary follicles grow, stromal cells next to the basement membrane of the follicle become aligned and form the theca cell layer. At the end of this stage, a vascular network is also developed (will be discussed in section VI) in the theca layer. The blood supply
not only provides nutrients and oxygen for the growth of the follicle, but also exposes the follicle to factors circulating in the blood stream, which influence follicle development. In the tertiary follicle, the granulosa and theca cells undergo further development, and a cell-free, fluid-filled cavity called an "antrum" (means cave in Greek) is formed. Therefore, the follicle at this stage is also called the antral follicle. Based on the appearance and the size of the antrum, the follicles at this stage can be further distinguished as "preantral" or "early antral". In the theca layer, the cells next to the basement membrane become epithelioid in appearance and form a so-called theca interna, while the peripheral cells, which form the theca externa, maintain their spindle shape. The theca interna cells undergo cytodifferentiation and acquire organelles characteristic of steroid-secreting cells such as mitochondria and smooth endoplasmic reticulum. As follicles continue to grow and synthesize hormones, they will reach the final stage called the Graafian follicle.

Taken together, follicular growth and development are characterized by proliferation of granulosa cells, differentiation of theca cells from the ovarian stroma, and deposition of a basement membrane separating the vascular theca from the avascular granulosa cell layer. As the follicular membrane expands within the limits of the ovarian stroma, the surface area of the follicle dramatically increases. For example, the bovine follicle increases approximately 360,000 fold from the primordial to the preovulatory stage. In primates, the mature follicle is almost 400 fold larger than the primordial follicle. Follicle development is accomplished in an extracellular environment that consists of collagen, laminin, and fibronectin. Thus, extensive tissue remodeling is required to accommodate the rapid cellular proliferation as follicles develop. Indeed, the composition of follicular basement membrane changes during bovine follicle development (Rodgers et al., 1998). In addition, modification of the surrounding ovarian stroma is also necessary for the growing follicle to reach a place on the surface of the ovary where the oocyte can be released at ovulation. Remodeling of the ECM during angiogenesis also occurs within the
theca layer. These structural reorganization events in the ovarian ECM are thought to be achieved, at least in part, by the MMPs/TIMPs system.

MMPs appear to play a role in follicular development of cattle. In the bovine ovary, MMPs constitute one family of the most relevant genes that are associated with differentiated follicles bearing developmentally competent oocytes (Robert et al., 2001). Both MMP-2 and MMP-9 activities were detected in bovine follicular fluid (Khandoker et al., 2001). In vitro studies on bovine follicles further indicate the expression of MMP-9 is related to follicle health, and thus can be used as a marker to assess follicle development (McCaffery et al., 2000). Of note, MMP-9 expression in the bovine follicle is augmented by ascorbic acid (Thomas et al., 2001), which has been postulated to be important for follicular health.

In developing rat follicles, MMP-2 and MMP-9 mRNAs are localized to the theca cells and the surrounding stroma (Liu et al., 1998; Curry et al., 2001). Consistent with its mRNA distribution, MMP-9 protein is restricted to theca and interstitial cells (Bagavandoss, 1998). However, MMP-2 immunoreactivity is observed in both theca-interstitial and granulosa cells, although the latter has a lower expression than the former (Bagavandoss, 1998). This discrepancy may be due to the inability (low sensitivity) of in situ hybridization to detect the low copies of MMP-2 mRNA in granulosa cells. Although MMP-2 mRNA is expressed in theca-interstitial cells, MT1-MMP, the activator of pro-MMP-2, is detected in both granulosa cells and theca-interstitial cells (Liu et al., 1998). Similar to MMP-2, the expression of MT1-MMP is also up-regulated by gonadotropins during follicle development (Liu et al., 1998). MMP-13 (collagenase-3) is also detected in theca-interstitial but not granulosa cells of rat growing follicles, suggesting a potential role of this MMP in follicular development (Balbin et al., 1996). Indeed, significant increases in MMP-13, MMP-2, and MMP-9 mRNA, and the corresponding collagenolytic and gelatinolytic activities, are observed during folliculogenesis induced by gonadotropin (Cooke et al., 1999). In the intact mouse follicle culture model, MMP-2 activity is up-regulated by ascorbic acid (Murray et al., 2001), suggesting that the promoting effects of this vitamin on follicle
integrity and survival may be mediated by regulating MMP activity. During gonadotropin-primed follicular development in rats, MMP-23 expression is switched from granulosa cells to theca-externa and the ovarian epithelium (Ohnishi et al., 2001). FSH, via the cAMP signal transduction pathway, reduced MMP-23 expression in granulosa cells, while the expression of MMP-23 is increased in theca-interstitial cells regardless of the presence of LH (Ohnishi et al., 2001). This indicates that MMP-23 is not only involved in follicle development but is also regulated in a cell specific manner.

Besides MMPs, TIMP-1 mRNAs are also localized to theca and stroma during follicle development in the rat (Curry et al., 2001). TIMP-1 protein is localized in the thecal cells of follicles and blood vessels of the ovary (Bagavandoss, 1998). The co-localization of MMPs and TIMPs indicates that the tissue remodeling events during follicular development are strictly regulated.

Among species with long reproductive cycles, TIMP-1 is also detected in ovine (Smith and Moor, 1991; Smith et al., 1993; Smith et al., 1994a; McIntush et al., 1996), bovine (Smith et al., 1996), porcine (Smith et al., 1994b; Driancourt et al., 1998; Shores and Hunter, 2000), primate (Chaffin and Stouffer, 1999), and human (Curry et al., 1988) follicles. TIMP-1 mRNA is increased in bovine (Smith et al., 1996) and ovine (Smith et al., 1994a) preovulatory follicles, while ovine TIMP-1 protein in the ovary (McIntush et al., 1996) is only observed after the gonadotropin surge. As suggested in a Sertoli cell model, TIMP-1 expression may be stimulated by FSH via a cAMP, PKA-dependent pathway. Furthermore, the PKC pathway also mediates signals that up-regulate TIMP-1 production (Ulisse et al., 1994).

TIMP-2 mRNA expression is observed in bovine (Smith et al., 1996), and ovine (Smith et al., 1995) follicles. In the latter, TIMP-2 is primarily expressed in theca cells, but its expression was not affected by the gonadotropin surge (Smith et al., 1995). However, after the gonadotropin surge, a remarkable increase in TIMP-2 expression is induced in the bovine ovary (Smith et al., 1996). TIMP-2 is also present in theca and granulosa cells, and follicular fluid of large and small
pig follicles (Shores and Hunter. 2000). TIMP-2 mRNA is expressed in the theca and stromal compartments during rat folliculogenesis (Curry et al., 2001). Although TIMP-2 expression can be up-regulated by FSH via a cAMP. PKA-dependent pathway, it is resistant to stimulation through the PKC pathway (Ulisse et al., 1994).

The localization of TIMP-3 mRNA exhibits a different pattern from that for TIMP-1 and -2. Although TIMP-3 mRNA is detected in theca and stroma of developing rat follicles, its expression in granulosa cells is only observed in certain follicles, but not in the follicles that are adjacent to them (Curry et al., 2001). In the mouse ovary, TIMP-3 mRNA is also expressed in the theca and granulosa cells of small and large follicles (Inderdeo et al., 1996). Similarly, TIMP-3 is observed in pig small and large follicles (Shores and Hunter, 2000). The study of TIMP-1, -2, and -3 production in pig follicles shows that the expression of these TIMPs is regulated by factors such as follicular size and the phase of follicular development (Shores and Hunter, 2000).

Although TIMP-4 is abundantly expressed in ovarian tissue (Leco et al., 1997), its localization in the follicular compartments has not been elucidated. Reverse zymography demonstrates that this inhibitor may be present in equine follicular fluid (Riley et al., 2001).

Because of their specialized ovarian structure and ovulation pattern, the equine gonads are a good model to study the tissue remodeling events in follicle growth and development. Different from the ovaries of other mammalian species, in which follicles can ovulate from any site on the ovarian surface, the follicles in equine ovaries migrate from the stroma to the ovulatory fossa, where ovulation occurs. Both MMP-2 and MMP-9 are present in equine follicular fluid. While MMP-2 is the predominant gelatinase, its level does not vary significantly during follicle development (Riley et al., 2001). However, MMP-9 increases dramatically, when follicles grow from <10mm to 11-20mm in diameter. Both these gelatinases are localized to the granulosa, theca, and stromal cells. Furthermore, all four tissue inhibitors of metalloproteinases (TIMPs 1-4) are also present in follicular fluid, but their levels do not change significantly during follicle development (Riley et al., 2001). Similar to the localization of MMP-2 and MMP-9, all these
TIMPs are immunolocalized to follicular granulosa and theca cells and the surrounding stromal cells. The co-localization of MMPs and TIMPs suggests that MMP activity is locally regulated during the tissue remodeling that occurs when follicles develop and migrate to the ovulation fossa in mares. Furthermore, accumulating evidence suggests that one of the roles of relaxin during ovarian follicle development and ovulation is to regulate proteolytic enzyme activity. In an in vitro equine ovarian stromal cell (EOSC) culture system, relaxin increases both latent and active MMP-2, latent MMP-9, TIMP-1, and TIMP-2 (Song et al., 1999). This suggests that relaxin may contribute to equine follicle growth and migration, and facilitate ovulation by modulating the degradation of ECM in ovarian stromal tissue. The growth factor, TGFβ, also plays an important role, as it significantly stimulates the activity of MMP-2 and MMP-9 and TIMP-1 production by EOSC (Song et al., 1999). The PKC signal transduction pathway may be involved in the stimulation of MMP-2, MMP-9, TIMP-2, and TIMP-3, since PMA (phorbol 12-myristate 13-acetate) increases the production of these enzymes and inhibitors (Song et al., 1999).

Follicles also undergo atresia, a process that involves apoptosis. Abnormal proteolytic degradation of the ECM may initiate an apoptotic signal or mediate the apoptosis process. In human ovary, low levels of MMP-1 in the apical wall of the follicle are associated with atresia. The level of stromelysin-1 (MMP-3) is inversely related to follicle size (Bogusiewicz et al., 2000). In the mouse model, although stromelysin-3 (MMP-11) is coordinately expressed with apoptotic follicles, the stromelysin-3-deficient mouse shows no difference in the apoptotic rates of follicles from the wild type (Hagglund et al., 2001). Therefore, stromelysin-3 may not be sufficient for inducing or completing follicle atresia, but it may be involved in this process. In non-atretic follicles, interstitial collagenase activity is high in granulosa and thecal cells and low in follicular fluid. Atresia is associated with declining collagenase activity in both cell types and increasing activity in follicular fluid (Garcia et al., 1997). Gelatinase activities are also correlated with follicular atresia. Specifically, active MMP-2 and pro-MMP-9 are present only in atretic follicular fluid (Khandoker et al., 2001).
The physiological roles of these metalloproteinases, therefore, may include their direct actions on ECM components for modulating the local extracellular milieu, and their indirect effects on cell proliferation by processing growth factors from their latent forms and/or modifying their receptors.

IV. MMPs/TIMPs in Ovulation

Although a variety of hypotheses has been proposed for the mechanism of ovulation, most of them, if not all, include a coordinated series of proteolytic degradative events which result in the breakdown of the follicle wall. As early as about one century ago (1916), Schochet pointed out the importance of proteolytic enzymes in the process of ovulation by degrading the ECM at the apex of a Graafian follicle. Numerous proteolytic enzymes were thereafter identified and characterized during the ovulation process (Espey, 1974; Parr, 1975; Tsafriri, 1995).

Besides oocyte maturation, deterioration of the follicle wall and follicle apex leads to ovulation (Thibault and Levasseur, 1988; Murdoch and McCormick, 1992). The apical wall of the preovulatory follicle, from the innermost to the outermost side, is composed of granulosa cells, basement membrane, theca interna, theca externa, tunica albuginea, a second basement membrane, and epithelium. During ovulation, which is initiated by a surge of the pituitary gonadotropin, luteinizing hormone (LH), all these layers of ECM and connective tissues have to be compromised for follicle rupture and expulsion of the oocyte. These events begin with alterations in the vasculature of the follicle, which results in the formation of an avascular area known as the macula pellucida or stigma (Parr, 1975). Then, the tunica albuginea is the first place where disintegration occurs, followed by the degradation of ECM in the epithelium. As a result, fibroblasts and epithelial cells are no longer in contact, and ultimately this leads to the loss of integrity of the whole surface epithelium. In some species such as mouse and rat, the outer layer of basement membrane, which lies beneath the surface epithelium, undergoes edema, which then causes fragmentation of the collagen matrix. The blood capillaries in the theca layer subsequently

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disintegrate and the blood seeps into the interstitium. As the time of ovulation approaches, the follicle expands and the number of layers of fibrils decreases over the apex, resulting in the deterioration of the basal lamina localized between granulosa and theca layers (Martin and Miller-Walker, 1983). Collectively, follicle wall breaching and the loss of apical integrity eventually lead to leakage of follicular fluid and release of the oocyte.

One of the major breakthroughs in the early studies of ovulation was accomplished with the demonstration that antral pressure does not increase prior to follicle rupture (Bronson et al., 1979). This observation, therefore, shifted the research focus to the deterioration and weakening of the follicle wall around the stigma area, which appears to be a necessary prerequisite to the ovulatory process (Parr, 1975; Martin and Miller-Walker, 1983). The degradation of the follicle wall is reflected by the changes in collagen content during follicle development, which increases with increasing follicle volume (Morales et al., 1983). However, right before ovulation, the collagen content decreases dramatically (Martin and Miller-Walker, 1983; Morales et al., 1983). All these matrix degradation events during ovulation may be a consequence of the proteolytic action of MMPs. For example, collagenase and gelatinase activities are associated with the apex of preovulatory follicles (Fukumoto et al., 1981; Murdoch and McCormick, 1992; Curry and Osteen, 2001).

Collagenase was the first proteolytic enzyme identified in the ovulation process. Collagenolytic activity is first detected in the rabbit and sow Graafian follicles during ovulation (Espey and Rondell, 1968). Higher collagenolytic activity is localized in the follicular apex area than the basal wall of the ovulatory follicles, suggesting degradation of extracellular matrix proteins may dictate the site of follicle rupture (Murdoch and McCormick, 1992). In the rat ovary, collagenase activity is correlated with the ovulatory process (Curry et al., 1985; Palotie et al., 1987; Woessner et al., 1989; Hirsch et al., 1993). An ovulatory dose of hCG increases interstitial collagenase (MMP-1) mRNA about twenty-five times in rat follicles, where it is predominantly localized in both granulosa cells of preovulatory follicles and the residual ovarian tissues (Reich.
et al., 1991). A continuous increase of neutral collagenase activity is observed in the follicular wall during the ovulatory process in humans (Yajima et al., 1980). Further investigation shows that granulosa cells are the major cellular source of collagenase, which is produced at maximal levels at ovulation and decreases abruptly thereafter (Fukumoto et al., 1981).

In the domestic fowl, collagenase activity is detected in the ovarian follicle wall (Fujii et al., 1981). Although there are no significant changes of collagenase activity observed up to the point of ovulation (Tojo et al., 1982), local administration of proteolytic enzymes induced the rupture of the ovarian follicle (Nakajo et al., 1973). LH and progesterone increased the total activity of neutral and acid proteases and collagenase, which are primarily concentrated in the stigma region (Ogawa and Goto, 1984). Retrospectively, the collagenase activity described at this stage is interstitial collagenase.

During ovulation of rabbit follicles, MMP-1 is expressed in theca interna, theca externa, interstitial cells and germinal epithelium. In addition, MMP-1 is also induced in the capillary lumina around the apex of preovulatory follicles and increases in granulosa and theca interna cells that are around the orifice of the ruptured follicles (Tadakuma et al., 1993).

The ovulatory process is regulated by multiple factors. Gonadotropin induces an increase in collagenolytic activity (Curry et al., 1985; Reich et al., 1985; Hirsch et al., 1993), which is reflected, in part, by the increase in interstitial collagenase expression at the transcriptional level (Reich et al., 1991). Collagenase 3 (MMP-13) is also highly expressed in thecal cells/stroma of rat antral follicles (Balbin et al., 1996), suggesting a plausible role for it during ovulation. It is up-regulated by LH via a tyrosine kinase-dependent pathway during ovulation in rats (Komar et al., 2001). Besides gonadotropins, a number of paracrine factors have regulatory effects on collagenase activity. Relaxin stimulates the release of collagenase activities from rat granulosa cells (Too et al., 1984). Prolactin, on the other hand, is unable to promote collagenolytic activities (Hirsch et al., 1993). Indomethacin (an inhibitor of cyclooxygenase) blocks the stimulatory effects of hCG on collagenase mRNA expression and collagenolytic activity (Reich et al., 1985;
Reich et al., 1991). In the ewe, indomethacin negates the stimulated collagenolysis in follicular tissue by gonadotropin (Murdoch et al., 1986), suggesting that prostaglandins are involved in enhancing collagenase activity. Prostaglandins have been shown to be stimulatory for proteolytic enzyme activity during ovulation in rabbits (Miyazaki et al., 1991). Furthermore, collagenase is also up-regulated by TNFα, which accumulates in the oocyte-cumulus cell complex (Johnson et al., 1999), implying that multiple cell types are involved in the regulation of collagenase activity during ovulation. In non-human primates, progesterone is indispensable for the stimulatory effects of gonadotropin on interstitial collagenase expression (Chaffin and Stouffer, 1999).

However, progesterone, prostaglandin E2, prostaglandin F2α, and 17β-estradiol have no effect on collagenase activity in the human follicle wall (Norstrom and Tjugum, 1986), but relaxin and oxytocin increase collagenase activity (Norstrom and Tjugum, 1986). Notably, oxytocin decreases collagen synthesis (Tjugum et al., 1986). Collectively, these results suggest that the regulation of collagenase activity is diverse and may be species dependent.

Type IV collagenolytic activity was first detected in human follicular fluid (Puistola et al., 1986). This activity increases as ovulation approaches, and decreases after follicle rupture (Puistola et al., 1986). In an in vitro culture model of rat follicles, type IV collagenase activity is stimulated by gonadotropin, showing a similar pattern of changes as type I collagenolytic activity (Palotie et al., 1987). Retrospectively, these type IV collagenolytic activities are attributed to gelatinases (A and B), as confirmed by gelatin zymography (Curry et al., 1992). The efficient degradation of the major component of the basement membrane, collagen type IV, by these activities may be pivotal for ovulation. Furthermore, type IV collagenolytic activity is also present within the follicular fluid and increases toward ovulation (Puistola et al., 1986). While interstitial collagenase increases at 3-6 hours after hCG administration (Reich et al., 1985; Reich et al., 1991), type IV collagenase expression, on the other hand, peaks at 9 hours after hCG treatment (Reich et al., 1991; Curry et al., 1992). Increased expression of MMP-9 in the late stage
of the ovulatory process is also observed in macaque (Chaffin and Stouffer, 1999). Furthermore, interstitial collagenase is expressed in both granulosa and residual cells, while type IV collagenase is restricted to residual (thecal-interstitial) cells (Reich et al., 1991). The aforementioned type IV collagenase activity is predominantly due to gelatinase B (MMP-9), which is highly expressed in the theca-interstitial compartment (Hurwitz et al., 1993; Curry et al., 2001). Taken together, these observations indicate that interstitial collagenase is expressed at the early stages of ovulation to loosen the connective tissues between the two basement membranes, while residual cells produce gelatinase B (type IV collagenase activity) at the final stages of ovulation to tear the major boundaries, the two layers of basal lamina, for oocyte extrusion. The abrupt decrease of this enzyme after the rupture of follicles further suggests its unique role during ovulation (Puistola et al., 1986). Other factors, such as interleukin-1 (IL-1), stimulate gelatinase B production by theca-interstitial cells (Hurwitz et al., 1993). However, transforming growth factor-beta 1 (TGF-β1) attenuates the stimulatory action of IL-1 (Hurwitz et al., 1993), suggesting a complex intraovarian regulatory loop for fine-tuning this MMP activity.

The importance of gelatinase A in the ovulatory process was recognized in the ewe, when immunization with the N-terminal peptide of inhibin α43 (αN) reduced the fertility. Specifically, latent and active MMP-2 levels increase as ovulation approaches (Russell et al., 1995; Gottsch et al., 2000), while immunization with αN significantly reduces gelatinase A activity (Russell et al., 1995). The increase in MMP-2 activity at ovulation, however, is abolished by administration of TNFα antiserum (Gottsch et al., 2000). When combined with the in vitro data that shows the stimulatory effects of TNFα on MMP-2 activity (Gottsch et al., 2000), these results indicate that MMP-2, which is regulated by inhibin and TNFα, is important for the tissue remodeling process during ovulation. In rats, relaxin stimulates the secretion of MMP-2 from theca-interstitial cells but not granulosa cells. This autocrine or paracrine action of relaxin on MMP-2 activity may contribute to its ability to facilitate ovulation (Hwang et al., 1996). Furthermore, MMP-2 and its
cell surface activator, MT1-MMP, are up-regulated in theca-interstitial cells of large preovulatory follicles after hCG stimulation (Liu et al., 1998). This indicates that the MT1-MMP/MMP-2 activation system may be associated with the tissue destruction events during ovulation. In the macaque, MMP-2 expression is also up-regulated via a progesterone-independent pathway during the ovulatory process (Chaffin and Stouffer, 1999).

