Research

Temporal and spatial expression of tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and -2) in the bovine corpus luteum

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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. In addition to regulating MMP activity, TIMPs are also involved in a variety of cellular processes, including cell proliferation and steroidogenesis. In a series of initial studies, we determined that matrix metalloproteinase inhibitory activity was present in protein extracts from early (4 days old, estrus = day 0), mid (10–12 days old) and late (16 days old) CL (n = 3 for each stage). Reverse zymography revealed four metalloproteinase inhibitory protein bands with relative molecular masses that are consistent with those reported for TIMP-1 to -4. In order to gain a better understanding of TIMPs and their role in luteal function, we further characterized this inhibitory activity with a particular focus on the temporal and spatial expression of TIMP-1 and TIMP-2 in the bovine CL. Northern blotting revealed that the TIMP-1 transcript (0.9 kb) was expressed at a higher (p < 0.05) level in early and mid cycle CL than in the late stage. In contrast, two TIMP-2 mRNA species, one major 1 kb species and one minor 3.5 kb species, were significantly (p < 0.05) increased in the mid and late cycle CL than in the early. Western blotting analyses demonstrated no differences in TIMP-1 (29 kDa) protein levels between early and mid stages, while its levels decreased (p < 0.05) from the mid to late stage CL. Conversely, TIMP-2 (22 kDa) protein was detected at a low level in the early CL, but significantly (p < 0.05) increased in the mid and late stages. Immunohistochemistry revealed that both TIMP-1 and -2 were localized to large luteal cells from all three ages of CL. TIMP-1 was also localized in capillary smooth muscle cells, while TIMP-2 was restricted to the endothelial cells in the capillary compartment. In conclusion, the different temporal expression patterns of TIMP-1 and TIMP-2 suggest that TIMP-1 may be important for luteal formation and development, while TIMP-2 may play significant roles during luteal development and maintenance. Furthermore, the distinct localization of these two inhibitors in the vascular compartment indicates that they may serve diverse physiological functions during different stages of luteal angiogenesis.

Background

The corpus luteum (CL) is a transient, dynamic endocrine gland, which develops from the postovulatory follicle [1]. Dramatic structural and functional changes are associated with the development, maintenance and regression of the CL [1]. These remodeling events require the participation of matrix metalloproteinases (MMPs), a growing family of zinc and calcium dependent proteolytic enzymes that collectively digest all known macromolecules constituting the extracellular matrix [2,3]. Generally, the catalytic activity of the MMPs is highly regulated at three levels, gene expression, proteolytic activation of latent proenzymes, and inhibition of activity by binding of endogenous tissue inhibitors of metalloproteinases (TIMPs) to the catalytic domain [2,4].

Four TIMPs, TIMP-1 [5,6], TIMP-2 [7,8], TIMP-3 [9], and TIMP-4 [10], have been identified. Among them, TIMP-1 and TIMP-2 are the two most studied inhibitors. TIMP-1, a glycoprotein with a molecular mass of approximately 29 kDa, was the first member of this family to be cloned [5]. TIMP-1 can bind the active forms of all known MMPs [4] and the latent form of MMP-9 [11]. In addition to suppressing the activity of MMPs, TIMP-1 also possesses mitogenic activity for a variety of cell types, such as gingival fibroblasts and erythroid precursor cells [12]. TIMP-1 may regulate steroidogenesis in that it stimulates progesterone production by rat granulosa cells [13]. In CL from a variety of species, the expression of TIMP-1 and TIMP-1-like proteins and messenger RNA has been determined, including cow [14,15], sheep [16], rat [17], mouse [18], monkey [19], and human [20].

TIMP-2 is an unglycosylated protein with an approximate molecular mass of 22 kDa [7,21]. TIMP-2 is also able to bind most active MMPs and inhibit their proteolytic activity [4]. Among the members of the MMP family, TIMP-2 preferentially binds to MMP-2 [21,22]. Paradoxically, however, TIMP-2 may also be involved in pro-MMP-2 activation by participating in the formation of a membrane type 1-MMP (MT1-MMP)/TIMP-2/pro-MMP-2 trimolecular complex on the cell membrane [23–25]. Similar to TIMP-1, TIMP-2 stimulates proliferation of a variety of cell types [26]. TIMP-2 mRNA expression has been determined in the sheep [27], cow [28], human [29], rat [30], and mouse [18] CL, while a TIMP-2-like protein was detected in the cow CL [15].

The variety and intensity of events that occur over the CL life span suggest a tightly regulated temporal and spatial interplay between MMPs and TIMPs. Our previous studies demonstrated that remodeling of the bovine CL involves a variety of MMP species, for example, MMP-2, MMP-9, and MT1-MMP. In the present study, we determined TIMP inhibitory activity and profiled the coordinate mRNA and protein expression patterns and cellular distribution of TIMP-1 and TIMP-2 in early, mid, and late stages of the bovine CL obtained over the estrous cycle.

Methods

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 by colpotomy 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. Based on progesterone concentrations determined by radioimmunoassay, the CL collected on day 16 of the estrous cycle were functionally active (8.99 ± 1.84; ng/ml ± SEM). The luteal tissue was subsequently used for total RNA and protein extraction. 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.