In addition to the two major MMP subgroups- collagenases and gelatinases, other MMPs may also participate in the tissue destruction process during ovulation. For example, gonadotropin induces increased expression of matrilysin in the macaque ovary (Chaffin and Stouffer, 1999). Although stromelysin-3 (MMP-11) is constitutively expressed during ovulation, maximal levels of MMP-19 in granulosa and theca-interstitial cells of large preovulatory and ovulating follicles are induced by hCG in the mouse ovary (Hagglund et al., 1999). In the porcine ovary, bradykinin up-regulates expression of MMP-3, MMP-20, and MT1-MMP in granulosa cells (Kimura et al., 2001). ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin-like motifs), which is a novel MMP-related proteolytic enzyme and which appears to be the regulatory target of progesterone, is induced in granulosa cells of mouse preovulatory follicles by LH (Espey et al., 2000; Robker et al., 2000).

Besides MMPs, the expression patterns of metalloproteinase inhibitors are also altered. A metalloproteinase inhibitor activity was first identified in human follicular fluid (Curry et al., 1988). Based on the characterized biochemical features, this metalloproteinase inhibitor activity is attributed to TIMP-1. Additionally, α-macroglobulins (α1-macroglobulin, α2-macroglobulin, and α1 inhibitor3), a group of plasma proteins with broad protease inhibitor activity, are also present in the follicular fluid of human and rat follicles (Curry et al., 1989; Curry et al., 1990; Zhu and Woessner, 1991). In the rat ovary, the activities of these α-macroglobulins increase as ovulation approaches (Zhu and Woessner, 1991). The absence of α2-macroglobulin mRNA in granulosa cells suggests that this serum-derived inhibitor is transported by the blood stream from
other sources in the body (Curry et al., 1990), while detection of TIMP-1 mRNA in granulosa cells indicates that this inhibitor may serve as a local regulator of proteolysis during ovulation (Curry et al., 1990; Hagglund et al., 1999; Curry et al., 2001). In addition to granulosa cells, TIMP-1 is also localized to theca-interstitial cells in the mouse ovary (Hagglund et al., 1999), suggesting that there is probably a species difference in the tissue distribution of this endogenous MMP inhibitor.

Similar to MMPs, the TIMPs are also regulated. TIMP-1 expression is dramatically increased in rat (Curry et al., 1989; Mann et al., 1991) and mouse (Hagglund et al., 1999) ovaries after hCG administration. In rat granulosa cells, a LH-cAMP-dependent protein kinase-A pathway or a cAMP-independent protein kinase-C pathway augments TIMP-1 expression (Mann et al., 1991; Mann et al., 1993), while neither prostaglandin nor estrogen affects the basal or LH-augmented TIMP-1 expression (Reich et al., 1991; Mann et al., 1993). In addition, calcium is also involved in modulating TIMP-1 expression, since the ionophore, A23187, decreases granulosa cell-derived TIMP-1 activity (Cannon et al., 1997). Although αN is involved in the regulation of MMP-2 during ovulation (Russell et al., 1995), the paracrine effects of this factor has not been determined for TIMP-1 (Dhar et al., 1998). In non-human primates, the stimulatory effects on TIMP-1 and TIMP-2 expression by gonadotropin are dependent on progesterone (Chaffin and Stouffer, 1999).

Proteolytic enzymes other than members of the MMP family, such as plasminogen and plasminogen activator, are also found in the follicle wall (Beers, 1975; Shimada et al., 1983; Hsueh et al., 1988; Liu et al., 1991; Chun et al., 1992; Hagglund et al., 1996; Liu, 1999). Inhibitors of plasminogen and plasmin significantly inhibit ovulation efficiency (Yoshimura and Wallach, 1987). Notably, the fertility in mice with a deficiency of either tPA or uPA is normal, while ovulation is reduced in mice lacking both PAs (Leonardsson et al., 1995). The plasmin inhibitor is effective only when it is administrated at the early stage (Woessner et al., 1989), while full inhibitory capability of the collagenase inhibitor on ovulation is observed when it is
administered as late as 7 hours after LH (Butler et al., 1991). These findings suggest that plasmin may serve as the activator for collagenases during ovulation.

All in all, ovulation is a complex physiological process involving a number of proteolytic enzymes and their corresponding inhibitors. MMPs and TIMPs undoubtedly play an important role in the proteolytic events during ovulation. Ovarian paracrine and autocrine factors, as well as gonadotropins, constitute a complex loop for regulating MMP activity. Further investigations on the temporal and spatial expression patterns, and regulatory mechanisms of proteolytic enzymes and endogenous inhibitors, are necessary for advancing our understanding of this exquisitely controlled tissue remodeling process.

V. MMPs/TIMPs in CL Formation, Maintenance, and Regression

The corpus luteum (CL) is a transient endocrine gland that secretes progesterone to establish and maintain pregnancy (Niswender and Nett, 1994). The CL also plays a central role in survival of the embryo, regulation of cyclicity, and controlling ovulation and length of the reproductive cycle. The CL develops from the remnants of the follicle following ovulation (Niswender and Nett, 1994). Dramatic structural and functional changes are associated with the development, maintenance and regression of the CL (Niswender and Nett, 1994). At the time of ovulation, the basement membrane breaks down, and the follicle wall folds toward the cavity of the ruptured follicle (Pederson, 1951). During this process, theca interna cells, fibroblasts and endothelial cells undergo mitosis, and migrate into the central region of the postovulatory follicle, contributing towards the formation of the CL (Parry et al., 1980; Cavender and Murdoch, 1988; O'Shea et al., 1989; Zheng et al., 1994). In addition, disruption of the basement membrane facilitates invasion of capillary vessels from the theca interna into the granulosa layer (Cavender and Murdoch, 1988). The latter, therefore, undergoes a striking transition from an avascular to a vascular compartment (Pederson, 1951; Ford et al., 1982; Wiltbank et al., 1988). This neovascularization provides a blood supply for the differentiated theca-derived small and granulosa-derived large
luteal cells (Dharmarajan et al., 1985; Wiltbank et al., 1988; Wiltbank et al., 1989), and is associated with increased progestin production by the CL (Smith et al., 1994c). During the mid-cycle phase of luteal development, neovascularization is slowed down and the integrity of the CL is maintained to provide a relatively steady environment for continued steroidogenesis (Jablonka-Shariff et al., 1993). Regression of the CL at the end of the cycle is also an example of a remarkable tissue reorganization process (Nett et al., 1976; Niswender and Nett, 1994; Murdoch, 1996).

These remodeling events in the CL require the participation of MMPs and TIMPs. TIMP-1 is one of the first members in this protein family identified to be closely associated with luteal physiology. In mouse adult tissues, the highest level of TIMP-1 was detected in the ovary, where the expression is predominantly restricted to the CL (Nomura et al., 1989; Edwards et al., 1992), indicating a significant role of this MMP inhibitor in luteal physiology. TIMP-1 immunoreactive staining is detected in rat (Bagavandoss, 1998), porcine (Tanaka et al., 1992), and ovine (McIntush and Smith, 1998) luteal capillaries. In addition, abundant expression of this inhibitor is predominantly localized in large luteal cells of species with relatively long luteal phases, such as sheep (McIntush and Smith, 1998), and pig (Tanaka et al., 1992). More specifically, TIMP-1 is packed in the secretory granules of large luteal cells (McIntush et al., 1996). While TIMP-1 mRNA does not vary in the ovine CL over the estrous cycle (Smith et al., 1994a), its levels are maximal in the early stage and decline with age of bovine (Freudenstein et al., 1990; Smith et al., 1996) and porcine (Pitzel et al., 2000) CL. Although TIMP-1 is most abundantly expressed in the CL (Tanaka et al., 1992) and thus the major secreted protein product of the sheep ovary (Smith and Moor, 1991; Smith et al., 1993), circulating concentrations of this protein remain at a consistent level over the estrous cycle (McIntush et al., 1997). In the bovine CL, immunoreactive TIMP-1-like protein is unchanged over the estrous cycle (Goldberg et al., 1996). The expression of TIMP-1 is also hormonally regulated (O'Sullivan et al., 1997). Triiodothyronine (T3) and
follicle stimulating hormone (FSH), alone and in combination, stimulate TIMP-1 production in human luteinized granulosa cells (Goldman et al., 1997).

The 5' non-coding region of bovine TIMP-1 is homologous to the protein coding region of the bovine StAR gene (Hartung et al., 1995), suggesting a possible role of TIMP-1 in steroid production. Indeed, TIMP-1 facilitates steroidogenesis in Leydig, granulosa, and theca cells in vitro (Boujrad et al., 1995; Nothnick et al., 1997; Shores and Hunter, 2000). In addition, TIMP-1 deficient mice have lower serum testosterone concentration in males (Nothnick et al., 1998), but normal serum 17β-estradiol and progesterone levels in females (Nothnick et al., 1997). Collectively, these data suggest that TIMP-1 may act as a co-regulator of steroidogenesis. However, the underlying mechanism of TIMP-1 in steroidogenesis and whether it is involved in luteal steroid production require further investigation.

There are two species of TIMP-2 mRNA; one migrates at 3.5 kb, while the other migrates at 1 kb. Both mRNA species are detected in rat (Nothnick et al., 1995; Simpson et al., 2001) CL. While a single 1 kb species is detected in ovine CL (Smith et al., 1995), the 3.5 kb species is the only TIMP-2 mRNA in human CL (Duncan et al., 1998). In the bovine CL, TIMP-2 transcripts and immunoreactive TIMP-2-like protein increase over the estrous cycle (Smith et al., 1996; Goldberg et al., 1996), while ovine luteal TIMP-2 mRNA expression in the early luteal phase is greater than the mid- and late stages (Smith et al., 1995). In contrast, TIMP-2 mRNA expression does not vary in the human CL during the menstrual cycle (Duncan et al., 1998). It has been shown that the large luteal cells of ovine CL are the cellular sources of TIMP-2 (Smith et al., 1995). However, TIMP-2 is localized to the theca-lutein cells and the surrounding connective tissue stroma in the human CL (Duncan et al., 1998). These data suggest that species differences may exist regarding the temporal and spatial expression patterns of TIMP-2.

The gelatinases are detected in the conditioned medium of luteal cells from the early bovine CL (Tsang et al., 1995) and luteinized bovine granulosa cells (Zhao and Luck, 1996). The early stage rat CL possesses higher levels of collagenase and gelatinase activities than any other stage.
MMPs play important roles in CL development and function. During CL formation, ewes immunized with the N-terminal peptide of inhibin α43-subunit (αN) manifested incomplete infolding of surrounding tissues and theca/vessel invaginations. The reduced fertility that resulted was attributed to decreased levels of MMP-2 (Russell et al., 1995). Furthermore, immunization with MMP-2 antibody caused incomplete CL formation in ewes (Gottsch et al., 2001). In human (luteinized) granulosa cells, levels of gelatinases are reduced while there is a concurrent increase in TIMP-1 production following exposure to hCG (Aston et al., 1996), suggesting one of the actions of gonadotropin (LH) in the CL is possibly to stabilize ECM remodeling at the early stages of CL development.

CL maintenance also includes active tissue remodeling events. MMP-2 and MMP-9 activities are present in the bovine CL throughout the estrous cycle (Goldberg et al., 1996). Higher levels of MMP-2, MMP-9, and MMP-1 gene expression are observed in the mid stage porcine CL than in the early stage (Pitzel et al., 2000). In the rat, MT1-MMP is constitutively expressed in CL throughout pseudopregnancy (Liu et al., 1999). While MMP-2 is mainly expressed during luteal development, MMP-13 (collagenase 3) is only expressed in the regressing CL (Liu et al., 1999). The MMP-2 protein is localized in endothelial and large luteal cells of the developing rat CL (Bagavandoss, 1998). Interestingly, MMP-9 is localized on the plasma membrane of luteal and interstitial cells in rat CL (Bagavandoss, 1998), indicating that this enzyme may perform pericellular proteolysis through its cell surface receptor, such as CD44 (Yu and Stamenkovic, 1999). Besides MMPs, other proteolytic enzymes, such as proteoglycanase, are also associated with luteal maintenance (Nothnick et al., 1996).

Extensive tissue deterioration occurs during CL regression (Niswender and Nett, 1994). During structural regression induced by PRL in the hypophysectomized rat, both latent and active forms of MMP-2 are increased (Endo et al., 1993). Similarly, enhanced MMP-2 activity and mRNA expression are also observed in GnRH agonist (GnRHa) (Goto et al., 1999) and GH (Kiya et al., 1999) induced structural regression. During rat luteal regression induced by
cloprostenol (a PGF$_{2\alpha}$ analog). MMP-13 is induced only after progesterone levels decreases (Liu et al., 1999), indicating that functional regression may initiate physiological signal(s) to bring about MMP expression, which are involved in structural regression. Indeed, the rise in MMP-2 activity is coincident with the nadir in progesterone during the early onset of luteolysis in ewes (Towle et al., 2002). In the human CL, an increase in MMP-2 activity may be associated with luteolysis (Duncan et al., 1998), since MMP-2 expression is decreased during luteal rescue (Duncan et al., 1998) or following treatment with hCG in luteinized human granulosa cells (Stamouli et al., 1996). In contrast, MMP-1 (collagenase 1) expression is not changed during luteal rescue (Duncan et al., 1998). During luteal regression of the pseudopregnant rat, collagenase mRNA levels are increased (Nothnick et al., 1996). Recently, this collagenase was most likely determined to be collagenase-3 (Liu et al., 1999).

TIMP-1 expression is decreased during PGF$_{2\alpha}$-induced luteal regression in the cow (Juengel et al., 1994) and the ewe (Towle et al., 2002). Interestingly, in the ewe, this occurs before the decline of progesterone and the rise in MMP-2 (Towle et al., 2002). During porcine luteal regression induced by PGF$_{2\alpha}$, the serum concentration of TIMP-1 is also significantly decreased (McIntush et al., 1997). A significant fall in TIMP-1 expression is also observed in marmoset CL during PGF$_{2\alpha}$ and GnRHa induced luteolysis (Duncan et al., 1996a). In these species, the reduced level of TIMP-1 may result in enhanced MMP activity, which favors ECM degradation during luteal regression. However, in the rat, TIMP-1 levels are increased during luteal regression (Liu et al., 1999). Furthermore, during rescue of the human CL, TIMP-1 and TIMP-2 expression do not vary (Duncan et al., 1996b; Duncan et al., 1998). Therefore, there may be species differences regarding the expression of TIMP-1 during luteal regression.

**VI. MMPs/TIMPs in Ovarian Angiogenesis**

- 45 -
Follicle development is accompanied by blood vessel recruitment (Cavender and Murdoch, 1988; Fraser and Lunn, 2000; Mattioli et al., 2001; Wulff et al., 2001). The rapid and hypertrophic growth of the CL, which requires an abundant supply of oxygen and nutrients, depends on concomitant vascular growth or angiogenesis (Reynolds et al., 2000). Angiogenesis, the formation of new capillaries from preexisting vessels, is critical for follicle development and CL formation. Angiogenesis is exquisitely orchestrated by the cell-cell and cell-matrix interactions in the capillary. The process of angiogenesis can be divided into the following steps: (1) production of angiogenic factors from the demand tissue; (2) spatiotemporal regulation of endothelial cell gene expression by these angiogenic factors; (3) fragmentation of existing capillary basement membrane; (4) migration of endothelial cells; (5) proliferation of endothelial cells; and (6) recruitment of pericytes and formation of capillary lumen (Moses, 1997; Klagsbrun and Moses, 1999).

MMPs are involved in various steps of the angiogenic process. MMP-deficient mice demonstrate delayed or reduced angiogenic events during normal development and tumor progression (Itoh et al., 1998; Vu et al., 1998). Furthermore, both endogenous and synthetic MMP inhibitors block angiogenesis in vivo and in vitro (Knauper et al., 1996b; Hiraoka et al., 1998). Thus, it seems likely that MMPs also play central roles in ovarian angiogenesis.

The interest in ovarian angiogenesis originates in the finding that this organ is a rich source of angiogenic factors (Gospodarowicz and Thakral, 1978; Frederick et al., 1984; Makris et al., 1984). Follicular fluid initiates angiogenesis (Frederick et al., 1984; Frederick et al., 1985), through its stimulatory effects, in part, on endothelial cell proliferation (Rose and Koos, 1988). In addition, both intact follicles and dispersed granulosa cells produce a vascular chemoattractant factor (Rone et al., 1993). Intense expression of vascular endothelial growth factor (VEGF) mRNA and protein is confined to granulosa and theca cells at the late stage of follicle development, consistent with the establishment of a vasculature around the follicle wall at this time (Ravindranath et al., 1992; Kamat et al., 1995). The importance of VEGF in follicle development is supported by the observation that VEGF-deficient mice exhibit reduced angiogenesis and follicle growth (Klagsbrun and Moses, 1999).
development is further established in the human ovary, where it is only expressed in healthy follicles, but not atretic ones (Gordon et al., 1996; Yamamoto et al., 1997). Furthermore, inhibition of ovarian angiogenesis by administration of a VEGF antibody in the late follicular phase of rhesus monkeys delays follicle development (Zimmermann et al., 2001). The expression of angiogenin, another potent angiogenic factor, is also closely correlated to follicle development (Lee et al., 1999). With the exception that TIMP-1 is localized in blood vessels surrounding developing follicles (Bagavandoss, 1998), little is known about the expression and localization of MMPs and TIMPs in capillaries around follicles. As in the case of tumor angiogenesis, however, MMPs and TIMPs produced by theca and interstitial cells may facilitate blood vessel recruitment during follicle development.

CL development is associated with extensive angiogenesis (Basset, 1943; Meyer and McGeachie, 1988; Tsukada et al., 1996). Right before ovulation, the basement membrane that separates the granulosa and theca layers is fragmented. At the same time, capillary basement membrane in the theca interna compartment is also broken down (Matsushima et al., 1996; Tsukada et al., 1996). Immediately following ovulation, endothelial cells migrate into the previously avascular granulosa layer to form new blood vessel sprouts. Accompanying this process, there are a series of changes in the microvascular extracellular matrix, including fibronectin, collagen type IV, and laminin (Matsushima et al., 1996; Amselgruber et al., 1999). However, there are contradictory reports regarding whether endothelial cells or pericytes lead the outgrowth of the newly formed vessels (Matsushima et al., 1996; Redmer and Reynolds, 1996; Amselgruber et al., 1999; Reynolds et al., 2000). This discrepancy may be due to technical limits of the research methods applied in the studies. Pericytes play important roles during capillary formation and vasculature survival (Nehls et al., 1992; Hirschi and D'Amore, 1996). Further investigation on the cellular angiogenic events during CL formation may provide valuable information regarding the interactions between endothelial cells and pericytes.
The luteal angiogenic process is so intense that endothelial cells make up 85% of the proliferative cell population during CL development (Reynolds et al., 1994). By mid cycle, endothelial cells are the most abundant cell type in the bovine CL, and constitute about 50% of total number of cells (Lei et al., 1991; Zheng et al., 1993; Zheng et al., 1994). At this stage, the CL is so vascularized that the majority of steroidogenic cells are in contact with one or more capillaries, and the blood flow in this gland is among the greatest of any tissue in the body (Reynolds et al., 1992; Redmer and Reynolds, 1996).

Consistent with this rapid development of the vasculature, various angiogenic factors are identified in the CL, including FGF (fibroblast growth factor), NGF (nerve growth factor), EGF (epidermal growth factor), IGF (insulin-like growth factor), TGF (transforming growth factor), VEGF (vascular endothelial growth factor), and angiopoietins (Gospodarowicz et al., 1977a; Gospodarowicz et al., 1977b; Gospodarowicz and Thakral, 1978; Gospodarowicz et al., 1985; Phillips et al., 1990; Reynolds et al., 1992; Kamat et al., 1995; Redmer and Reynolds, 1996; Laitinen et al., 1997; Abulafia and Sherer, 2000). Among these factors, heparin-binding factors, such as FGFs and VEGFs, have been postulated to be key regulators (Redmer and Reynolds, 1996; Reynolds et al., 2000). VEGF is expressed in the primate and human CL from early to late stages, but is absent in regressing tissues (Ravindranath et al., 1992; Gordon et al., 1996). In addition, total angiogenic activity is present in the bovine CL throughout the estrous cycle, but there is a slight increase with age (Redmer et al., 1988; Reynolds et al., 2000). Therefore, it may not be appropriate to view the mid-stage CL as non-angiogenic (Goede et al., 1998). Indeed, in the human CL, although the total volume of luteal cells decreases in the late stage, the vascular volume progressively grows until the late regressing stage (Gaytan et al., 1999). This is consistent with the observation in the bovine CL, where blood vessel regression lasts several weeks after luteolysis (Augustin et al., 1995). It has been postulated that the vasculature in the early stages of luteolysis may serve as a route for degradation products destined for breakdown and excretion (Redmer et al., 1988). Besides the endometrium, the CL also exhibits blood vessel regression.
Endothelial cell detachment and vessel occlusion are two major phenomena during luteal blood vessel regression (Modlich et al., 1996). These phenotypic changes of luteal endothelial cells may be mainly related to changes in their cell surface adhesion molecules (Augustin et al., 1995).

MMPs and TIMPs may participate in the regulation of the luteal angiogenic process. In the rat (Bagavandoss, 1998) and sheep (McIntush et al., 1996), TIMP-1 is localized in luteal capillaries, suggesting that this endogenous MMP inhibitor may participate in maintaining the integrity of the capillary. Administration of a MMP-2 antibody in ewe results in abnormal luteal vasculature and incomplete CL formation (Gottsch et al., 2001), implicating the critical role of this enzyme in luteal angiogenesis.

Ovarian angiogenesis shares similarities with tumor angiogenesis. For example, in both processes, angiogenesis is viewed as a response to nutrient deficiency, and the same regulators and angiogenic factors, such as hypoxia-inducible factor 1 (HIF-1) and VEGF, are involved (Waleh et al., 1995; Neeman et al., 1997). Therefore, elucidation of the molecular mechanisms of ovarian angiogenesis and the normal capillary degeneration process during luteolysis, especially those that involve MMPs and TIMPs, may provide insights toward a better understanding of tumor angiogenesis.

VII. Perspectives

Ovarian physiological events, such as follicle development, ovulation, corpus luteum development, and angiogenesis, require the participation of proteolytic enzymes and their endogenous inhibitors, such as MMPs and TIMPs, respectively, to orchestrate the associated dynamic tissue remodeling that occurs. By modifying the extracellular milieu, MMPs and TIMPs modulate the cellular or biochemical properties of cells, facilitating proliferation, growth, or differentiation. Beyond this, MMPs also affect other physiological events by liberating growth factors and cytokines from their biologically inactive forms, or modifying the receptors for growth factors or cytokines. In addition, TIMPs may directly modulate cellular behaviors, such as
steroidogenesis and proliferation. However, a great deal of work remains before the physiological functions of MMPs and TIMPs in the reproductive system are fully elucidated. From the above, it is clear that the ovary is an excellent research model for studying the roles of MMPs and TIMPs during normal tissue remodeling and angiogenesis. The information obtained will perhaps prove valuable in designing therapeutic strategies for physiological processes associated with infertility or for pathological conditions, such as cancer, retinopathies, and rheumatoid arthritis.
#### Table 1-1 The MMP family

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<th>MMP number</th>
<th>Trivial name</th>
<th>Other names</th>
<th>EC number</th>
<th>Subfamilies</th>
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Note: 1. Asterisks denote names that are not longer used.
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D/O = domain organization. Letters denote arrangement of domains described in Figure 1-1.
Figure 1-1 Domain Organization of the MMPs

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<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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- Signal Peptide
- Catalytic domain
- Hemopexin-like domain
- Transmembrane domain
- Glycophosphatidyl inositol-anchoring domain
- Cysteine/proline rich IL-1 receptor like sequence
- Vitronectin-like sequence

- Furin-like recognition site
- Fibronectin type II-like insert
- Collagen type V-like insert
- Cytoplasmic tail
- Hinge region

Note: Letters (A-I) denote domain organization.

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1. Abbreviations: CAM: cell adhesion molecules; GROα: growth related oncogene α; PF-4: platelet factor 4; CTAP-III/NAP-2: connective tissue activating protein III/neutrophil-activating peptide-2; SDF: Stromal cell-derived factor; COMP: cartilage oligomeric matrix protein; Cell-surface (tTG): cell surface tissue transglutaminase; TNFα: Tumor necrosis factor α; TGFβ: Transforming growth factor β; IL: Interleukin; HBEGF: Heparin-binding epidermal growth factor; FGFR: Fibroblast growth factor receptor; ILR: Interleukin receptor; MCP: Monocyte chemoattractant protein.

Table 1-4 Biochemistry and molecular features of TIMPs

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<td>TIMP-4</td>
<td>23-24</td>
<td>unresolved</td>
<td>diffusible</td>
<td>3p25</td>
<td>1.2 kb</td>
</tr>
</tbody>
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CHAPTER II

BOVINE MATRIX METALLOPROTEINASE-2 (MMP-2): MOLECULAR CLONING, EXPRESSION IN THE CORPUS LUTEUM, AND INVOLVEMENT IN ANGIogensis

Abstract

Angiogenesis and other tissue remodeling events in the corpus luteum (CL) are mediated by matrix metalloproteinases (MMPs). Previously, we cloned the bovine homolog of membrane-type 1 metalloproteinase (MT1-MMP), and showed that active MT1-MMP is correlated to MMP-2 activity in the CL during the estrous cycle. The aims of the present study were to clone the bovine MMP-2 gene, to investigate its temporal and spatial expression in three ages of CL obtained over the bovine estrous cycle, and to determine its potential roles in endothelial cells during capillary tube formation. The bovine MMP-2 cDNA, which was isolated from a UNI-ZAP II capillary endothelial cell cDNA library, encoded a protein of 661 amino acids. Luteal tissues were collected from non-lactating dairy cows on days 4, 10, and 16 (n=3/day; day 0 = estrus) of the estrous cycle. Northern blotting and Western blotting revealed that the levels of MMP-2 mRNA (3.1kb) and immunoreactive pro-MMP-2 protein (68 kDa) did not differ (P>0.05) in any age of CL examined. In addition to large luteal cells, MMP-2 was localized in endothelial cells in all ages of CL by immunohistochemistry. The potential roles of MMP-2 in angiogenesis were evaluated using bovine capillary endothelial (BCE) cells in a Matrigel capillary tube-formation assay. Tube formation by BCE cells was remarkably suppressed by a neutralizing antibody against MMP-2. Taken together, these data suggested that the activity of MMP-2 and its localization in endothelial cells may be critical for CL development, e.g., by regulating capillary tube formation.
Introduction

The formation and development of the corpus luteum (CL) include extensive matrix remodeling events. After ovulation, the basement membrane degrades, and the follicle wall folds toward the cavity of the collapsed follicle (Niswender and Nett, 1994; Smith et al., 1994). Fibroblasts and luteinized theca interna cells proliferate and migrate into the cavity of the ruptured follicle, where they mix with luteinized granulosa cells (Pederson, 1951). In addition, endothelial cells from capillaries in the theca interna compartment invade the granulosa layer to form new blood vessels (Plendl, 2000). This process of new capillary formation is termed angiogenesis (Moses, 1997). These tissue remodeling events likely require the participation of a large family of zinc and calcium dependent proteolytic enzymes, matrix metalloproteinases (MMPs), which collectively degrade all known components of the extracellular matrix (ECM) (Massova et al., 1998; Smith et al., 1999; Curry and Osteen, 2001).

Among the more than 25 members of the MMP family, only a few have been studied in the CL. The patterns of expression and activities of MMP-2 and MMP-9 have been investigated in bovine (Tsang et al., 1995; Goldberg et al., 1996; Zhang et al., 2002), ovine (Russell et al., 1995; Towle et al., 2002), porcine (Pitzel et al., 2000), rat (Nothnick et al., 1996; Bagavandoss, 1998; Liu et al., 1999), and human (Duncan et al., 1998) CL. Furthermore, MMP-1 (collagenase-1) expression was detected in rat (Nothnick et al., 1996) and human (Duncan et al., 1998) CL, while MMP-13 expression was found in bovine (Zhang and Tsang; unpublished data), ovine (Ricke et al., 2002), and rat (Liu et al., 1999) CL. Within this select group of enzymes, the action of MMP-2 is most notable because its reduced levels impair CL formation (Russell et al., 1995). In part, this may be attributed to the broad substrate specificity of MMP-2, especially its efficient degradative action on type IV collagen, the major component of the basement membrane (Yu et al., 1998). Additionally, administration of a MMP-2 antibody blocks vasculature formation.
during CL development, supporting the potential role of this enzyme in blood vessel recruitment (Gottsch et al., 2001).

Another distinguishing feature of MMP-2 is its activation process. Most MMPs are secreted as zymogens and then cleaved extracellularly into their active forms by serine proteinases (Murphy et al., 1999). However, pro-MMP-2 is activated, instead, on the cell surface by membrane-type 1 metalloproteinase (MT1-MMP) (Murphy et al., 1999). Previously, we cloned the bovine MT1-MMP gene, and demonstrated that the active form of MT1-MMP is correlated to MMP-2 activity in three ages of CL obtained over the estrous cycle (Zhang et al., 2002).

However, much remains to be learned about MMP-2, especially in domestic ruminants, before we can elucidate its role or function in the CL. Therefore, in the present study, we have cloned the bovine MMP-2 cDNA, and profiled its temporal and spatial expression patterns in CL obtained during the estrous cycle. The role of MMP-2 during angiogenesis was studied by using an in vitro capillary tube formation assay.

**Materials and Methods**

1. **Molecular Cloning and Sequence Analysis**

A bovine UNI-ZAP II cDNA library was constructed using RNA isolated from BCE cells of the adrenal cortex with Oligo(dT) primers. The primers for cloning bovine MMP-2 were designed based on the conserved catalytic region of known MMP-2 sequences of other species. The cDNA encoding the catalytic domain of bMMP-2 was directly amplified from this cDNA library using the polymerase chain reaction (PCR). Successive rounds of PCR were performed using new primers designed based on the obtained sequence data to clone the rest of bMMP-2 cDNA. PCR products were cloned into PCR TA-cloning vector (Invitrogen, Carlsbad, CA) for subsequent DNA sequencing.
Sequencing of cDNA was performed using PCR-based automated sequencing methods (Biopolymer Facility and DNA Sequencing Core Facility, Children's Hospital, Boston, MA). Protein database search was assessed with advanced BLAST service provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) with default settings. DNA and protein sequence analysis was conducted using the MacVector 6.0 DNA sequence analysis software package (Accelrys Inc, San Diego, CA). Multiple alignment analysis was performed using Genetyx Sequence Analysis Software (Software Development Company, Tokyo, Japan).

2. Tissue Distribution of Bovine MMP-2

For Northern blot analysis, a cDNA fragment consisting of mostly the 5'-untranslated region (UTR) of bMMP-2 was used to generate cDNA probes, which were random primer-labeled with α-32P-dCTP using the Ready Prime cDNA Labeling Kit (Amersham, Piscataway, NJ). Poly(A)' RNA from different bovine tissues was purified using a modified guanidinium thiocyanate method followed by poly (A)' RNA selection with two rounds of oligo(dT)-cellulose columns. After separation with 1.0% (w/v) agarose electrophoresis containing formaldehyde, RNA was transferred to nylon membranes (Schleicher & Schuell, Keene, NH). A Northern blot of 2.5 µg poly(A)'-RNA was hybridized overnight at 65° C with the radiolabeled probes at a concentration of 2×10^7 cpm/10 ml. The blot was washed to a final stringency of 0.1×SSC and 0.1% SDS (w/v) at 65° C. Hybridized probes were detected with autoradiography.

3. Animals and Tissue Collection

All animal procedures in the present study were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Hampshire. Corpora lutea were collected from regularly cycling, non-lactating dairy cows housed at the University of New Hampshire Dairy Teaching and Research Center. Luteal tissues were
removed on days 4, 10, and 16 of the estrous cycles (day 0 = estrus; n=3/day). For day 4 CL, the ovary was removed by colpotomy from animals under an epidural anesthesia [2% mepivacaine hydrochloride (w/v); 0.01 mL/kg body weight; Upjohn, Kalamazoo, MI]. The CL tissues were then dissected from the ovarian stroma. The day 10 and 16 CL tissues were removed from the ovary by enucleation.

4. Expression of MMP-2 in the Bovine CL during the Estrous Cycle

Total RNA was extracted from luteal tissues using TRIZOL (GIBCO-BRL, Carlsbad, CA) according to the manufacturer’s instructions. Twenty micrograms of total RNA were fractionated on 1.0% (w/v) agarose gels containing formaldehyde and were transferred onto Hybond™-N nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were first incubated in pre-hybridization buffer [50% (v/v) formamide, 5xSSC, 0.2% (w/v) SDS, and 2% (w/v) blocking reagent] for two hours at 42°C. Hybridization was carried out in the pre-hybridization buffer containing MMP-2 cDNA probes at 50°C overnight. These cDNA probes were labeled with digoxigenin (DIG)-dUTP using the DIG DNA random-primed Labeling Kit (Roche Molecular Biochemicals, Indianapolis, IN). The blots were then washed twice at room temperature in 2xSSC with 0.1% (w/v) SDS, followed by higher stringency washes in 0.2xSSC with 0.1% (w/v) SDS at 65°C. Hybridized probes were detected using an anti-DIG antibody conjugated to alkaline phosphatase (used at 1:5000; Roche Molecular Biochemicals, Indianapolis, IN) in 1% (w/v) blocking reagent. The signals were detected by CSPD (Roche Molecular Biochemicals, Indianapolis, IN), a chemiluminescent substrate for alkaline phosphatase. After visualization, the membranes were subsequently stripped according to manufacturer’s instructions, and re-hybridized with cyclophilin cDNA probes.

5. Western Blot Analysis

- 62 -
Total protein was extracted from luteal tissues as previously described (Zhang et al., 2002). Equal amounts of protein were subjected to SDS-PAGE on 10% (w/v) polyacrylamide gels and were transferred to Protran® nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Nonspecific binding sites were blocked by incubating the membranes with 5% (w/v) nonfat dry milk in TBST buffer [10mM Tris-HCl, 150mM NaCl, 0.05% (v/v) Triton X-100, pH 8.0]. Membranes were then incubated with rabbit anti-human MMP-2 antibody (used at 1:5000; Chemicon, Temecula, CA). After extensive washes with TBST buffer (five times, 15 minutes per wash), HRP-conjugated anti-rabbit-IgG (1:10,000) was applied onto membranes as a secondary antibody. The signals were detected with the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer’s instructions.

6. Immunohistochemistry

Frozen sections (6μm) were prepared from luteal tissues collected at three stages of the estrous cycle using a Cryotome (Shandon, Pittsburgh, PA). Sections were fixed in cold acetone for 5 minutes. The endogenous peroxidase activity was quenched by incubating sections in 0.03% (v/v) H₂O₂; for 30 minutes at room temperature. Nonspecific sites were blocked by 10% (w/v) BSA before incubating the slides with a mouse anti-human MMP-2 antibody (1:250; Oncogene Research Products, San Diego, CA). Following incubation with the biotin-conjugated goat anti-mouse IgG secondary antibody (1:2000; Pierce, Rockford, IL), MMP-2 was visualized using VECTASTAIN® Elite ABC Kits (Vector Laboratories, Burlingame, CA). Sections were subsequently counterstained with Gill’s 2 hematoxylin (Shandon, Pittsburg, PA). For each tissue sample, a consecutive section placed on the same slide was used as a negative control, with BSA substituting for the primary antibody. For each tissue (3 corpora lutea for each age), 10 to 20 sections were stained. A minimum of 20 areas per section was examined.
7. BCE Cell Culture and Gelatin Zymographic Analysis

SDS substrate zymography electrophoresis was performed based on a previously described method with modifications (Tsang et al., 1995; Zhang et al., 2002). Medium conditioned by BCE cells were mixed with non-reducing SDS sample buffer before fractionation in 10% (w/v) polyacrylamide gels containing 0.1% (w/v) gelatin (Bio-Rad, Hercules, CA). After electrophoresis, gels were washed twice with 2.5% (v/v) Triton X-100 (15 minutes per wash), followed by incubation with substrate buffer [50 mM Tris-HCl, 5 mM CaCl₂, 1 μM ZnCl₂, and 0.02% (w/v) NaN₃, pH7.6] at 37°C for 24 hours. The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (BioRad, Hercules, CA), and the gelatinolytic activities were shown as clear zones against a blue background.

To verify that bMMP-2 is a bona fide metal-dependent proteinase, zymogram gels were incubated with substrate buffer containing a MMP-specific inhibitor 1,10 phenanthroline (10 mM; Sigma, St. Louis, MO). The latent and active forms of MMP-2 were distinguished by incubating BCE conditioned medium with 1 mM 4-aminophenylmercuric acetate (APMA) (Sigma, St. Louis, MO), an organomercurial activator of MMPs, at 25°C for 30 minutes before zymographic analysis.

8. In Vitro Capillary Tube Formation Assay

The in vitro capillary tube formation assay was carried out as previously described (Kubota et al., 1988). Cell culture surfaces were pre-coated with Matrigel (Becton Dickinson, Waltham, MA) at a density of 100 μl/cm² growth area. After incubating at 37°C for 30 minutes, culture surfaces were washed with PBS. BCE cells were plated at 25,000 cells/cm² growth area in growth medium that contains 5% (v/v) fetal bovine serum (FBS) and 1% (v/v) glutamine penicillin-streptomycin (GIBCO-BRL, Carlsbad, CA).
To assess the potential function of MMP-2 in angiogenesis, the capillary tube formation assay was carried out under conditions that MMP-2 activity was neutralized by an anti-MMP-2 antibody (1:100) (Albini et al., 1991; Pauly et al., 1994; Kenagy et al., 1997), which was kindly provided by Dr. William Stetler-Stevenson at NIH. In the negative control, normal mouse serum was added in the same dilution.

9. Data Analysis

Densitometric intensities of bands on Northern and Western blots were determined by UN-SCAN-IT gel™ automated digitizing system (Silk Scientific, Orem, UT). Data were analyzed by ANOVA, followed by Tukey’s test for multiple comparisons.

Results

1. Cloning and Sequence Analysis of Bovine MMP-2

Bovine MMP-2 was PCR-cloned from a cDNA library constructed using mRNA isolated from BCE cells of the adrenal cortex. Compiled cDNA sequences of bovine MMP-2 were aligned using MacVector sequence analysis software to generate a cDNA of 2169 basepairs (bp), including an open reading frame (ORF) of 1986 bp, a 5'-untranslated region (UTR) of 181 bp, and a partial 3'-UTR of 70 bp (Figure 1A; GenBank accession number AF290428).

The predicted bovine MMP-2 protein is composed of 661 amino acids with a calculated molecular mass of about 74 kDa. From the amino terminus, bovine MMP-2 is composed of the hydrophobic signal domain (M1 to A30), the pro-domain (A31 to N110), the catalytic domain (Y111 to G447), the hinge region (A448 to L469), and the hemopexin domain (C470 to C661) (Figure 1B). The catalytic domain contains a conserved sequence HEXGHXXGXXHS/T, which functions as the binding site for the catalytic zinc atom (HEFGHAMGLEHS in the bovine MMP-2). In addition, the catalytic domain of bovine MMP-2 is interrupted by three fibronectin type II-like repeats, which function as binding sites for gelatin and serve as a hallmark of gelatinases. Multiple
sequence alignment analysis demonstrated that bovine MMP-2 shares 94.7%, 94.1%, 93.5%, 93%, and 95.9% sequence identity to its human, rat, mouse, rabbit, and pig homologs, respectively (Figure 1C).

2. Tissue Distribution of Bovine MMP-2

Expression of bMMP-2 in various bovine tissues was analyzed by Northern blotting (Figure 2A). Bovine MMP-2 is encoded by a single mRNA species of approximately 3.1 kb. The density of Northern blots was evaluated by scanning densitometry analysis, and relative expression levels of MMP-2 mRNA in different tissues are presented as percentage of density compared to that in the lung, which had the strongest expression and was assigned as 100% (Figure 2B). A moderate expression of MMP-2 was also detected in the kidney (50.0%), heart (30.2%) and spleen (15.0%). However, there were extremely low and non-detectable levels of MMP-2 mRNA in the brain (6.7%) and in the liver (0%), respectively.

3. Expression and Localization of MMP-2 in the Bovine CL

The pattern of MMP-2 expression in the early, mid-, and late stage CL during the estrous cycle was investigated at the mRNA level by Northern blot analysis (Figure 3A), and at the protein level by Western blot analysis (Figure 4A). Northern blot analysis detected a single 3.1 kb MMP-2 transcript in all CL tissues. No apparent differences in the levels of bMMP-2 mRNA were observed among these three ages of CL (p>0.05). Western blot analysis detected an approximately 69 kDa protein band, which corresponds to the latent form of MMP-2, in all three ages of CL (Figure 4A). Densitometric analysis demonstrated that the level of the latent MMP-2 did not change (p>0.05) during the estrous cycle (Fig 4B).

The spatial pattern of MMP-2 expression in the bovine CL was studied by immunohistochemistry. In early (d4), mid (d10), and late (d16) cycle CL (Figure 5A-C, respectively), positive staining for MMP-2 was localized in endothelial and large luteal cells.
Large (steroidogenic) luteal cells were identified based on cell size and presence of lipid droplets. Endothelial cells were identified by staining a consecutive section with an antibody for Factor XIII, an endothelial cell marker (Figure 5E). No staining was observed when the primary antibody was omitted (Figure 5D and 5F).

4. Detection of MMP-2 Activity in Bovine Capillary Endothelial (BCE) Cells

MMP-2 activity in BCE cells was studied using gelatin zymography. A 68 kDa gelatinolytic activity, which corresponds to the protein size of MMP-2, was readily detected in serum-free medium conditioned by BCE cells (Figure 6, lane 1). Under the cell culture conditions, the majority of gelatinase activity expressed by BCE cells is in the latent form. This was validated by incubation of BCE conditioned medium with 1.0 mM APMA, which resulted in a conversion of the 68 kDa proMMP-2 to the 62 kDa active forms (Figure 6, lane 2). The detected gelatinolytic activity was from an MMP because its activity was completely inhibited by an MMP-specific inhibitor, 1.10-phenanthroline (Figure 6, lane 3).

5. MMP-2 is Required for In vitro Capillary Tube Formation

The role of MMP-2 during angiogenesis was studied by using an in vitro capillary tube formation system. As shown in Figure 7A, BCE cells formed extensive capillary-like structures when they were plated on Matrigel. The most extensive tube formation was observed at 8 to 12 hours after cell plating and was dependent on the presence of 5% serum (data not shown). When incubated with a monoclonal MMP-2 antibody at 1:100 dilution, BCE cells showed reduced degree of in vitro capillary tube formation (Figure 7B).

Discussion

In addition to their involvement in pathological extracellular degradation events such as tumor invasion and metastasis, matrix metalloproteinases (MMPs) have been shown to be...
important in normal tissue remodeling processes that relate to female physiology, including follicle development, ovulation, CL development and regression, and implantation (Smith et al., 1999; Curry and Osteen, 2001). However, information regarding detailed expression profiles of these enzymes and their physiological functions, especially in species with long luteal phases, is limited. In the present study, we reported the full length cDNA sequence of bovine MMP-2, its expression and localization in the bovine CL, and its involvement in capillary formation. The open reading frame of the bovine MMP-2 gene encodes a protein consisting of 661 amino acids, showing a high degree of identity to its human, rat, mouse, rabbit, and pig homologs. This indicates that this enzyme is highly conserved among mammals. Bovine MMP-2 possesses the classical domain organization of gelatinases, including the three head-to-tail repeats of fibronectin type II-like inserts. These inserts function as binding sites for gelatin and are critical for the proteinase activity on ECM substrates such as casein, gelatin, and type IV collagen (Murphy et al., 1994; Shipley et al., 1996). The tissue distribution pattern of bovine MMP-2 is not only similar to that observed in mouse (Gack et al., 1994), but is also correlated to its membrane-associated activator, MT1-MMP (Zhang et al., 2002). Thus, in cattle, the coordinated expression of these two MMPs in multiple tissues suggests that MMP-2 is activated similarly in a variety of cell types.

Only a few studies have reported the activity and expression patterns of MMP-2 in CL obtained during the estrous cycle or undergoing regression. The MMP-2 transcript and protein are highly expressed during the early stages of rat CL development (Liu et al., 1999), while significant increases are observed in the late rather than the early and mid stages in the human CL (Duncan et al., 1998). In the present study, MMP-2 transcript and pro-MMP-2 protein were constitutively expressed in early, mid, and late stages of the bovine CL. Furthermore, enhanced MMP-2 expression is associated with luteolysis in sheep (Towle et al., 2002), human (Duncan et al., 1998), and rat (Endo et al., 1993). Eight hours after a systemic infusion of the luteolysin, PGF_{2a}, MMP-2 activity is significantly increased (Towle et al., 2002). PGF_{2a} also induces high
production of MMP-2 by porcine luteal cells (Pitzel et al., 2000). When human chronic
gonadotropin (hCG) is given to rescue the human CL from regressing, MMP-2 expression is
reduced (Duncan et al., 1998). Collectively, these data indicate that there are species differences
in the expression patterns of MMP-2 in the CL. Furthermore, the degradation of the ECM
mediated by MMP-2 may be important for the involution of the CL.

The temporal expression of MMP-2 follows a pattern similar to its endogenous activator.
MT1-MMP. While the latent forms of MMP-2 and MT1-MMP remain unchanged during the
bovine estrous cycle, the activities of both enzymes paralleled each other, being low in the early
stage CL, but significantly enhanced in the mid and late stages (Zhang et al., 2002). Previously,
zymography revealed the presence of active MMP-2, which was validated by incubation with
APMA to distinguish between latent and active forms of MMP-2 (Zhang et al., 2002). Thus, our
inability to detect the active form of MMP-2 in immunoblots in the present study was most likely
the result of using a heterologous antibody. Besides MMP-2, MT1-MMP also activates MMP-13
(collagenase 3). MMP-13 was expressed at a constant level in the bovine CL during the estrous
cycle (Zhang and Tsang; unpublished data). However, in the rat CL, which predominantly
expresses MMP-2 at the early stage, MT1-MMP is constitutively expressed in all stages
examined, while MMP-13 is highly expressed in the late stage (Liu et al., 1999). Following
PGF$_{2}a$-induced luteolysis in sheep. MMP-2 and MMP-13 mRNA are increased by 6 hours, while
MT1-MMP mRNA is increased by 15 minutes, and remains elevated through 48 hours (Ricke et
al., 2002). Thus, there is also species variation in the interplay between MMP-2 and MMP-13,
and their activator in vivo. MT1-MMP.

Although MMP-2 transcripts are localized in human theca-lutein cells (Duncan et al.,
1998), MMP-2 mRNA and activity has been predominantly observed in large luteal cells of a
variety of species. Rat luteinized granulosa cells express MMP-2 mRNA (Curry et al., 2001),
while immunoreactive MMP-2 is detected in luteal cells of developing rat CL (Bagavandoss,
1998). In addition. MMP-2 mRNA and activity are detected in porcine large luteal cell cultures
In the present study, immunohistochemistry demonstrated that large (steroidogenic) luteal cells expressed MMP-2, which is consistent with our previous observation that MMP-2 activity is present in conditioned medium of bovine luteal cell cultures (Tsang et al., 1995). The co-localization of MMP-2 and MT1-MMP in large luteal cells suggests that activation of pro-MMP-2 is locally regulated. For example, MMP-2, per se., binds to cell membranes via the integrin αvβ3 (Brooks et al., 1996), promoting localized pericellular proteolysis to accommodate the increasing size of large luteal cells during CL development (Schwall et al., 1986; Zheng et al., 1994). As proposed by Smith et al. (Smith et al., 1999), the molecular regulation of the luteinization process and the changes in the biochemical properties of luteal cells are possibly attributed to dynamic interactions between the ECM and the cell.

Another example of cell-cell and cell-matrix interactions is angiogenesis, a hallmark of CL development (Basset, 1943; Reynolds et al., 2000). Angiogenesis is a multi-step process that involves the interplay between angiogenic factors, endothelial cell migration and proliferation, and ECM remodeling, which ultimately lead to recruitment of pericytes and formation of capillary lumen (Moses, 1997; Klagsbrun and Moses, 1999). As a highly vascularized gland, the CL is a rich source of angiogenic factors (Reynolds et al., 2000). Over the course of CL development, 85% of proliferative cells are endothelial cells (Reynolds et al., 1994), which are the most abundant cell type in the mid cycle CL (Lei et al., 1991; Zheng et al., 1993).

Extracellular proteolysis, which requires the participation of MMPs, is an integral part of the angiogenic process (Moses, 1997). Besides the large luteal cells, MMP-2 was also localized in endothelial cells of the bovine CL. In the rat CL, MMP-2 is also detected in the same cellular compartments (Bagavandoss, 1998). The in vivo cellular localization of MMP-2 is supported by a recent in vitro study, showing that MMP-2 was the predominant gelatinase in CL-derived endothelial cells (Zhang et al., unpublished data). The present study also showed that an MMP-2 antibody inhibits capillary tube formation by endothelial cells, an indispensable step in angiogenesis. Taken together, these data suggest that MMP-2 activity may be necessary for
neovascularization (Hiraoka et al., 1998). In ewes, administration of a MMP-2 antibody results in incomplete CL formation, whereby the build up of normal vasculature is blocked (Gottsch et al., 2001). In the same animal model, immunization of the N-terminal peptide of the inhibin α43-subunit (αN) also causes incomplete CL development, concurrent with reduced MMP-2 activity (Russell et al., 1995). In addition, MMP-2 activity is critical for angiogenic processes in other tissues. For example, the MMP-2 gene knockout mouse shows remarkable resistance to tumor-induced angiogenesis (Itoh et al., 1998), while corneal angiogenesis is diminished in MMP-2-deficient mice (Kato et al., 2001).

MMP-2 may play multiple roles during angiogenesis. The cleavage of plasminogen generates a 38 kDa internal fragment, which is called angiostatin and has inhibitory action on angiogenesis (O'Reilly et al., 1999). MMP-2 also cleaves big ET-1 into an active and potent vasoconstrictor, ET-1 (Fernandez-Patron et al., 1999). Moreover, the cleavage of calcitonin gene-related peptide (CGRP), a potent vasodilator, by MMP-2, remarkably reduces its vasodilatory potency (Fernandez-Patron et al., 2000). In addition to its degradation on ECM components, therefore, MMP-2 may regulate angiogenesis and vascular function via diverse pathways.

Considering our previous observation that active MT1-MMP is correlated to MMP-2 activity (Zhang et al., 2002), the constitutive expression of MMP-2 transcript during the estrous cycle suggests that MMP-2 activity is predominantly regulated at the level of activation in the bovine CL. In addition, expression of MMP-2 in large luteal cells may facilitate pericellular proteolysis of the ECM during CL development. Furthermore, localization of MMP-2 in endothelial cells and inhibition of capillary tube formation by a MMP-2 antibody indicate that this enzyme may be critical for angiogenesis in the CL.
Figure 2-1. Sequence analysis of bovine MMP-2. A) Nucleotide sequence of the bovine MMP-2 cDNA and its deduced amino acid sequence. The protein sequence is shown in uppercase letters and the DNA sequence in lowercase. While the numbers on the left are for the amino acid sequence, the ones on the right are for the cDNA sequence. B) Schematic diagram showing the domain organization of bovine MMP-2. Signal, Pro, Cat, Hinge, and Hex represent the signal peptide, propeptide, catalytic, hinge, and hemopexin/vitronectin domains, respectively. The fibronectin type II-like inserts and the Zn$^{2+}$ binding consensus site in the catalytic domain are also indicated. C) Multiple sequence alignment of bovine MMP-2 and its homologs from other species. The numbers in the boxes indicate the percentages of amino acid sequence identity in corresponding domains. The overall homology percentages for the entire molecule are shown on the right. GenBank accession numbers of the sequences analyzed are bovine MMP-2 AAG28169, human MMP-2 A28153, mouse MMP-2 A42496, rat MMP-2 S34780, rabbit MMP-2 S70365, and pig MMP-2 AAK97133.
Figure 2-2. Tissue distribution of bovine MMP-2 mRNA expression. A) Northern blot analysis of MMP-2 mRNA expression in various bovine tissues, including the brain, heart, kidney, liver, lung, and the spleen. A single transcript of approximately 3.1 kb is marked by the arrow on the left. B) Relative expression levels of MMP-2 mRNA in different bovine tissues. Densitometric intensities of MMP-2 Northern blots were determined by UN-SCAN-IT digitizing systems (Salk Scientific, Orem, UT), and are presented in percentages as compared to that in the lung, which was assigned as 100%.
MMP-2 mRNA expression in the bovine CL

A)

B)

Figure 2-3. MMP-2 mRNA expression in luteal tissues. A). Northern blotting detected a 3.1 kb MMP-2 mRNA species in the early (E; day 4), mid- (M; day 10), and late (L; day 16) stage CL (upper panel). The same membranes were stripped and hybridized with a cyclophilin probe as a control for RNA loading (lower panel). B). Relative levels of MMP-2 mRNA are presented as ratios of the densitometric value of MMP-2 to cyclophilin. Dissimilar letters denote significance at p<0.05.
MMP-2 protein expression in the bovine CL

A) This particular MMP-2 antibody detected the pro- (~69 kDa) but not the active form of MMP-2 in the early (E: day 4), mid (M; day 10), and late (L; day 16) stage CL. Molecular masses (kDa) of prestained protein standards (Bio-Rad Laboratories, Hercules, CA) are shown on the left. HT denotes conditioned medium of HT1080 cells. B) Densitometric analysis showed that the levels of pro-MMP-2 did not vary (p>0.05) among different stages of CL. Dissimilar letters denote significance at p<0.05.

Figure 2-4. Representative Western blot of MMP-2 in bovine CL obtained over the estrous cycle. A) This particular MMP-2 antibody detected the pro- (~69 kDa) but not the active form of MMP-2 in the early (E: day 4), mid (M; day 10), and late (L; day 16) stage CL. Molecular masses (kDa) of prestained protein standards (Bio-Rad Laboratories, Hercules, CA) are shown on the left. HT denotes conditioned medium of HT1080 cells. B) Densitometric analysis showed that the levels of pro-MMP-2 did not vary (p>0.05) among different stages of CL. Dissimilar letters denote significance at p<0.05.

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MMP-2 localization in the bovine CL

Figure 2-5. Immunohistological staining of MMP-2 in the early, mid-, and late stage CL. MMP-2 protein expression was localized in endothelial (black arrows) and large luteal cells (white arrows) of the early (A; day 4), mid (B; day 10), and late (C; day 16) stages of CL. Endothelial cells were identified (black arrows) based on staining with Factor VIII (E). No staining was observed in the negative controls (D and F, for MMP-2 and Factor VIII, respectively), in which the primary antibody was replaced by 10% BSA. Magnification is at 400×.
Figure 2-6. Gelatin zymographic analysis of medium conditioned by BCE cells. A major 68 and a faint 62 kDa band, which correspond to pro and active MMP-2, respectively, were observed in medium conditioned by BCE cells (lane 1). Incubation with APMA converted the 68 kDa pro-MMP-2 to its 62 kDa active form (lane 2). All zones of clearance disappeared following incubation with 1, 10-phenanthroline. The molecular masses (kDa) of Perfect Protein™ Marker (Novagen, Madison, WI) are indicated on the left.
Effects of MMP-2 antibody on in vitro capillary tube formation of BCE cells

Figure 2-7. Effects of a neutralizing antibody against MMP-2 on in vitro capillary formation of BCE cells. Phase contrast photography of capillary-like tube formation of BCE cells cultured on Matrigel under control conditions (A), or in the presence of a neutralizing anti-MMP-2 antibody (1:100; B).
CHAPTER III

BOVINE MEMBRANE-TYPE 1 MATRIX METALLOPROTEINASE (MT1-MMP):
MOLECULAR CLONING AND EXPRESSION IN THE CORPUS LUTEUM

Abstract
Matrix metalloproteinase-2 (MMP-2) is produced as a zymogen, which is subsequently, activated by membrane-type 1 metalloproteinase (MT1-MMP). The objectives of the present study were to clone bovine MT1-MMP and to investigate its expression in the corpus luteum (CL). CL were harvested from nonlactating dairy cows on day 4, 10, and 16 of the estrous cycle (day 0=estrus; n=3 for each age). The bovine MT1-MMP cDNA contained an open reading frame of 1749 base pairs, which encoded a predicted protein of 582 amino acids. Northern blotting revealed no differences (p>0.05) in MT1-MMP mRNA levels between any ages of CL. Western blotting demonstrated that two species, the latent form (~63 kDa) and the active form (~60 kDa) of MT1-MMP, were present in CL throughout the estrous cycle. Active MT1-MMP was lower (p<0.05) in the early CL than the mid- and late stages, where MMP-2 activity, as revealed by gelatin zymography, was also elevated. Furthermore, immunohistochemistry revealed that MT1-MMP was localized in endothelial, large luteal, and fibroblast cells of the CL at different stages. Taken together, the differential expression and localization of MT1-MMP in the CL suggest that it may have multiple functions throughout the course of the estrous cycle, including activation of pro-MMP-2.

Introduction
The corpus luteum (CL) is a dynamic, transient endocrine gland, which develops from the postovulatory follicle (Niswender and Nett, 1994). In addition to functional changes, the
development, maintenance and regression of the CL are associated with structural events such as tissue remodeling and angiogenesis (Smith et al., 1999; Reynolds et al., 2000). It is believed that this tissue remodeling process includes breakdown and resynthesis of extracellular matrix (ECM) components (Luck and Zhao, 1995; Smith et al., 1999), which require the participation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Hulboy et al., 1997; McIntush and Smith, 1998; Smith et al., 1999). Although the literature is still emerging, a few of the secreted MMPs and TIMPs have been investigated in CL and follicles of species with long luteal phases, including the bovine (Juengel et al., 1994; Tsang et al., 1995; Goldberg et al., 1996; Smith et al., 1996; Zhang and Tsang, 2000b). ovine (Murdoch et al., 1986; Smith et al., 1994; Smith et al., 1995), porcine (Pitzel et al., 2000), human (Duncan et al., 1998), and macaque (Chaffin and Stouffer, 1999).

Matrix metalloproteinases are secreted as latent proenzymes, which are proteolytically cleaved to their active forms (Birkedal-Hansen, 1995; Massova et al., 1998). It is proposed that some serine proteinases, such as trypsin, neutrophil elastase and plasmin, can initiate the extracellular activation process of pro-MMPs by a "cysteine switch" mechanism (Van Wart and Birkedal-Hansen, 1990). Unlike other secreted MMPs, however, pro-MMP-2 is inefficiently activated by this proteolytic activation mechanism, because its sequence is not susceptible to cleavage by serine proteinases (Collier et al., 1988). Instead, pro-MMP-2 activation is localized on the cell surface (Murphy et al., 1992b), where membrane-type 1 matrix metalloproteinase (MT1-MMP) has been identified as its activator in placenta and several different kinds of tumor cells (Sato et al., 1994; Sato et al., 1997a).

Currently, six different membrane-type MMPs (MT1-MT6 MMP) have been identified (Ravanti and Kahari, 2000; Vu and Werb, 2000) with MT1-MMP being the most well studied. Similar to other non-membrane type MMPs, MT1-MMP is characterized by having a signal peptide at the amino terminus, followed by a pro-peptide, catalytic domain, hinge region, and hemopexin-like domain at the C terminus (Sato et al., 1994; Massova et al., 1998). In contrast to
secreted MMPs. MT1-MMP contains a distinct transmembrane domain, which has about 25 hydrophobic amino acid residues, enabling it to be expressed on the cell surface (Massova et al., 1998). The human MT1-MMP nucleic acid and protein sequence was the first to be deduced (Sato et al., 1994), and subsequently, rat (Okada et al., 1995a), mouse (Apte et al., 1997), and rabbit (Sato et al., 1997b) MT1-MMPs were cloned and sequenced.

MT1-MMP possesses a variety of functions. As a matrix-degrading enzyme, MT1-MMP has collagenolytic and gelatinolytic activities during connective tissue remodeling (Imai et al., 1996; Ohuchi et al., 1997). With the exception of MT4-MMP, the most well known role of MT-MMPs, including MT1-MMP, is their ability to activate pro-MMP-2, a key enzyme associated with a variety of pathological and physiological processes. Thus, it is not surprising that MT1-MMP expression is correlated with wound healing (Ravanti and Kahari, 2000) and a variety of disease states including tumor invasion and metastasis (Polette and Birembaut, 1998), and rheumatoid arthritis (Yamanaka et al., 2000). Interestingly, MT1-MMP expression is also associated with several physiological processes, including bone resorption, implantation, and mammary gland development (Vu and Werb, 2000). In rats and mice, MT1-MMP mRNA is expressed during follicular development, ovulation, and formation and regression of the CL (Liu et al., 1998; Goto et al., 1999; Hagglund et al., 1999; Liu et al., 1999).

In order to establish the presence and activity of MT1-MMP in a domestic ruminant, the objectives of the present study were to clone and sequence the bovine specific MT1-MMP, and determine the pattern of mRNA and protein expression in relation to MMP-2 activity in early, mid, and late stage CL obtained throughout the estrous cycle.

**Materials and Methods**

1. **Molecular Cloning and Sequencing of Bovine MT1-MMP cDNA**

A bovine UNI-ZAP II cDNA library was constructed using RNA isolated from bovine capillary endothelial cells of adrenal cortex with Oligo(dT) primers. The catalytic domain of
bovine MT1-MMP cDNA was amplified from this library using PCR primers designed according to the conserved regions of MT1-MMP of other species (Massova et al., 1998). The remaining cDNA was amplified using primer-walking. PCR products were subsequently cloned into the PCR TA-cloning vector (Invitrogen. Carlsbad, CA) for DNA sequencing. cDNA sequencing was performed with PCR-based automated sequencing methods (Biopolymer Facility and DNA Sequencing Core Facility. Children’s Hospital. Boston. MA). Protein database searches were assessed by using the BLAST service of the National Center for Biotechnology Information (Bethesda. MD). DNA and protein sequence analyses were conducted using the Mac Vector 6.0 DNA sequence analysis software package (Kodak. Rochester. NY).

2. Animals and Tissue Collection

Corpora lutea were collected from regularly cycling. nonlactating dairy cows that were housed at the University of New Hampshire Dairy Teaching and Research Center. Luteal tissues were removed on days 4, 10, and 16 of the estrous cycle (day 0 = estrus; n=3 per day). For day 4 CL, the ovary was removed by colpotomy after the cow received an epidural anesthetic [2% (v/v) mepivacaine hydrochloride: 0.01 mL/kg BW: (Upjohn. Kalamazoo. MI)], and the CL was then dissected from the ovarian stroma. The day 10 and 16 CL were removed from the ovary by enucleation. All animal experimentation protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Hampshire.

3. Tissue Distribution of MT1-MMP

In order to determine the tissue distribution of MT1-MMP, poly(A)+ RNA from bovine liver. brain. kidney. lungs. heart and spleen was purified using a modified guanidinium thiocyanate method (Chomczynski and Sacchi. 1987) followed by poly(A)+ RNA selection with two rounds of oligo(dT)-cellulose columns. Poly(A)+ RNA (2.5μg) was fractionated on 1% formaldehyde agarose gels before transfer onto nylon membranes (Schleicher & Schuell. Keene.
A 700bp cDNA probe, corresponding to nucleotides 900 to 1600 of bovine MT1-MMP coding sequence, was labeled with $^{32}$p-dCTP using the Rediprime™ II Random Prime Labeling System (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were then hybridized overnight at 65°C with radiolabelled probes at a concentration of $2 \times 10^7$ cpm/10ml, followed by washing with $1 \times$ SSC (150mM sodium chloride, 15mM sodium citrate, pH 7.0) at room temperature and $0.1 \times$ SSC at 65°C.

4. Northern Blotting of Luteal Tissues

For analysis of luteal tissues, the same cDNA probe described above was labeled with digoxigenin (DIG)-dUTP using the DIG DNA Labeling Kit (Roche Molecular Biochemicals, Indianapolis, IN). Total RNA was isolated from luteal tissues using the guanidine thiocyanate-phenol acid extraction protocol as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Following quantification by absorbance at 260nm, 20µg of total RNA was fractionated on 1% (w/v) agarose gels containing formaldehyde and transferred onto Hybond™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by capillary blotting using $20 \times$ SSC (3M sodium chloride, 300mM sodium citrate, pH 7.0). After an overnight transfer, nucleic acids were cross-linked with a UV Crosslinker (Hoefer, San Francisco, CA). Prehybridization was performed at 50°C in standard hybridization buffer containing 50% (v/v) formamide, $5 \times$ SSC, 0.2% (w/v) SDS, and 2% (w/v) blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN) for 2 hours. The membranes were then hybridized in the same buffer containing DIG-labeled MT1-MMP cDNA probe overnight at 65°C. The blots were washed twice at room temperature in $2 \times$ SSC in 0.1% (w/v) SDS, followed by higher stringency washes with $0.1 \times$ SSC in 0.1% (w/v) SDS at 65°C. The membranes were then equilibrated in washing buffer [0.1M maleic acid, 0.15M NaCl, 0.3% (v/v) Tween-20, pH 7.5] for 1 minute, followed by a 30-minute incubation in 1% (w/v) blocking solution (in washing buffer).
membranes were then incubated in the same blocking solution containing an anti-DIG antibody conjugated to alkaline phosphatase (1:5000; Roche Molecular Biochemicals, Indianapolis, IN). After two rinses with washing buffer, the blots were incubated with CSPD (Roche Molecular Biochemicals, Indianapolis, IN), a chemiluminescent substrate for alkaline phosphatase. Signals were visualized after development of Kodak XAR-5 films by a Konica Medical Film Processor (Tokyo, Japan).

5. Western Blot Analysis

Finely minced luteal tissue was homogenized in an extraction buffer [50mM Tris-HCl, 150mM NaCl, 0.02% (w/v) sodium azide, 10mM EDTA, 1% (v/v) Triton X-100, 10μg/ml aprotinin, 1μg/ml pepstatin A, 1μg/ml aminoethyl benzenesulphonyl fluoride] described by Lehti et al. (Lehti et al., 1998) with minimal modification (pH 7.5). A ratio of 0.125g luteal tissue and 1.0 ml extraction buffer was maintained. Following extraction, the samples were centrifuged at 800g for 10 minutes at 4°C to enable collection of the supernatant fraction.

To assess the protein expression pattern of MT1-MMP in different ages of CL, equivalent amounts of tissue extracts were subjected to SDS-PAGE on 10% (w/v) polyacrylamide gels. Fractionated proteins were then electrophoretically transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Nonspecific binding sites were blocked with 5% nonfat powdered dry milk in TBST buffer [0.01M Tris-HCl, 0.15M NaCl, 0.05% (v/v) Triton X-100, pH 8.0] for 2 hours. A rabbit anti human MT1-MMP polyclonal antibody (1:500; Chemicon International Inc, Temecula, CA), which recognizes the conserved hinge region of the bovine molecule, was diluted in 5% nonfat dry milk in TBST. applied onto the membranes, and incubated overnight at room temperature. The membranes were then washed five times, 15 minutes each, with TBST before a 1 hour incubation with anti-rabbit IgG conjugated to horseradish peroxidase (1:15,000; Pierce, Rockford, IL) at room temperature. After another five
washes as described above, the blots were developed with the use of SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer’s instructions. The protein bands were finally visualized after exposure to Kodak XAR-5 film (Kodak, Rochester, NY). Low range Prestained SDS-PAGE Standard (Bio-Rad Laboratories, Hercules, CA) was run in adjacent lanes.

6. Gelatin Zymography

Gelatinolytic activity in luteal tissue samples was detected using a modified protocol that was previously described (Goldberg et al., 1996). Briefly, equivalent amounts of tissue extracts were electrophoresed on 10% polyacrylamide gels impregnated with 0.5mg/ml gelatin. After electrophoresis, SDS was removed from gels by two 15-minute washes with 2.5% (v/v) Triton X-100, and then incubated overnight at 37°C in substrate buffer (50mM Tris-HCl, 5mM CaCl₂, 50mM NaCl, pH 8.0). The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA) for 30 minutes, and destained with distilled H₂O. Gelatinolytic activity was revealed as clear bands against a blue-stained background. Perfect Protein™ Markers (Novagen, Madison, WI) and a positive control, the conditioned medium of HT1080 cells, were run in adjacent lanes.

In order to verify that the gelatinolytic activities detected were metalloproteinases, gels were incubated with substrate buffer containing 10mM 1,10-phenanthroline (Sigma, St Louis, MO), a specific MMP inhibitor and a zinc ion chelator. By interfering with the zinc-containing active site of MMPs, zones of clearance are prevented from forming. Furthermore, in order to distinguish between the latent and active forms of MMPs, samples and conditioned medium of HT1080 cells were incubated with 2mM p-aminophenylmercuric acetate (APMA; Sigma, St Louis, MO) for 2 hours at 37°C prior to zymography. This results in the cleavage of latent MMPs...
to their "active" lower molecular weight forms, which may undergo further processing, i.e.,
autolytic cleavage.

7. Immunohistochemistry

Tissue sections (6μm) were cut onto Superfrost slides (Fisher Scientific, West Chester,
PA), and air-dried at room temperature for 30 min. Sections were then fixed in cold acetone for
10 min, and washed with phosphate-buffered saline (PBS, pH 7.4). Endogenous peroxidase
activity was quenched by incubating sections with 0.3% (v/v) hydrogen peroxide for 30 min.
Nonspecific binding was blocked for 30 min by 5% (w/v) BSA. Sections were subsequently
incubated at room temperature for 1 hour with a 1:250 dilution of a polyclonal rabbit anti-MT1-
MMP (Chemicon International Inc. Temecula, CA). After washing, slides were incubated with
biotin conjugated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA), before
visualization with the VECTASTAIN® Elite ABC Kit (Vector Laboratories, Burlingame, CA)
according to the manufacturer's instructions. For each tissue sample, an adjacent section placed
on the same slide was used as a negative control, with BSA substituting for the primary antibody.
After completion of the color reaction, the slides were counter-stained with Gill 2 Hematoxylin
(Shandon, Pittsburgh, PA). For each tissue (3 corpora lutea for each age), 10 to 20 sections were
stained. A minimum of 20 areas per section was examined.

8. Densitometry

Northern blots, Western blots, and zymograms were analyzed with UN-SCAN-IT gel™
automated digitizing system (Silk Scientific Inc. Orem, UT). For Northern blots, densities of
MT1-MMP and 28S rRNA were used to calculate the MT1-MMP/28S rRNA density ratio for
each sample. Similarly, for zymography, levels of latent and active MMP2, and the HT1080
positive control were used to calculate the MMP2/HT1080 ratio for each sample.
9. Statistics

Each sample was run in triplicate. Quantitative values were expressed as means ± SEM. All data were analyzed by ANOVA, followed by Tukey's test of pairwise comparisons to determine differences between the three age groups of CL. A value of p<0.05 was considered significant.

Results

1. Sequence Analysis of Bovine MT1-MMP

The bovine MT1-MMP cDNA contained an open reading frame of 1749 bp (GenBank accession number AF290429: July 27th, 2000), which encoded a protein of 582 amino acids (Figure 1A). All three characteristic regions of the MT-MMP subfamily, the 11-amino-acid insertion (IS-I) between the propeptide and catalytic domains, the 8-amino-acid (IS-II) insertion in the catalytic domain, and the 75-amino-acid (IS-III) at the C terminus, were also present at these positions in bovine MT1-MMP (Figure 1B). Alignment of the predicted protein sequences revealed significant homology between the bovine MT1-MMP protein and other species, with rat being the highest (95.9%), followed by human (95.7%), mouse (95.0%), and rabbit (92.3%) (Figure 1C).

2. Tissue Distribution of MT1-MMP mRNA

Northern blotting revealed a 3.5 kb transcript in a variety of bovine tissues, with the strongest signal in the lungs, followed by spleen, kidney, heart, and liver. The signal in the brain was undetectable (Figure 2).

3. Expression of MT1-MMP in Bovine Corpus Luteum
A single 3.5 kb transcript was detected by Northern blotting in luteal tissues collected at early, mid-, and late stages of the estrous cycle (Figure 3A). However, the levels of MT1-MMP mRNA, expressed as a ratio of the MT1-MMP band density for each CL sample to its corresponding 28S rRNA band density, were similar (p>0.05) among the three stages (Figure 3B).

MT1-MMP was studied in bovine CL by Western blot analysis. In all samples, two bands with a relative molecular mass of ~63 kDa, consistent with the latent form of MT1-MMP, and ~60 kDa, consistent with the active form of MT1-MMP, were observed (Figure 4A). The level of active MT1-MMP protein in the d4, early CL was significantly lower (p<0.05) than mid- and late stages (Figure 4B).

4. Expression of MT1-MMP is Correlated to Pro-MMP-2 Activation

MMP-2 activity in the bovine CL during the estrous cycle was determined by gelatin zymography (Figure 5A). Visual observation revealed an ~68 (pro-MMP-2) and ~62 kDa (active MMP-2) species in all luteal samples. While the intensity of the ~68 kDa species did not vary, the intensity of the ~62 kDa species in the d4, early CL appeared to be less than the other two older stages. Incubation of samples with 1. 10-phenanthroline prevented the appearance of gelatinolytic zones of clearance (data not shown), indicating that these clear zones on the zymograms reflected MMP activities. Lastly, the decrease in band intensity of the ~68 kDa band after treatment of luteal samples and conditioned medium of HT 1080 cells with APMA for two hours at 37°C suggested that this enzyme species was the latent form of MMP-2 (Figure 5C).

Densitometric analysis revealed that the levels of the ~68 kDa pro-MMP-2 were similar (p>0.05; data not shown) among the three stages of CL. However, the level of the ~62 kDa active form of MMP-2 increased during the progression of the estrous cycle, being significantly greater (p<0.05) in d10 and d16 CL than in the early stage (Figure 5B).
5. Cellular Distribution of MT1-MMP

Immunohistochemistry was used to investigate the cellular localization of MT1-MMP in the bovine CL. Overall, the cellular distribution of MT1-MMP varied throughout the life span of the CL. In the early (Figure 6A) and mid-cycle (Figure 6B) CL, MT1-MMP was localized in endothelial cells, and on the cell membrane and cytoplasm of large luteal cells, consistent with cellular processing of the MT1-MMP protein in cytoplasmic organelles before becoming resident on the cell membrane. In the late stage CL (Figure 6C), the expression of MT1-MMP was predominantly observed in fibroblasts, which were identified based on cell shape. However, endothelial and large luteal cells were weakly stained in the late stage CL. No staining was observed when the primary antibody was omitted (Figure 6D).

Discussion

To date, MT1-MMP nucleotide and predicted protein sequences are known for the human (Sato et al., 1994), mouse (Apte et al., 1997), rat (Okada et al., 1995a), and rabbit (Sato et al., 1997b), while there are no reports, to our knowledge, regarding its sequence and expression in agriculturally important species such as domestic ruminants. In the present study, we report the bovine-specific MT1-MMP complementary nucleotide sequence and demonstrate its expression and localization in the bovine CL. The bovine MT1-MMP cDNA consisted of 1746 bp nucleotides, which encode a 582 amino-acid protein. Sequence alignment indicated that the bovine MT1-MMP was highly similar (>92%) to the rat, human, mouse, and rabbit homologs. In addition, bovine MT1-MMP shares the characteristics typical of other membrane-type metalloproteinases, e.g., the 11 amino acids insertion (IS-I) between the propeptide and the catalytic domains that serves as a putative convertase recognition site, the RXKR motif (IS-II) in the catalytic domain, and the transmembrane segment (IS-III) (Murphy et al., 1999a). These results strongly suggest that this molecule is highly conserved among the mammals investigated thus far. Furthermore, as in the human (Sato et al., 1994) and the rabbit (Sato et al., 1997b),
bovine MT1-MMP had broad tissue distribution, suggesting multiple sites of action in these species.

Since information regarding MT1-MMP and ovarian function is limited, the remainder of the present study focused on the bovine CL. Previously, MT1-MMP transcripts have been shown to be present in mouse ovaries (Hagglund et al., 1999) and in developing and regressing rat CL (Goto et al., 1999; Liu et al., 1999). Here, we found that MT1-MMP mRNA was constitutively expressed in all three stages of luteal development, however, the level of active MT1-MMP protein was greater in the mid- and late stage bovine CL than that in the early stage. A major function of MT1-MMP is to activate latent MMP-2. In contrast to other MMPs, the activation process of pro-MMP-2 is unusual in that it is not through the extracellular proteolytic cleavage by serine proteinases (Murphy et al., 1999b), but rather the activation of pro-MMP-2 is localized on the cell membrane (Murphy et al., 1992b). A ternary molecular complex model is hypothesized in which TIMP-2 binds to MT1-MMP forming a cell surface "co-receptor" for pro-MMP-2 (Butler et al., 1998; Zucker et al., 1998). This cell surface bound pro-MMP-2 is subsequently activated by the adjacent active MT1-MMP (Zucker et al., 1998; Stetler-Stevenson, 1999). Indeed, in rats (Liu et al., 1998), MT1-MMP and MMP-2 mRNA are coordinately expressed and localized during follicular development and ovulation. In the bovine CL, we demonstrated that active MMP-2 enzyme levels correlated to that of active MT1-MMP. Thus, together with our observation that TIMP-2 was also coordinately expressed (Zhang and Tsang, 2001), we suggest that in the bovine CL, as is the case in other tissues, MT1-MMP is an endogenous activator of pro-MMP-2.

The cellular localization of MT1-MMP in luteal tissue was also investigated. The formation and development of the CL are associated with tissue remodeling and angiogenesis (Smith et al., 1999; Reynolds et al., 2000). Angiogenesis is the formation of new capillaries from pre-existing vessels. This complex, multifactor-regulated process requires angiogenic factors to stimulate production of MMPs, which degrade the basement membrane surrounding endothelial cells (Moses, 1997; Klagsbrun and Moses, 1999). This alteration of the ECM leads to endothelial...
cell proliferation and migration. As vessels extend, additional MMPs are required to break down the ECM, accommodating the growth of sprouting vessels (Klagsbrun and Moses, 1999). MT1-MMP might mediate these processes because angiogenesis is impaired in MT1-MMP deficient mice (Zhou et al., 2000), and it is highly associated with malignant tumor angiogenesis (Hiraoka et al., 1998). In the present study, MT1-MMP was localized in endothelial cells of the young bovine CL, where it might directly digest ECM proteins (Imai et al., 1996; Ohuchi et al., 1997; Butler et al., 1998). Indeed, co-incubation of purified MT1-MMP and collagen types I, II, and III before SDS-PAGE (Ohuchi et al., 1997) yielded cleavage products, while very weak gelatinolytic activity by MT1-MMP mutants lacking a transmembrane domain was observed using zymography (Imai et al., 1996). This may explain the lack of a readily detectable band in zymograms of our luteal tissue samples. Besides MT1-MMP, MMP-2 is also required for angiogenesis (Moses, 1997). In an in vivo tumor system, MMP-2 activity is necessary for the switch to the angiogenic phenotype during tumor development (Fang et al., 2000). Consistent with these findings is the fact that injection of ewes with a MMP-2 antibody results in defects of the CL vasculature (Gottsch et al., 2001). Together with our preliminary finding that MMP-2 is localized in bovine luteal endothelial cells (Zhang and Tsang, 2001), MT1-MMP might mediate the angiogenic process by activating pro-MMP-2, which in turn digests collagen type IV, a major component of the basement membrane (Moses, 1997). Thus, following ovulation, when the follicle is transformed to become the richly vascularized CL, MT1-MMP might have a dual role as a proteolytic enzyme and an activator of pro-MMP-2.

As the CL develops, ongoing angiogenesis is necessary to establish and maintain the mature vascular network (Dickson et al., 2001). In fact, the density of the vasculature is highest in mid-cycle cow (Zheng et al., 1993) and mature human (Gaytan et al., 1999) CL, correlating with the high metabolic rate, and the high rate of blood flow and progesterone production by the CL (Bruce and Moor, 1976; Zheng et al., 1993). Progesterone either decreases (Jaggers et al., 1996; Zhang et al., 2000c) or increases (Chaffin and Stouffer, 1999; Luo and Liao, 2001) MMP activity
and angiogenesis. In part, this may be attributed to cell type or species differences. In the present study, the active form of both MT1-MMP and MMP-2 enzymes increased in mid- and late stage CL, consistent with the need for MMP production and activity for vascular maintenance.

In addition to endothelial cells, MT1-MMP was prominently expressed on the membranes of mid-cycle large luteal cells. Accompanying the development of the luteal vasculature, there is also a rapid increase in luteal weight and size (Zheng et al., 1994), although this increase is primarily attributed to the proliferation of non-luteal cells such as fibroblasts and endothelial cells (Farin et al., 1986; Lei et al., 1991), and small luteal cells (Zheng et al., 1994). The sizes of large luteal cells also enlarge from the early to mid-cycle stage (Schwall et al., 1986; Lei et al., 1991). To accommodate this growth, MT1-MMP might act directly or indirectly through activation of locally produced pro-MMP-2 to commence the pericellular degradation of the ECM (Hiraoka et al., 1998). The activated MMP-2 may then bind to luteal cell membranes through an integrin such as α,β3 (Brooks et al., 1996). Thus, in large luteal cells, MT1-MMP and its associated actions might facilitate cell-matrix and cell-cell interactions, which ultimately could regulate steroidogenesis (Smith et al., 1999).

At the end of estrous cycle, luteolysis ensues, whereby luteal progesterone production (Niswender and Nett, 1994), size and weight (Niswender and Nett, 1994; Zheng et al., 1994) decrease dramatically. There is also apoptosis of luteal cells (Juengel et al., 1993). In the present study, MT1-MMP was localized in fibroblasts of the late stage, day 16 CL. At this time, these cells also undergo proliferation (Lei et al., 1991). This switch in cellular distribution of MT1-MMP, when compared to the young and mid-cycle CL, might occur in anticipation of the functional and structural changes that are soon to follow. Indeed, fibroblast MT1-MMP might participate in the luteolytic process by degrading connective tissue ECM as part of the structural demise of the CL.

Although the literature base is relatively small, there are reports of differences or consistent trends in expression patterns of MMPs and TIMPs in species with long luteal phases.
Similar to the present study, high MMP-2 activity is observed in the late stage human CL (Duncan et al., 1998), and in mid and late stages of the porcine CL (Pitzel et al., 2000). When MT1-MMP is low, MMP-9 (Goldberg et al., 1996) and TIMP-1 mRNA (Smith et al., 1996) are increased early in the cycle, while TIMP-2 protein (Goldberg et al., 1996; Zhang and Tsang, 2001) and mRNA (Smith et al., 1996; Zhang and Tsang, 2001) along with MT1-MMP (present study) are increased during the mid-luteal phase in the bovine. In the human CL, MMP-9 peaks in early and late stages, but TIMP-1 and -2 do not change over the luteal phase (Duncan et al., 1998). In contrast, TIMP-1 and -2 mRNA levels decline with age of the porcine CL, while MMP-1 and -9 are highest in the late phase (Pitzel et al., 2000). However, in the sheep CL, TIMP-2 mRNA in the early phase is greater than the late phase (Smith et al., 1995), but TIMP-1 mRNA does not vary over the cycle (Smith et al., 1994). Collectively, among the non-laboratory mammals, a number of MMPs, including MT1-MMP in the bovine, and TIMPs, may work in concert to regulate remodeling of the vasculature and the ECM during the life span of the CL.

In summary, the present study reports the cDNA and predicted protein sequences of bovine MT1-MMP and the pattern of mRNA and protein expression in CL harvested over the bovine estrous cycle. Active MT1-MMP and active MMP-2 protein levels were coordinately increased in mid- and late stage CL, supporting the role of MT1-MMP as an activator of pro-MMP-2 in vivo. The localization of MT1-MMP in endothelial cells, in large luteal cells, and in late cycle fibroblasts was associated with the angiogenesis and structural remodeling that occur over the life span of the CL. Collectively, these data indicate that MT1-MMP might act on the ECM to modulate the local environment, which in turn may influence vascular development and the function of luteal cells, e.g., hormone biosynthesis, by affecting intercellular and cell-matrix communication.
**Figure 3-1.** Sequence and homology analysis of bovine MT1-MMP. A) Nucleotide sequence of bovine MT1-MMP cDNA and its deduced amino acid sequence. The protein sequence is shown in uppercase letters and the DNA sequence in lowercase. While the numbers on the left are for the amino acid sequence, the ones on the right are for the cDNA sequence. B) Alignment of predicted MT1-MMP amino acid sequences of bovine, human, mouse, rat, and rabbit. The asterisks indicate the amino acid residues that are conserved among the five MT1-MMPs. The consensus sequences of the convertase recognition site (IS-I), the catalytic domain insertion (IS-II), and transmembrane domain (IS-III) are shaded. The signal peptide, propeptide, catalytic, hinge, hemopexin-like, and transmembrane domains are also indicated. There was a 95.7%, 95%, 92.3%, and 95.9% homology of bovine MT1-MMP to human, mouse, rabbit, and rat MT1-MMP, respectively. C) Homology analysis of bovine MT1-MMP domains with other species. The signal peptide (Signal), propeptide (Pro), catalytic (Cat), hinge (Hinge), hemopexin (Hex), and transmembrane (TM) domains are indicated. Species names appear on the left. The whole molecule (Overall) and individual domains of MT1-MMPs are compared. The numbers represent percentages. The identity scores are determined by setting the bovine MT1-MMP as 100 percent.
Figure 3-2. Tissue distribution of bovine MT1-MMP mRNA. For Northern analysis, mRNA was extracted from bovine brain, heart, kidney, liver, lung, and spleen before probing with bovine MT1-MMP. The positions of 28S and 18S rRNA are indicated on the left. A single 3.5 kb transcript was detected (arrow).
MT1-MMP mRNA expression in the bovine CL

Figure 3-3. Expression of MT1-MMP mRNA in different ages of bovine CL. A) The upper panel shows that a single 3.5 kb MT1-MMP transcript (arrow) was detected in early (E; day 4), mid-(M; day 10), and late (L; day 16) cycle CL. Corresponding positions of 28S and 18S rRNA are indicated on the left. The lower panel shows ethidium bromide staining of 28S rRNA. B) The levels of MT1-MMP mRNA, expressed as the densitometric ratio of MT1-MMP to 28S rRNA, were not different (p>0.05) among any age of CL. Dissimilar letters denote significant difference at p<0.05.
MT1-MMP protein expression in the bovine CL

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<td>51.8</td>
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**A)**

In early (E; day 4), mid (M; day 10), and late (L; day 16) cycle CL, the ~63 kDa latent and the ~60 kDa active MT1-MMP species were observed.

**B)** Densitometric analysis (average total pixels x 10^6) showed that the active form of MT1-MMP in early (day 4) CL was significantly lower (p<0.05) than in mid (day 10) and late (day 16) CL. Dissimilar letters denote significant difference at p<0.05.

Figure 3-4. Expression of MT1-MMP protein in different ages of bovine CL. A) In early (E; day 4), mid (M; day 10), and late (L; day 16) cycle CL, the ~63 kDa latent and the ~60 kDa active MT1-MMP species were observed. B) Densitometric analysis (average total pixels x 10^6) showed that the active form of MT1-MMP in early (day 4) CL was significantly lower (p<0.05) than in mid (day 10) and late (day 16) CL. Dissimilar letters denote significant difference at p<0.05.
MMP-2 activity in the bovine CL

A

MW HT E M L

75 kDa

50 kDa

35 kDa

Pro-MMP-2

Active MMP-2

B

Densitometric Ratio

Early Mid Late

C

1 2 3 4 5 6 7 8 9

75 kDa

50 kDa

35 kDa

20 kDa

Samples: MW HT HT E E M M L L

APMA: - - + - + - + - +

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Figure 3-5. Gelatin zymographic analysis of MMP-2 activity in bovine CL. A) Perfect Protein™ Markers (Novagen, Madison, WI) were loaded in lane 1 (MW). The positive control, PMA (Phorbol 12-myristate 13-acetate)-stimulated HT1080 conditioned medium (HT), was loaded in lane 2. Representative early (E; day 4), mid (M; day 10), and late (L; day 16) luteal samples were loaded in lanes 3 to 5, respectively. The arrows on the right indicate the pro- and active forms of MMP-2 in the positive control and CL samples. The relative molecular masses of protein markers are indicated on the left. B) The active form of MMP-2, expressed as the densitometric ratio of CL samples to HT 1080 conditioned medium, was significantly lower (p<0.05) in early (day 4) cycle CL than in mid (day 10) and late (day 16) stages. Dissimilar letters denote significant difference at p<0.05. C) Treatment with APMA. Samples of HT1080 conditioned medium (HT) and representative early (E; day 4), mid (M; day 10), and late (L; day 16) CL extracts were incubated at 37°C with (+) or without (-) 2mM APMA before zymographic analysis. Perfect Protein™ Markers were loaded in lane 1. The numbers on the left indicate relative molecular mass (kDa). Arrows indicate the pro- and active forms of MMP-2.
Cellular localization of MT1-MMP in the bovine CL

Figure 3-6. MT1-MMP immunohistochemistry in the bovine CL. A) In the early (day 4) cycle CL, positive staining is observed in endothelial (black arrows), and the cell membrane and cytoplasm of large luteal (white arrows) cells (×400). B) In the mid (day 10) cycle CL, the staining is localized on large luteal cell plasma membranes and cytoplasm (white arrows), and endothelial cells (black arrows; ×400). C) In the late (day 16) stage CL, there was predominant staining in fibroblasts (white arrowheads; ×400), and weak staining in endothelial (black arrows) and large luteal (white arrows) cells. D) Negative control of a representative section of mid (day 10) cycle CL. No positive staining was observed (×400).
CHAPTER IV

TEMPORAL AND SPATIAL EXPRESSION OF TISSUE INHIBITORS OF METALLOPROTEINASES 1 AND 2 (TIMP-1 AND -2) IN THE BOVINE CORPUS LUTEUM

Abstract

The matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), may mediate the dramatic structural and functional changes in the corpus luteum (CL) over the course of its life span. Besides regulating MMP activity, TIMPs are involved with a variety of cellular processes, including cell proliferation and steroidogenesis. In order to gain a greater understanding of the role of TIMPs in luteal function, we investigated the temporal and spatial expression of TIMP-1 and TIMP-2 in the bovine CL. Luteal tissues were collected from early (4 days old, estrus=day 0), mid (10-11 days old) and late (16 days old) phases (n=3 for each phase) of the estrous cycle. Northern blotting revealed that the TIMP-1 transcript (0.9kb) was expressed at a higher (p<0.05) level in early and mid cycle CL than in the late stage. On the other hand, two TIMP-2 mRNA species, one major 1 kb species and one minor 3.5kb species, were significantly increased (p<0.05) in the mid and late cycle CL than in the early. Reverse zymography demonstrated four metalloproteinase inhibitory protein bands with relative molecular masses that are consistent with these reported for TIMP-1 to -4. TIMP-1 was the major species in the bovine CL. Western blotting showed no differences in TIMP-1 (29 kDa) protein levels between early and mid
phases, while its levels decreased (p<0.05) from the mid to late stage CL. Conversely, TIMP-2 (22 kDa) protein was low in the early CL, but significantly increased (p<0.05) in the mid and late stages. Immunohistochemistry revealed that both TIMP-1 and -2 were localized in large luteal cells from all three ages of CL. Furthermore, TIMP-1 was also localized in vascular smooth muscle cells, but TIMP-2 was restricted to the endothelial cells in the capillary compartment. Overall, these results suggested that the variations in TIMP-1 and -2 expression may suggest diverse roles for these multifunctional MMP inhibitors in the physiology of the CL during the estrous cycle.

Introduction

The corpus luteum (CL) is a transient, dynamic endocrine gland, which develops from the postovulatory follicle (Niswender and Nett, 1994). Dramatic structural and functional changes are associated with the development, maintenance and regression of the CL (Niswender and Nett, 1994). These remodeling events require the participation of matrix metalloproteinases (MMPs), a large family of zinc and calcium dependent proteolytic enzymes that collectively digest all known macromolecules constituting the extracellular matrix (Matrisian, 1990; Massova et al., 1998). Generally, the catalytic activity of the MMPs are highly regulated at three levels, gene expression, proteolytic activation of proenzymes, and inhibition by binding of endogenous tissue inhibitors of metalloproteinases (TIMPs) to the catalytic domain (Matrisian, 1990; Woessner and Nagase, 2000).

So far, four TIMPs, including TIMP-1 (Carmichael et al., 1986), TIMP-2 (Stetler-Stevenson et al., 1989), TIMP-3 (Pavloff et al., 1992), and TIMP-4 (Greene et al., 1996), have been identified. TIMP-1, a glycoprotein with a molecular mass of approximately 29kDa, is the first member of this family to be cloned (Carmichael et al., 1986). TIMP-1 can bind the active
forms of all known MMPs (Woessner and Nagase, 2000). In addition, TIMP-1 also binds the latent form of MMP-9 (Itoh et al., 1995). Besides its capacity to inactivate the activity of MMPs, TIMP-1 also possesses mitogenic activity to a variety of cell types, such as gingival fibroblasts and erythroid precursor cells (Docherty et al., 1985; Hayakawa et al., 1992). In addition, TIMP-1 may regulate steroidogenesis (Boujrad et al., 1995). In CL from a variety of species, protein and messenger RNA expression of TIMP-1 have been observed, including cow (Freudenstein et al., 1990; Goldberg et al., 1996), sheep (Smith et al., 1994), rat (Nothnick et al., 1995), mouse (Inderdeo et al., 1996), monkey (Duncan et al., 1996a), and human (Duncan et al., 1996b).

Different from TIMP-1, TIMP-2 is an unglycosylated protein with an approximate molecular mass of 22 kDa (Stetler-Stevenson et al., 1989; Goldberg et al., 1989). TIMP-2 binds most active MMPs and inhibits their proteolytic activity (Woessner and Nagase, 2000). Among all members of the MMP family, TIMP-2 preferentially binds to MMP-2 (Goldberg et al., 1989; Olson et al., 1997). Paradoxically, however, TIMP-2 is also involved in pro-MMP-2 activation by forming a MT1-MMP/TIMP-2/pro-MMP-2 tri-molecular complex on the cell membrane (Deryugina et al., 1997; Cao et al., 1998; Butler et al., 1998). Similar to TIMP-1, TIMP-2 possesses potent stimulatory activity on proliferation of a variety of cell types (Hayakawa et al., 1994). TIMP-2 mRNA expression was observed in the sheep (Smith et al., 1995), cow (Smith et al., 1996), human (Duncan et al., 1998), rat (Simpson et al., 2001), and mouse (Inderdeo et al., 1996) CL, while a TIMP-2-like protein was detected in the cow CL (Goldberg et al., 1996).

Because the variety and intensity of remodeling events that occur over the life span of the CL suggest a temporal and spatial interplay between MMPs and TIMPs, we presently investigated the expression patterns and cellular distribution of TIMP-1 and TIMP-2 in the early, mid, and late developmental stages of bovine CL obtained over the estrous cycle.

**Materials and Methods**

1. **Animal Model and Tissue Collection**
Corpora lutea were collected from cyclic, nonlactating dairy cows, which were housed at the University of New Hampshire Dairy Teaching and Research Center. CL were removed on day 4, 10, and 16 of the estrous cycle (day 0 = estrus; n=3 per day). For day 4 CL, the ovary was removed by an ecraseur after the cow received an epidural anesthetic [2% (w/v) mepivacaine hydrochloride; 0.01 mL/kg BW: Upjohn, Kalamazoo, MI], and the CL was then dissected from ovarian stroma. The day 10 and 16 CL were enucleated from the ovarian stroma. All animal experimentation protocols in the present study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Hampshire.

2. Northern Blotting

TIMP-1 and TIMP-2 mRNA expression in the CL were studied by Northern blotting as previously described (Zhang et al., 2002). Briefly, total RNA was extracted from luteal tissues using TRIZOL (GIBCO-BRL, Carlsbad, CA) according to the manufacturer’s instructions. Twenty micrograms of total RNA were fractionated on 1.0% (w/v) agarose gels containing formaldehyde and were transferred onto Hybond™-N nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were first incubated in pre-hybridization buffer [50% (v/v) formamide, 5×SSC, 0.2% (w/v) SDS, and 2% (w/v) blocking reagent] for two hours at 42°C. Hybridization was carried out in hybridization buffer containing TIMP-1 (Waterhouse et al., 1990), TIMP-2 (Stetler-Stevenson et al., 1989), or cyclophilin (a generous gift from Dr. Robert Thompson, University of Michigan) cDNA probes at 50°C overnight. These cDNA probes were labeled with digoxigenin (DIG)-dUTP using the DIG DNA random-primed Labeling Kit (Roche Molecular Biochemicals, Indianapolis, IN). The blots were then washed twice at room temperature in 2×SSC with 0.1% (w/v) SDS, followed by higher stringency washes in 0.2×SSC with 0.1% SDS at 65°C. Hybridized probes were detected using an anti-DIG antibody conjugated to alkaline phosphatase (used at 1:5000; Roche Molecular Biochemicals, Indianapolis,
IN) in 1% (w/v) blocking reagent. The signals were detected by CSPD (Roche Molecular Biochemicals, Indianapolis, IN), a chemiluminescent substrate for alkaline phosphatase. The blots were visualized by developing Kodak XAR-5 films with a Konica Medical Film Processor (Tokyo, Japan).

3. Reverse Zymography

In order to simultaneously identify total TIMP proteins in the bovine CL, reverse zymographic analysis was performed. Equivalent aliquots (15 μl) of CL tissue extracts were mixed with loading buffer [2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 50 mM Tris-HCl, pH 6.8] and applied to 12% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS and 0.5 mg/ml gelatin. After electrophoresis, gels were rinsed twice with 2.5% (v/v) Triton X-100, followed by incubation with conditioned medium of fibrosarcoma HT1080 cells for 4 hours. After rinsing, gels were incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl₂, pH 8.0). Subsequently, gels were stained for 30 minutes with 0.1% (w/v) Coomassie Blue G-250, and destained with a solution of 30% (v/v) methanol and 10% (v/v) glacial acetic acid. TIMPs were visualized as blue bands on a clear background. Prestained SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA) were run along side samples as molecular weight references.

4. Western Blotting Analysis

To investigate the protein expression of TIMP-1 and TIMP-2 in different ages of CL, equivalent amounts of tissue extracts were subjected to SDS gel electrophoresis on 12% (w/v) polyacrylamide gels under reducing conditions before transfer onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Prestained SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA) were also loaded on the gel in an adjacent lane. Nonspecific binding sites were
blocked with 5% (w/v) nonfat powdered milk in TBST [0.01 M Tris-HCl, 0.15 M NaCl, and 0.05% (v/v) Tween-20, pH 8.0] for 2 hours. A mouse anti human TIMP-1 monoclonal antibody (1:100; Oncogene Research Products, Cambridge, MA) in 5% nonfat powdered milk in TBST was added onto the membrane, and allowed to incubate overnight at room temperature. The membranes were then washed five times with TBST (each time for 15 minutes), and then incubated for 1 hour with goat anti-mouse IgG conjugated to horseradish peroxidase (1:15,000, Pierce, Rockford, IL) at room temperature. After five 15-minute washes, the blots were developed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, IL) according to the manufacturer’s instructions. The protein bands were finally visualized after development of Kodak XAR film (Konica Medical Film Processor, Tokyo, Japan).

5. Immunohistochemistry

Frozen tissue sections (6 μm) were cut onto Superfrost®/Plus slides (Fisher Scientific, Pittsburg, PA), and air-dried at room temperature for 30 min. Sections were fixed in cold acetone for 10 min, and then washed with phosphate-buffered saline (PBS, pH 7.4). Endogenous peroxidase activity was quenched by incubating with 0.3% (v/v) hydrogen peroxide for 30 min. Nonspecific binding was blocked for 30 min by 5% (w/v) BSA. Sections were subsequently incubated at room temperature for 1 hour with a 1:40 dilution of mouse anti human TIMP-1 monoclonal antibody (Oncogene Research Products, Cambridge, CA) or with a 1:50 dilution of mouse anti human TIMP-2 monoclonal antibody (Oncogene Research Products, Cambridge, CA). After washing, slides were incubated with goat anti-mouse IgG followed by VECTASTAIN® Elite ABC Kit (Vector Laboratories, CA), according to the manufacturer’s instructions.

To further validate the localization of TIMP-1 in vascular smooth muscle cells (VSMC), consecutive sections were also stained with an antibody against α-actin, a cellular marker for
VSMC. For every tissue, an adjacent section placed on the same slide was used as a negative control, where BSA substituted for the primary antibody.

For each tissue (3 corpora lutea for each age), 10 to 20 sections were stained. A minimum of 20 areas per section was examined.

6. Data analysis

Intensities of Northern and Western blots were determined by UN-SCAN-IT gel™ automated digitizing system (Silk Scientific, Orem, UT). The data were analyzed by ANOVA, followed by Tukey’s test for multiple comparisons.

Results

1. TIMP-1 and TIMP-2 mRNA Expression in the Bovine CL during the Estrous Cycle

Northern blotting demonstrated that a single TIMP-1 transcript (0.9kb) was present in all three stages of CL (Figure 4-1A). The 18S rRNA was used to normalize sample loading. The densitometric ratio of TIMP-1 mRNA to 18S rRNA was high in the early and mid-cycle CL but decreased significantly (p<0.05) in the late stage (Figure 4-1B).

Two TIMP-2 mRNA species, a major one at 1kb and a minor one at 3.5 kb, were present in all ages of CL (Figure 4-2A). In contrast to the expression pattern of TIMP-1 mRNA, both species of TIMP-2 mRNA, shown as the densitometric ratio of TIMP-2 mRNA to a housekeeping gene cyclophilin, were low in the early stage, but increased significantly (p<0.05) in the mid and late cycle CL (Figure 4-2B).

2. Metalloproteinase Inhibitor Activities in the Bovine CL

Reverse zymography revealed four protein bands in luteal tissues. These four protein bands had relative molecular masses of ~30kDa, ~27kDa, ~24kDa, and ~22kDa, corresponding
those reported for TIMP-1. -3, -4, and -2, respectively (Figure 4-3). TIMP-1 was the predominant TIMP present in the bovine CL.

3. TIMP-1 and TIMP-2 Protein Levels in the Bovine CL

Western blotting revealed an approximately 30kDa immunoreactive TIMP-1 protein in all ages of CL examined (Figure 4-4A). The level of TIMP-1 protein was not different between the early and mid-cycle CL (p=0.324), but it was significantly decreased (p<0.05) in the late stage CL (Figure 4-4B). A 22 kDa TIMP-2 immunoreactive protein was also observed in all three ages of CL (Figure 4-5A). The TIMP-2 protein level in the mid and late cycle CL was significantly greater (p<0.05) than in the early stage (Figure 4-5B).

4. Cellular Localization of TIMP-1 and TIMP-2 Proteins in the Bovine CL

Immunohistochemistry demonstrated that TIMP-1 was present in both large luteal cells and vascular smooth muscle cells (Figure 4-6 B, D, and F). The localization of TIMP-1 in VSMC compartments was validated with staining for α-actin, a cellular marker for VSMC. Although large luteal cells from all three stages of CL expressed TIMP-1, visual observations revealed that the highest level of expression was in cells of the mid-cycle CL. TIMP-1 was expressed in vascular smooth muscle cells of the early and late, but not the mid cycle CL (Figure 4-6 B, D and F). Vascular smooth muscle cells were identified by staining with α-actin (Figure 4-6 G). In all ages of CL. TIMP-2 was consistently localized in the endothelial and large luteal cells (Figure 4-6 A, C and E). No positive signals were observed in sections devoid of primary antibody (Figure 4-6 H).

Discussion

Accumulating evidence supports the notion that matrix metalloproteinases (MMPs) play important roles during CL development and demise. Once activated, these enzymes are strictly
regulated by a group of endogenous inhibitors, the TIMPs. Furthermore, the two most studied members in this TIMP family, TIMP-1 and TIMP-2, possesses a variety of other functions. Therefore, it is necessary to determine the temporal and spatial expression patterns of these two inhibitors before their physiological roles in the CL can be elucidated.

In the present study, TIMP-1 mRNA was highly expressed in the early and mid cycle bovine CL, but was decreased in the late stage. This finding was similar to previous observations by others (Freudenstein et al., 1990; Smith et al., 1996). Likewise, in the porcine CL, TIMP-1 transcript was also highly expressed in the early stage, and is slightly reduced as the estrous cycle progresses before it is significantly decreased in the regressing stage (Pitzel et al., 2000). In contrast, TIMP-1 mRNA expression in the ovine (Smith et al., 1994) and human (Duncan et al., 1998) CL does not change throughout the estrous or menstrual cycle, respectively. In the pseudopregnant rat, yet a different pattern emerges, whereby the strongest TIMP-1 mRNA expression is observed during CL formation and regression (Liu et al., 1999). Clearly, there are species differences with regard to the temporal expression of the TIMP-1 transcript in the CL. Despite these differences, we chose to pursue our studies in cows. In particular, as a relevant animal model for humans, cows have a relatively long cycle length, during which a single ovum is shed. Also, a single CL provides sufficient tissue for multiple analyses.

The expression of a TIMP-1 specific protein in three ages of CL was also demonstrated in the present study. This is consistent with our previous study that showed the presence of a TIMP-1-like protein in CL obtained over the estrous cycle (Goldberg et al., 1996). Furthermore, reverse zymography showed that TIMP-1 was the predominant TIMP in the bovine CL, similar to the finding in sheep (Smith et al., 1993). Lastly, the patterns of TIMP-1 protein and mRNA paralleled each other, being high in the early and mid stages, but decreased in the late cycle CL. The high levels of TIMP-1 in the early and mid cycle CL may be needed to regulate the extensive tissue remodeling events that occur during CL formation and development. The reduced TIMP-1 mRNA and protein expression in the late stage CL may portend the decline of this inhibitor.
observed during luteolysis in bovine (Juengel et al., 1994), ovine (Towle et al., 2002), porcine (Pitzel et al., 2000), and primate (Duncan et al., 1996a) CL. Collectively, these data implicate TIMP-1 might be an important player in the physiology of the CL.

The TIMP-1 protein was localized in large luteal cells of early, mid, and late stages of the bovine CL. A similar observation was reported in the ovine CL where TIMP-1 and oxytocin were co-localized in secretory granules of large luteal cells (McIntush et al., 1996). In addition, TIMP-1 expression was also detected in isolated ovine (Smith et al., 1994) and porcine (Pitzel et al., 2000) large luteal cells and luteinized human granulosa cells (Aston et al., 1996). The presence of TIMP-1 in steroidogenic cells may be associated with its ability to enhance steroid production (Boujrad et al., 1995). In part, this may be related to a 124 bp-nucleotide sequence similarity between the protein coding region of the bovine steroidogenic acute regulatory (StAR) gene and the 5' non-coding region of TIMP-1 (Hartung et al., 1995). Additionally, although female mice lacking the TIMP-1 gene do not show detectable differences in serum estradiol-17β concentrations, the TIMP-1 deficient male mice produce higher levels of total serum testosterone than the wild type (Nothnick et al., 1997; Nothnick et al., 1998). Furthermore, cell culture studies demonstrated that TIMP-1 increases estradiol-17β production by granulosa cells from both TIMP-1 deficient and wild-type mice (Nothnick et al., 1997), and enhances estradiol-17β and progesterone production from porcine thecal cells (Shores and Hunter, 2000). Therefore, the strong expression of TIMP-1 in large luteal cells may be related to its collateral role in steroidogenesis.

Because of the predominant expression of TIMP-1 in large luteal cells, this inhibitor is used as a cellular marker for this cell type (Haworth et al., 1998). However, other cell types in the CL are also positive for TIMP-1 expression. For example, the present study showed that TIMP-1 was also localized in the vascular smooth muscle cells (VSMC) of the bovine CL. Although the cellular source was not specified, the capillary compartments in ovine (McIntush et
al., 1996) and rat (Bagavandoss, 1998) CL also stain positively for TIMP-1. The localization of TIMP-1 in the vascular smooth muscle compartment supports its potential role during angiogenesis and vascular maintenance. Indeed, over-expression of TIMP-1 in VSMC by exogenous gene transfer reduces VSMC cell proliferation and migration (Forough et al., 1996; George et al., 1998). In addition, stimulation of TIMP-1 expression impairs angiogenesis in a variety of tumor types (Martin et al., 1999; Guedez et al., 2001; Bloomston et al., 2002).

Therefore, TIMP-1 acts as a negative regulator of blood vessel formation (Moses et al., 1990). During early CL development, extensive angiogenesis occurs, and theca-derived luteal cells and fibroblasts invade through the breached basement membrane into the cavity of the ruptured follicle. These early events in the angiogenic process require net proteolytic/MMP activity. In the last step of angiogenesis, recruitment and maintenance of pericytes (VSMC) are critical for vascular maturation and survival (Nehls et al., 1992; Hirschi and D'Amore, 1996). Thus, the high level of TIMP-1 expression in VSMC in 4-day old CL may provide an environment where MMP action is inhibited as the vasculature matures. As the CL ages, its structure remains relatively stable. Although angiogenesis is ongoing, it is slowed-down during CL maintenance (Tsukada et al., 1996; Amselgruber et al., 1999; Dickson et al., 2001). This may be the reason for the absence of TIMP-1 in the VSMC compartment at this stage of the bovine estrous cycle. In the 16-day old CL, TIMP-1 was detected again in the VSMC compartment. This localized expression of TIMP-1 is consistent with the view of Redmer et al. (1988), who proposed that maintenance of the vasculature may be necessary for the transport of degraded products during luteolysis, which ultimately results in a massive decrease in CL size and weight as regression ensues.

Although TIMP-1 was the most abundant TIMP in luteal tissues, the other three TIMPs were also detected in the bovine CL by reverse zymography. Among them, we chose to focus our attention on TIMP-2. It has been reported that TIMP-2 is involved in the pro-MMP-2 activation process by binding a MT1-MMP to form a "co-receptor" for pro-MMP-2 on the cell surface (Butler et al., 1998). This bound pro-MMP-2 is then presented to an adjacent MT1-MMP for

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activation (Deryugina et al., 1997; Butler et al., 1998). Although a transmembrane-deleted MT1-MMP activates pro-MMP-2 without the participation of TIMP-2 (Pei and Weiss, 1996), TIMP-2 deficient mice have a dramatically reduced ability to activate pro-MMP-2 (Wang et al., 2000). In the present study, both TIMP-2 mRNA and protein levels were low in the early CL, but significantly increased in the mid and late stages. This expression pattern was similar to those for active MT1-MMP and MMP-2 (Zhang et al., 2002), suggesting that the MT1-MMP/TIMP-2/pro-MMP-2 tri-molecular system may be available for MMP-2 activation in vivo in the bovine CL. The coordinate expression of these three molecules is also observed during embryo development (Apte et al., 1997), which supports the presence of this activation system in a variety of tissue types.

In addition to the temporal correlation of TIMP-2 with active MT1-MMP and MMP-2 expression in the CL, TIMP-2 was also co-localized with these two molecules in endothelial and large luteal cells. TIMP-2 has a variety of functions in endothelial cells. On the one hand, its stoichiometrically correlated expression with MT1-MMP and pro-MMP-2 may facilitate the activation process of pro-MMP-2, which is critical for the angiogenesis process. On the other hand, inhibition of angiogenesis by TIMP-2 cannot be excluded, because over-expression of TIMP-2 blocks vascular smooth muscle cell invasiveness (Cheng et al., 1998) and reduces angiogenic ability (Li et al., 2001). The latter observation is due, in part, to down-regulation of vascular endothelial cell growth factor (VEGF) (Hajitou et al., 2001). Furthermore, TIMP-2 also possesses growth inhibitory ability on endothelial cells (Murphy et al., 1993a).

The localization of TIMP-2 in large luteal cells may also contribute to the activation of pro-MMP-2 in this cell type by assembling the trimeric complex on the cell membrane. The in situ activated MMP-2 then binds onto integrin α,β3, where localized pericellular proteolysis ensues. This resulting degradation of the ECM is needed to accommodate the enlargement of large luteal cells from the early to mid and late stages (Schwall et al., 1988; Zheng et al., 1994). Although there is no direct evidence showing the involvement of TIMP-2 in steroidogenesis, the
dynamic interactions between large luteal cells and their local ECM niche may induce biochemical changes related to the steroid biosynthetic process in this cell type (Smith et al., 1999). For example, disruption of the links between ECM, integrins, and the cytoskeleton (Murdoch et al., 1996) may perturb the intracellular transport of substrates, such as cholesterol, for steroidogenesis (Niswender et al., 2000). However, additional in vitro and in vivo studies are needed to elucidate the physiological roles of these TIMPs in this endocrine gland.

In summary, the present study demonstrated that the temporal and spatial expression of TIMP-2 was correlated to the expression patterns of active MT1-MMP and MMP-2. This suggested that these three molecules might be coordinately expressed in the bovine CL to facilitate the activation of pro-MMP-2 in vivo, and in turn coordinate physiological processes such as enlargement of large luteal cells and luteal angiogenesis. The expression pattern of TIMP-1 was temporally and spatially distinct from TIMP-2. The predominant expression of TIMP-1 in the CL suggested that it might be a key regulator in CL physiology.
Figure 4-1. TIMP-1 mRNA expression in the bovine CL. A) Northern blotting of TIMP-1 mRNA in early (E), mid (M), and late (L) stage CL is shown in the upper panel. The arrow indicates the 0.9kb TIMP-1 transcript. The ethidium bromide stained 18S rRNAs in corresponding luteal samples are shown in the lower panel. B) Changes in TIMP-1 mRNA, shown as a densitometric ratio of TIMP-1 mRNA to 18S rRNA, are illustrated. Dissimilar letters denote significant difference at p<0.05.
TIMP-2 mRNA in the bovine CL

A

E  M  L

TIMP-2

3.5 kb
1 kb

cyclophilin

B

\[
\begin{align*}
\text{Densitometric value} & \quad \text{Early} \quad \text{Mid} \quad \text{Late} \\
\hline
\text{3.5kb} & a' \quad a' \quad b' \\
\text{1kb} & a \quad b \quad b \\
\end{align*}
\]

Ages of CL

Figure 4-2. TIMP-2 mRNA expression in the bovine CL. A) The upper panel shows Northern blotting of TIMP-2 mRNA in early (E), mid (M), and late (L) bovine CL. The arrows indicate the 3.5 and 1 kb species. The lower panel is the same membrane hybridized with a human cyclophilin probe. B) Densitometric ratios of the two TIMP-2 mRNA bands to cyclophilin in corresponding stages are shown. Dissimilar letters denote significant difference at p<0.05.
Activities of tissue inhibitors of metalloproteinases (TIMPs) in the bovine CL

Figure 4-3. Reverse zymographic analysis of tissue inhibitors of metalloproteinases (TIMPs). Inhibitor activities in the early (E), mid (M), and late (L) stages of bovine CL are shown. Lane 1 indicates the Prestained SDS-PAGE standards (STD; Bio-Rad Laboratories, Hercules, CA). Their corresponding molecular masses (kDa) are indicated on the left. Four bands possessing MMP inhibitory activities (indicated by arrows on the right) were observed in the luteal samples. These bands correspond to TIMP-1 (~30kDa), TIMP-3 (~27kDa), TIMP-4 (~24kDa), and TIMP-2 (~22kDa), respectively.
TIMP-1 protein expression in the bovine CL

Figure 4-4. TIMP-1 protein expression in the bovine CL during the estrous cycle. A) A representative Western blot of TIMP-1 in early (E), mid (M), and late (L) CL collected over the estrous cycle. Conditioned medium of HT1080 cells (HT) was loaded in the first lane and was used as a positive control. The arrow indicates the 30 kDa immunoreactive TIMP-1 protein. B) TIMP-1 protein levels in different stages are presented as a ratio of band intensity in luteal samples to that in HT1080 conditioned medium. Dissimilar letters denote significant difference at p<0.05.
TIMP-2 protein expression in the bovine CL

Figure 4-5. TIMP-2 protein expression in the bovine CL throughout the estrous cycle. A) A representative Western blot of TIMP-2 in the bovine CL. Molecular masses (kDa) of protein standards are shown on the left. As indicated by the arrow, a single 22 kDa TIMP-2 immunoreactive protein band is present in early (E), mid (M), and late (L) stages of CL. B) Densitometric analysis of TIMP-2 protein expression among different stages of CL. Dissimilar letters denote significant difference at p<0.05.
Cellular localization of TIMP-1 and -2 in the bovine CL
Figure 4-6. Immunohistochemistry of TIMP-2 (A, C, E; ×400) and TIMP-1 (B, D, F; ×200) in the early (A and B), mid (C and D), and late (E and F) stages of CL. Vascular smooth muscle cells were identified by staining with α-actin (G; ×200). Positive staining (red color) is indicated by white arrows, black arrows, and white triangles in large luteal cells, endothelial cells, and vascular smooth muscle cells, respectively. In a representative negative control (H; ×400), no positive staining was observed.
CHAPTER V

REGULATION OF MATRIX METALLOPROTEINASE 2 (MMP-2) EXPRESSION IN ENDOTHELIAL AND LUTEAL CELLS BY CYTOKINES

Abstract

Our previous studies showed that MMP-2 activity varies in day 4, day 10, and day 16 bovine CL obtained over the estrous cycle. We also observed that MMP-2 is localized in endothelial and large luteal cells. Although cytokines regulate progesterone and prostaglandin production by the corpus luteum (CL), little is known about their effects on MMPs in the ovary. Thus, the aim of the present study was to investigate cytokine regulation of MMP-2 expression by endothelial and luteal cells. In experiment I, luteal cells were isolated from mid cycle bovine CL (n=3). Dissociated cells were then incubated with 1, 10, or 100 ng/ml tumor necrosis factor (TNFα) for 12 hours or with 100 ng/ml TNFα for 6, 12, 24, and 48 hours. Zymography revealed that the two highest doses of TNFα increased (p<0.05) the level of pro-MMP-2 above the 1 ng/ml treatment and control groups. In addition, pro-MMP-2 also increased in a time-dependent manner, being the highest at 48 hours. Using concentrated medium, Western blotting showed that active MMP-2 was present in cells treated with the 100 ng/ml dose of TNFα. Furthermore, the level of active MMP-2 was the highest (p<0.05) at 48 hours. In experiment II, endothelial cells were isolated from cow CL during early pregnancy. Luteal-derived endothelial cells were treated with either 100 ng/ml TNFα, 200 IU/ml interferon γ (IFNγ), or a combination of these two cytokines for 24 hours. Zymographic analysis revealed that TNFα stimulated, while IFNγ inhibited latent MMP-2 expression. Furthermore, when combined, IFNγ attenuated the stimulatory effect by TNFα, and the level of latent MMP-2 being not different from controls.
Together, these data suggest that MMP-2 expression in the CL may be regulated, in part, by cytokines.

**Introduction**

Matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent proteolytic enzymes that have been shown to be important in ovarian physiology, including follicular development, follicle atresia, ovulation, corpus luteum development, and ovarian angiogenesis (Smith et al., 1999). Among the more than 25 members that have been identified in this family, matrix metalloproteinase 2 (MMP-2; gelatinase A) is one of the most well studied. This may be due to its broad substrate specificity on ECM proteins. In addition to its efficient degradation on gelatins, MMP-2 also cleaves type I (Aimes and Quigley, 1995), type V (Okada et al., 1990), type VII (Seltzer et al., 1989), and type X (Gadher et al., 1989; Welgus et al., 1990) collagens, and other ECM components such as elastin (Senior et al., 1991), laminin (Imai et al., 1995b; Giannelli et al., 1997), vitronectin (Giannelli et al., 1997), and aggrecan (Fosang et al., 1992). In particular, MMP-2 efficiently hydrolyzes soluble collagen type IV, which is one of the major barriers encountered during structural remodeling in a variety of tissues. Furthermore, this proteolytic enzyme affects cell behaviors by regulating the availability of growth factors, cytokines, and their receptors (McCawley and Matrisian, 2001). For example, proteolytic cleavage of biologically inactive pro-transforming growth factor (TGF) β1 and β2 (Imai et al., 1997), pro-tumor necrosis factor α (TNFα) (McGehee et al., 1994), and pro-interleukin 1β (IL1β) (Schonbeck et al., 1998) by MMP-2 yields the active molecules. Also, N-terminal processing of chemokines, such as monocyte chemoattractant protein 3 (MCP-3) and stromal cell derived factor (SDF) 1α and 1β results in loss of biological activity (McQuibban et al., 2000; McQuibban et al., 2001). Furthermore, MMP-2 acts on the Val368-Met369 peptide bond of the fibroblast growth factor receptor type 1 (FGFR1) ectodomain to release a soluble extracellular...
domain, which modulate the mitogenic activities of FGF (Levi et al., 1996). Together, these properties of MMP-2 establish its complex but critical roles in tumor invasion and metastasis. Likewise, it may also be necessary for normal physiological processes, such as ovulation, and CL development.

MMP-2 has been identified in the bovine (Tsang et al., 1995; Goldberg et al., 1996; Zhang et al., 2002), ovine (Russell et al., 1995; Towle et al., 2002), porcine (Pitzel et al., 2000), rat (Nothnick et al., 1996; Bagavandoss, 1998; Liu et al., 1999), and human (Duncan et al., 1998) CL. In ewes, MMP-2 activity is critical for CL development and luteal neovascularization (Russell et al., 1995; Gottsch et al., 2001). Increased MMP-2 expression or activity is also associated with luteal regression in a variety species, including rat (Endo et al., 1992), sheep (Reike et al., 2002; Towle et al., 2002), and human (Duncan et al., 1998). Although luteinizing hormone (LH) has no effect on MMP-2 expression by luteal cells isolated from 4 day old bovine CL (Tsang et al., 1995), its expression in vivo is augmented by prolactin and PGF_2 alpha in rat (Endo et al., 1992) and sheep (Towle et al., 2002) CL, respectively.

The regulation of MMP-2 expression, which is unique and complex, occurs at the activational or translational level. Recently, we showed that MMP-2, and membrane type 1 MMP (MT1-MMP) and tissue inhibitor of metalloproteinases 2 (TIMP-2), the two key regulators that activate it, are co-localized in endothelial and large luteal cells of the ovine CL (Zhang and Tsang, 2001; Zhang et al., 2002). MMP-2 can also be regulated at the transcriptional level. Because it lacks well characterized regulatory elements in the promoter region, MMP-2 has been long viewed intractable to either stimulatory or inhibitory reagents (Mauviel, 1993). In part, this may be the reason for the paucity of in vitro studies on the regulation of MMP-2 expression in the ovary. However, a recent study demonstrated that the MMP-2 promoter has a GC-rich region, even though it lacks a typical TATA box (Qin et al., 1998). Further sequence analysis revealed that a number of cis-acting regulatory elements, including p53, AP-1, AP-2, Sp1, Ets-1, C/EBP, CREB, and PEA3, are present in the up-stream regulatory region of the MMP-2 gene (Bian and...
Sun, 1997; Qin et al., 1998). Therefore, these elements could be involved in the regulation of MMP-2 by growth factors and cytokines. Notably, the CL is a potentially rich source of a variety of cytokines (Pate, 1995; Terranova and Rice, 1997; Pate and Landis Keyes, 2001). Furthermore, cytokines participate in CL physiology by being involved in steroidogenesis, apoptosis, angiogenesis, and luteolysis (Pate and Landis Keyes, 2001).

Among the cytokines identified in the CL, TNFa and interferon γ (IFNγ) have been implicated in regulating endothelial and luteal cell physiology. Towards this end, the aim of the current study was to investigate regulation of MMP-2 by TNFa and IFNγ in luteal and endothelial cells isolated from bovine CL.

**Materials and Methods**

1. **Isolation and culture of luteal cells**

 Corpora lutea were collected from regularly cycling, nonlactating dairy cows on day 10 of the estrous cycle (day 0 = estrus; n=3). After removing connective tissues, CL were dissociated as previously described (Tsang et al., 1995). Briefly, luteal tissues were minced before placing into a spinner flask containing 25ml of Ham’s F-12, with 1% bovine serum albumin (BSA), and Type I collagenase (Worthington Biochemical, Lakewood, NJ: 200 units/g tissue) for one hour at 37°C. During this period, tissues were triturated every 10 minutes. After one hour, dissociated cells were centrifuged sequentially at 190g, 110g, and 80g to remove collagenase, connective tissues and other tissue debris. Cell viability and number were determined by trypan blue exclusion and counting on a hemacytometer, respectively.

 Luteal cells (1×10⁶) were seeded in T25 flasks containing 4 ml Ham’s F-12 culture medium, supplemented with insulin/selenium/transferring (ITS: Upstate Biotechnology, Lake Placid, NY) and gentamicin (30μg/ml: GIBCO-BRL, Paisley, PA). After an overnight incubation, unattached cells were removed by rinsing with fresh Ham’s F-12 medium. Then, for experiment
1. Luteal cells were treated with 1, 10, or 100 ng/ml TNFα (n=3 per dose; R&D Systems, Minneapolis, MN) for 12 hours. For experiment II, luteal cells were treated with TNFα (100ng/ml) and incubated for 6, 12, 24, or 48 hours. Cell number was determined before treatment and at the end of each time point.

2. Endothelial cell isolation and culture

Endothelial cells from early pregnant cow CL were provided by Dr. Bo Rueda at the Massachusetts General Hospital and Dr. John Davis at the University of Nebraska. The isolation, purification, and characterization of these luteal-derived endothelial cells were commercially accomplished by BioWhittaker Molecular Applications (Walkersville, MD). Endothelial cells were then cultured in 24 well plates (5000 cells/cm²) in EGM-2 MV medium (BioWhittaker, Walkersville, MD) containing 3% fetal bovine serum (FBS) and a cocktail of growth factors (BioWhittaker, Walkersville, MD) based on the manufacturer’s recommendations. After the cells attained 90% confluency, the spent medium was replaced with serum-free EGM-2 MV before they were treated with 100ng/ml TNFα (Upstate Biotechnology, Lake Placid, NY), 200 units/ml IFNγ (a generous gift from Dr. Dale Godson), or a combination of both for 24 hours (n=3 wells per treatment). Cell numbers were determined at the end of the treatment period.

3. Gelatin zymography

MMP-2 activity was determined by zymographic analysis on 10% acrylamide SDS gels containing 0.5mg/ml gelatin (Zhang et al., 2002). Conditioned medium of luteal and endothelial cell cultures was normalized to cell number. Equivalent aliquots of conditioned medium were loaded into the gels. Perfect Protein™ Markers (Novagen, Madison, WI) and a positive control, the conditioned medium of HT1080 cells, were run in adjacent lanes.
Following electrophoresis, SDS was removed by rinsing twice with 2.5% (v/v) Triton X-100 (15 minutes per wash). The gel was then incubated in substrate buffer (0.05M Tris-HCl, 5mM CaCl₂, 0.05M NaCl, pH 8.0) at 37°C for 18 hours, followed by staining with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA) for 30 minutes. Gelatinolytic activities were identified as clear zones against a blue background after destaining with ddH₂O.

4. Western blotting

Conditioned medium from luteal cell cultures was concentrated 20 times by using Microcon® YM-3 Centrifugal Filter Devices (Millipore, Bedford, MA). Aliquots of concentrated medium equivalent to 10,000 cells were used for Western blot analysis as previously described (Zhang et al., 2002). Briefly, media were separated on 12% acrylamide SDS gels under reducing condition, which was accomplished by including 10mM (final concentration) dithiothreitol (Sigma, St Louis, MO) in the loading buffer and boiling the samples for 3 minutes prior electrophoresis. Proteins were then electrophoretically transferred onto Protran® nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Nonspecific binding sites on the membranes were blocked by incubation with 5% (w/v) nonfat dry milk in TBST buffer (10mM Tris, 150mM NaCl, 0.05% Tween-20, pH 8.0) for 2 hours at room temperature. A mouse anti human MMP-2 monoclonal antibody (1µg/ml in 5% nonfat dry milk; Oncogene Research Products, San Diego, CA) was incubated with membranes overnight at 4°C. After four washes with TBST buffer (15 minutes per wash), a horse radish peroxidase (HRP) conjugated goat anti mouse IgG antibody (1:10,000; Pierce, Rockford, IL) was applied onto membranes for 1 hour. Immunoreactive protein bands were visualized with SuperSignal® West Pico chemiluminescent substrate (Pierce, Rockford, IL) according to manufacturer’s instructions.
5. Statistical analysis

Densitometric intensities of bands on zymograms and Western blot membranes were analyzed by UN-SCAN-IT gel™ automated digitizing system (Silk Scientific, Orem, UT). The data were analyzed by ANOVA. The Dunnet test was used for comparisons between treatment and control groups, while the Bonferroni test was used for comparisons among different treatments (Zar. 1999). A value of p<0.05 was considered to be significantly different.

Results

1. Dose dependent responses of MMP-2 expression to TNFα in luteal cells.

Zymographic analysis of luteal cell conditioned media revealed two major gelatinolytic species with relative molecular masses of 88 and 68 kDa, which correspond to MMP-2 and MMP-9 family members, respectively (Figure 5-1A). While latent MMP-2 and MMP-9 were the major forms observed, less intense bands of active MMP-2 and MMP-9 were also detected. Densitometric analysis demonstrated that the level of latent MMP-2 increased coordinately with TNFα concentration. Compared to the control group, latent MMP-2 was significantly increased (p<0.05) in cells treated with 10 ng/ml and 100 ng/ml TNFα (Figure 5-1B). Interestingly, latent MMP-9 also increased in a similar manner (data analysis not shown).

Western blotting of concentrated luteal cell conditioned medium demonstrated that active MMP-2 was only detected in the 100 ng/ml TNFα treatment group, while latent MMP-2 was present in all samples (Figure 5-2A). Furthermore, levels of active and latent MMP-2 were the greatest (p<0.05) in the 100 ng/ml of TNFα treatment group, while no differences were observed among all other groups.

2. Time dependent responses of MMP-2 expression to TNFα in luteal cells.
The stimulatory effect of TNFα (100ng/ml) on MMP-2 expression in luteal cells was shown to be time dependent. Gelatin zymography revealed that latent MMP-2 was increased with length of incubation time (Figure 5-3A). While no significant differences were detected between the 6- and 12-hour groups (Figure 5-3B), latent MMP-2 at 24- and 48-hours was greater than the earlier time points. Furthermore, when compared to their contemporaneous control group, latent MMP-2 expression in the presence of TNFα was significantly greater (p<0.05) only at the 24 and 48-hour time points (Figure 5-3B).

Western blotting confirmed the pattern of change observed in zymograms, whereby latent MMP-2 increased with incubation time. While latent MMP-2 was lowest and greatest at the 6- and 48-hour time points, respectively, there was no difference between the 12- and 24- hour time points. Also, latent MMP-2 in cells treated with TNFα was greater in contemporaneous controls at all time points except at 6-hours. Furthermore, active MMP-2 was detected in cells treated with 100 ng/ml TNFα at all time points (Figure 5-4A). Notably, active MMP-2 was significantly increased only at the 48-hour time point (Figure 5-4B).

3. Differential regulation of MMP-2 expression by TNFα and IFNy in luteal-derived endothelial cells.

In luteal-derived endothelial cells, zymography demonstrated that MMP-2 was the predominant gelatinase, since MMP-9 was undetectable. Furthermore, latent MMP-2 was the major form, while minor active MMP-2 was observed in all groups (Figure 5-5A). Treatment with TNFα significantly increased (p<0.05), while IFNy inhibited (p<0.05) MMP-2 expression. When combined, IFNy attenuated the stimulatory effect of TNFα alone, and the level of latent MMP-2 expression was similar to the control group (p>0.05) (Figure 5-5B).

Discussion
MMP-2 is critical for ovulation, luteal angiogenesis, and CL development (Gottsch et al., 2000; Gottsch et al., 2001). Unlike other MMPs, the activation of pro-MMP-2 is accomplished on the cell surface by the coordinate interactions between MT1-MMP and TIMP-2 (Nagase, 1997). The regulation of MMP-2 gene expression is also unique among MMPs due to its atypical promoter organization (Qin et al., 1999). Little is known regarding the regulation of this enzyme in the ovary. LH has no effects on MMP-2 expression by luteal cells from 4 day old bovine CL (Tsang et al., 1995), while estradiol up-regulates MMP-2 production by human granulosa-lutein cells (Puistola et al., 1995). In equine ovarian stromal cells, transforming growth factor β (TGFβ) stimulates MMP-2 activity (Song et al., 1999). In addition, a number of cytokines exhibit differential regulatory effects on MMP-2 expression. For example, TNFα up-regulates MMP-2 activity in ovine follicular explants, while incubation with TNFα antiserum reduces MMP-2 activity (Gottsch et al., 2000). Furthermore, intrafollicular administration of TNFα antiserum blocks ovulation. Although the un-ruptured follicles, nonetheless, underwent luteinization, a fully formed CL with accompanying vasculature was not observed (Gottsch et al., 2000). These data suggest that MMP-2, under the regulation of TNFα, may be critical for CL formation and angiogenesis.

We previously reported that MMP-2 is localized to large luteal cells and endothelial cells in the bovine CL (Zhang and Tsang, 2001). In the present study, we investigated the regulation of MMP-2 expression in these two cell types by TNFα and/or IFNγ. In bovine luteal cells, TNFα stimulated MMP-2 expression in a dose and time dependent manner. Similarly, TNFα also stimulates MMP-2 expression in porcine large luteal cells (Pitzel et al., 2000). The CL produces TNFα. Macrophages are the major sources of TNFα in the porcine CL (Zhao et al., 1998), while large luteal cells also secrete TNFα at a significantly higher level than endothelial and small luteal cells (Zhao et al., 1998). In both porcine and bovine CL, the large luteal cells are positive for TNFα receptor type I (TNFR I) mRNA expression (Richards and Almond, 1994; Sakamoto et
al., 2000a). Considering these data, we postulate that MMP-2 production may be regulated by TNFα in a paracrine and/or autocrine manner.

Of interest, the present study demonstrated that only the highest dose of TNFα (100 ng/ml) stimulated pro-MMP-2 activation, even though the level of pro-MMP-2 increased with increasing concentration of TNFα. This indicated that TNFα may have multiple cellular signal transduction pathways to regulate MMP-2 expression and activity. On the one hand, TNFα may work through transcriptional activators, such as p53 and/or NFκB, to stimulate MMP-2 gene expression (Marti et al., 1993; Bian and Sun, 1997), which results in the increase of pro-MMP-2. On the other hand, TNFα may modulate activation of MMP-2 via a nongenomic pathway. For instance, efficient activation of MMP-2 is accomplished on the cell membrane by assembling a trimeric complex comprising MT1-MMP, TIMP-2, and pro-MMP-2 (Cao et al., 1998; Butler et al., 1998). In the bovine CL, we reported the coordinate expression of MT1-MMP and TIMP-2 with MMP-2 activity (Zhang and Tsang, 2001; Zhang et al., 2002). Since this activation is performed on the cell membrane, it can be greatly affected by factors that modulate membrane trafficking, including fluidity, stability, and other biophysical features (Lehti et al., 2002). Indeed, ConA stimulates pro-MMP-2 activation by shifting endogenous MT1-MMP from the intracellular compartment to the cell surface (Jiang et al., 2001). Also, binding of TNFα to its membrane receptor (TNFR I) induces activation of sphingomyelinases, which hydrolyze N-acylsphingosin-1-phosphorylcholine, a phospholipid preferentially found in the plasma membrane of mammalian cells, to ceramide (Kronke, 1999). The latter can act as a second messenger to trigger a variety of cellular functions. Notably, conversion of sphingomyelin, a sphingolipid situated predominantly in the outer part of the cell membrane, to ceramide, a second messenger, may affect the biophysical structure of plasma membranes. In addition, TNFα also increases PGF₂α production by activating phospholipase A2 enzymes, which liberate arachidonic acid (ACA) from membrane phospholipids for the synthesis of eicosanoids (Townson and Pate, 1996; Sakumoto et al., 2000b).
Therefore, one of the cellular functions of TNFα might be through sphingomyelinase/sphingomyelin and/or PLA2/ACA pathway(s) to affect the oligomerization of MT1-MMP on the cell surface, which then modulates pro-MMP-2 activation (Lehti et al., 2002). In the present study, induction of pro-MMP-2 activation by 100 ng/ml TNFα was observed as early as 6 hours after treatment, indicating that it is likely acting via a nongenomic pathway rather than being regulated at the transcriptional level. By using inhibitors against different second messengers, studies are underway to further pinpoint the detailed cellular signal transduction events triggered by TNFα for MMP-2 expression and activation.

Similar to luteal cells, MMP-2 expression in endothelial cells was also enhanced by TNFα treatment. Luteal-derived endothelial cells have greater levels of TNFRI mRNA than luteal cells (Sakumoto et al., 2000a), suggesting that endothelial cells are also a major target of TNFα. Indeed, injection of the rabbit CL with TNFα induced blood vessel regression, a process characterized by strictures, obstructions, and rugged surfaces on the vessels (Nariai et al., 1995). This is similar to the vessel regression observed during luteolysis in cows (Modlich et al., 1996).

Therefore, one action of increased TNFα during the late luteal phase and in the regressed CL may be to stimulate MMP-2 expression and activation in endothelial cells. The increased MMP-2 activity allows endothelial cells to degrade the surrounding ECM proteins, to detach from the underlying basement membrane (Modlich et al., 1996), and to initiate blood vessel regression.

In contrast to TNFα, IFNγ inhibited MMP-2 expression by luteal-derived endothelial cells. In addition, co-treatment with TNFα and IFNγ resulted in MMP-2 expression that is similar to controls, indicating possible interactions between the second messengers induced by these two cytokines. IFNγ has anti-proliferative effects on luteal-derived microvascular endothelial cells (Fenyves et al., 1994). IFNγ also up-regulates endothelial cell expression of E-cadherin (Fenyves et al., 1993), a cell adhesion molecule. This is consistent with its ability to increase intercellular junctions in an in vitro cell culture system (Ricken et al., 1996). Taken together, IFNγ may play
an important role in vasculature maintenance by down-regulating MMP-2 expression, inhibiting endothelial cell proliferation, and reinforcing intercellular junctions between endothelial cells. These biological functions of IFNγ on luteal-derived endothelial cells may contribute to its luteotrophic effects (Prakash et al., 1997).

Although the physiological roles of IFNγ and TNFα on luteal physiology are very complex, they have been postulated to play important roles in luteolysis (Pate, 1994). IFNγ mRNA levels decline in late diestrus and early luteolysis (Petroff et al., 1999), although its receptor in the CL remains to be elucidated. TNFα mRNA levels in the CL do not vary throughout the estrous cycle (Petroff et al., 1999; Sakamoto et al., 2000a). However, the TNFα protein significantly increases from the mid to late stage (Shaw and Britt, 1995; Sakamoto et al., 2000a). Although the concentration of TNFα receptors (TNFR I) is significantly lower in the late stage than in others, high affinity binding sites for TNFα are detected in the regressed CL (Sakamoto et al., 2000a). Collectively, decreased IFNγ expression, increased TNFα level, and appearance of high affinity TNFα receptor may act in concert to increase MMP-2 expression and activation, which we have observed in the regressing bovine CL (Zhang et al., unpublished observation).

In summary, the present study demonstrated the stimulatory effects of TNFα on MMP-2 expression and activation in luteal cells and luteal-derived endothelial cells. In contrast, IFNγ attenuated MMP-2 expression in endothelial cells. The regulatory effects of these cytokines on MMP expression may contribute to their roles in modulating CL physiology, i.e., angiogenesis, vascular maintenance, and luteolysis.
Dose dependent responses of MMP-2 expression in luteal cells to TNFα

Figure 5-1. Zymographic analysis of medium conditioned by bovine luteal cells treated with different concentrations of TNFα for 12 hours. A) In this representative zymogram, Perfect Protein™ Marker (Novagen, Madison, WI) molecular weight standards are listed on the left and shown in lane 2 (M). In lane 1, HT stands for the conditioned medium of HT1080 cells. In lane 3, C denotes the control culture medium from luteal cells in the absence of treatment. In lanes 4-6, the concentrations of TNFα are indicated on the top. B) Densitometric analysis of pro-MMP-2. Dissimilar letters denote significance at p<0.05.
Dose dependent responses of MMP-2 expression in luteal cells to TNFα

**TNFα (12h)**

**A**

![Western blot image](image)

- 113kDa
- 92kDa
- 52kDa
- 35kDa
- 28.9kDa
- 20kDa

HT C 1 10 100 (ng/ml)

- Pro-MMP-2
- Active MMP-2

**B**

![Bar graph image](image)

<table>
<thead>
<tr>
<th>Control</th>
<th>1ng/ml</th>
<th>10ng/ml</th>
<th>100ng/ml</th>
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<tr>
<td>Latent</td>
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<tr>
<td>Active</td>
<td>a</td>
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Latent MMP-2: □
Active MMP-2: □

Figure 5-2. Immunoreactive MMP-2 in luteal cells treated with different concentrations of TNFα.

A) Representative Western blot of latent and active MMP-2. Molecular masses of Prestained protein standards (Bio-Rad Laboratories, Hercules, CA) are listed on the left. In lane 1, HT is the conditioned medium of HT1080 cells. In lane 2, C represents the control group. Lanes 3-5 are samples from luteal cells treated with 1, 10, or 100 ng/ml of TNFα for 12 hours. The latent and active MMP-2 bands are indicated on the right. B) Densitometric analysis of latent (□) and active (□) MMP-2 among different groups. Dissimilar letters denote significance at p<0.05.
Figure 5-3. Time dependent responses of MMP-2 expression in bovine luteal cells treated with TNFα (100 ng/ml). A) Representative zymogram of luteal cells incubated in the absence (-) and presence (+) of TNFα. Perfect Protein™ Marker (Novagen, Madison, WI) molecular weight standards are indicated on the left and shown in lane 1 (M). In lane 10, conditioned medium from HT1080 cells (HT) were loaded. Samples incubated for 6-, 12-, 24-, or 48-hours were loaded in lanes 2-9. Arrows indicate pro- and active MMP-2. B) Densitometric analysis of latent MMP-2. Dissimilar letters denote significance (p<0.05) among different incubation times following TNFα treatment. Asterisks represent significant difference (p<0.05) between respective control and TNFα treatment groups at each time point.
Figure 5-4. Time course responses of latent and active MMP-2 in bovine luteal cells treated with TNFα. A) Representative Western blot of conditioned medium from luteal cells in the absence (-) and presence (+) of TNFα. Molecular masses of Prestained protein standards (Bio-Rad Laboratories, Hercules, CA) are indicated on the left. In lane 1, HT represents the conditioned medium of HT1080 cells. Samples incubated for 6-, 12-, 24-, or 48-hour were loaded in lanes 2-9. Latent and active MMP-2 bands are indicated by arrows on the right. B) Statistical analysis of active and latent MMP-2 levels. Dissimilar letters denote significance (p<0.05) among different incubation times following TNFα treatment. Asterisks represent significant difference (p<0.05) between respective control and TNFα treatment groups at each time point.
Regulation of MMP-2 expression in luteal-derived endothelial cells by cytokines

A) Representative zymogram of conditioned medium of luteal-derived endothelial cells. Molecular masses of Perfect Protein™ Markers (Novagen, Madison, WI) are indicated on the left and shown in lane 1 (M). In lane 2, HT represents HT1080 conditioned medium. In lanes 3-6, control and cytokine treatments, TNFα (100ng/ml) and IFNγ (200IU/ml), alone or in combination, are shown.

B) Densitometric analysis of MMP-2 levels. Dissimilar letters denote significance at p<0.05.

Figure 5-5. MMP-2 expression in luteal-derived endothelial cells in response to TNFα and IFNγ, alone and in combination. A) Representative zymogram of conditioned medium of luteal-derived endothelial cells. Molecular masses of Perfect Protein™ Markers (Novagen, Madison, WI) are indicated on the left and shown in lane 1 (M). In lane 2, HT represents HT1080 conditioned medium. In lanes 3-6, control and cytokine treatments, TNFα (100ng/ml) and IFNγ (200IU/ml), alone or in combination, are shown. B) Densitometric analysis of MMP-2 levels. Dissimilar letters denote significance at p<0.05.
SUMMARY AND CONCLUSIONS

In the present studies, we cloned the full length cDNAs of the bovine matrix
metalloprotease-2 (MMP-2) and membrane type 1 metalloprotease (MT1-MMP) genes.
Multiple alignment analysis indicated that the predicted proteins for these two molecules have a
high level of sequence identity with their homologs from other mammalian species, suggesting
that they have been conserved through evolution. Although the transcript levels of both molecules
were unchanged in three ages of bovine corpus luteum (CL) obtained over the estrous cycle, their
protein levels varied. While latent MT1-MMP was higher (p<0.05) in the early CL than the other
two stages, the active MT1-MMP was significantly increased from the early stage to the mid and
late cycle CL. In spite of the fact that the level of pro-MMP-2 remained constant in all ages of CL
evaluated, the level of active MMP-2 in the mid and late stages was significantly higher than that
in the early CL. In addition, MT1-MMP and MMP-2 were localized to endothelial and large
luteal cells. Furthermore, MT1-MMP expression was also detected in the fibroblast-like cells of
the late cycle CL. Thus, the correlation between active MT1-MMP and active MMP-2 throughout
the estrous cycle and their co-localization in the same cellular compartments suggests that MT1-
MMP may serve as the in vivo activator for pro-MMP-2.

Recent studies report that the activation process of pro-MMP-2 by MT1-MMP is
regulated by an endogenous tissue inhibitor of metalloproteinas 2 (TIMP-2). This inhibitor
bridges these two MMPs by binding MT1-MMP on the cell surface via its N-terminus and pro-
MMP-2 at its C-terminal domain. This assembled trimeric complex presents pro-MMP-2 to an
adjacent, active MT1-MMP, which cleaves the N-terminal pro-peptide domain of pro-MMP-2,
initiating its activation. In order to determine whether TIMP-2 is coordinately expressed with
MT1-MMP and MMP-2, we investigated the temporal and spatial expression of TIMP-1 and
TIMP-2 in the bovine CL. TIMP-1 mRNA and protein were expressed at higher levels in the
early and mid stages than the late cycle CL. However, TIMP-2 mRNA and protein expression was low in early CL, but significantly increased in the mid and late stages, corresponding to the changes observed for active MT1-MMP and MMP-2 proteins. Furthermore, TIMP-2 was also localized in endothelial and large luteal cells in bovine CL, while TIMP-1 was localized in large luteal cells, and in vascular smooth muscle cells in the early and late stage CL. These data indicated that the MT1-MMP/TIMP-2/pro-MMP-2 system might be available for pro-MMP-2 activation in the bovine CL. In addition, the localization of TIMP-1 in the vascular smooth muscle cell compartment suggested that it might have a role in luteal angiogenesis and vascular maintenance.

Besides its distinct activation mechanism, MMP-2 also possesses a different promoter organization from other MMPs. The last part of our experiments was to initiate studies on the regulation of MMP-2 in luteal and endothelial cells by using in vitro cell culture systems. TNFα stimulated MMP-2 expression and activation in luteal cells in a time and dose dependent manner. Although TNFα increased MMP-2 expression in luteal-derived endothelial cells, IFNγ inhibited its expression. In the presence of both cytokines, IFNγ attenuated the stimulatory effects of TNFα, reducing MMP-2 expression to control levels.

Collectively, the in vivo data suggest that the MT1-MMP/TIMP-2/pro-MMP-2 system might be available in the CL for pro-MMP-2 activation. The localization of these three molecules in the same cellular compartments further support their coordinated actions during turnover of the CL extracellular matrix, such as during enlargement of large luteal cells and luteal angiogenesis. Furthermore, localization of TIMP-1 in vascular smooth muscle cells suggests that it is another player participating in the regulation of angiogenesis and vascular maintenance. Lastly, the in vitro study using luteal and endothelial cells indicated that the cytokines, TNFα and IFNγ, might serve as local regulators of MMP-2 expression and activation.
The data provided by the present study on the expression, activation, and regulation of MMP-2 will enable us to conduct future experiments to continue elucidating the roles of this and other MMP enzymes in angiogenesis and the physiology of the corpus luteum.
LIST OF REFERENCES


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Zhang B, Tsang PCW. (2001) Are matrix metalloproteinase 2 (MMP-2) and tissue inhibitor of metalloproteinases 2 (TIMP-2) coordinately expressed with membrane type 1 metalloproteinase (MT1-MMP) in the bovine corpus luteum (CL) during the estrous cycle? Biol Reprod 64 (Suppl 1):236


APPENDIX

APPROVAL OF ANIMAL PROTOCOLS BY THE UNH INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

University of New Hampshire

Office of Sponsored Research
Service Building
51 College Road
Durham, New Hampshire 03824-3585
(603) 862-3564 FAX

<table>
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<tr>
<th>LAST NAME</th>
<th>Tsang</th>
<th>FIRST NAME</th>
<th>Paul</th>
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The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a project report with regard to the involvement of animals before that date. If your project is still active, you may apply for extension of IACUC approval through this office.

The appropriate use and care of animals in your project is an ongoing process for which you hold primary responsibility. Changes in your protocol must be submitted to the IACUC for review and approval prior to their implementation. If you have questions or concerns about your project or this approval, please feel free to contact the Regulatory Compliance Office at 862-2003 or 862-3536.

Please note: Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Giadi Porsche, UNH Health Services.

Please refer to the IACUC # above in all correspondence related to this project. The IACUC wishes you success with your research.

For the IACUC,

[Signature]

John A. Lithvitis, Ph.D.
Chair

cc: File

ORI APPL 12/17/1999