Radiometric MMP Assay

Total protein was extracted from luteal tissues in a lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 0.02% (w/v) sodium azide, 10 mM EDTA, 1% (v/v) Triton X-100, 10 μg/ml aprotinin (Sigma, St Louis, MO), 1 μg/ml aminoethyl benzenesulphonyl fluoride (Sigma, St Louis, MO)] which we previously described [31]. Equivalent amounts of luteal protein extracts were added to wells of 96-well microtiter plates containing polymerized ^{14}C-acetylated collagen in the presence of a known amount of MMP-1 activity from the conditioned medium of bovine corneal explants [32]. The plates were then incubated at 37°C for 2.5 hours, after which the supernatant containing degraded ^{14}C-acetylated collagen fragments was collected and counted in a scintillation counter. A unit is defined as the amount of protein required to inhibit one unit of corneal collagenase by 50%.

Reverse Zymography

In order to simultaneously distinguish the activity of TIMP proteins in bovine CL, reverse zymographic analysis was performed. Equivalent aliquots 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 human fibrosarcoma HT1080 cells, which is a rich source of various MMPs, for 4 hours to degrade

gelatin. After rinsing, gels were incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl$_2$, 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. Whereas the MMPs present in the HT-1080 conditioned medium digest the gelatin within the gel, the TIMPs in the luteal samples inhibit MMP action, and were visualized as dark bands on a clear background. Prestained SDS-PAGE protein standards (Bio-Rad Laboratories, Hercules, CA) were run in an adjacent lane.

Northern Blotting
TIMP-1 and TIMP-2 mRNA expression in the CL was studied by Northern blotting as described previously [31]. 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 the hybridization buffer containing TIMP-1 [33], TIMP-2 [7], or cyclophilin (a generous gift from Dr. Robert Thompson, University of Michigan) cDNA probes at 55°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).

Western Blotting Analysis
Equivalent amounts of tissue protein 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 protein standards (Bio-Rad Laboratories, Hercules, CA) were also loaded 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. Two primary antibodies, mouse anti-human TIMP-1 monoclonal antibody (Oncogene Research Products, Cambridge, MA) and mouse anti-human TIMP-2 monoclonal antibody (Oncogene Research Products, Cambridge, MA), were used at 1 µg/ml and 2 µg/ml, respectively. After an overnight incubation at 4°C, the membranes were washed five times with TBST (each time for 15 minutes), followed by a 1-hour incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (1:15,000, Pierce, Rockford, IL) at room temperature. The blots were then washed with TBST, and developed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), according to the manufacturer's instructions.

Immunohistochemistry
Frozen tissue sections (6 µm) were cut onto Superfrost®/Plus slides (Fisher Scientific, Pittsburg, PA). Sections were air-dried at room temperature for 5 min before a 10-min fixation in cold acetone. Endogenous peroxidase activity was quenched by incubating with 0.3% (v/v) hydrogen peroxide for 30 min. Nonspecific binding was blocked by 5% (w/v) BSA for 30 min. 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 determine the presence of vascular smooth muscle cells (VSMC) and endothelial cells in the luteal tissue, consecutive sections were also stained with an antibody against α-actin and von Willebrand Factor (VWF), cellular markers for VSMC and endothelial cells, respectively [34]. 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 was examined.

Data Analysis
Intensities of Northern and Western blots, in which each sample was run in triplicate, were determined by UN-SCAN-IT™ digitizing software system (Silk Scientific, Orem, UT). The data were analyzed by ANOVA, followed by Tukey's test for multiple comparisons.

Results
Metalloproteinase Inhibitor Activities in the Bovine CL
The radiometric MMP assay for TIMP activity revealed that the protein extract from all three ages of CL possessed
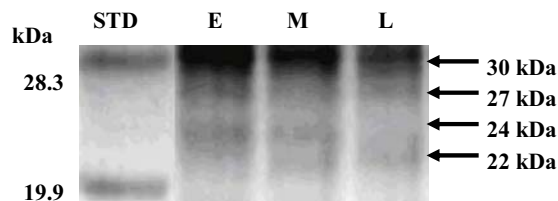
Figure 1
Reverse zymographic analysis of tissue inhibitors of metalloproteinases (TIMPs). Inhibitor activities in the early (E), mid (M), and late (L) stages of bovine CL are shown. Lane 1 indicates the prestained SDS-PAGE standards (STD; Bio-Rad Laboratories, Hercules, CA). Their corresponding molecular masses (kDa) are indicated on the left. Four bands possessing MMP inhibitory activities (indicated by arrows on the right) were observed in luteal samples. These bands correspond to TIMP-1 (~30 kDa), TIMP-3 (~27 kDa), TIMP-4 (~24 kDa), and TIMP-2 (~22 kDa), respectively.

significant metalloproteinase inhibitory activity (inhibitory units per gram total protein ± SEM; day 4, 22,933 ± 1703; day 10, 24,066 ± 33; day 16, 22,166 ± 2938). However, there was no significant difference (P > 0.05) among the three stages of CL studied. Based on these observations, reverse zymography was then used to distinguish metalloproteinase inhibitory activities in luteal tissues. Four protein bands were identified, and they migrated at ~30 kDa, ~27 kDa, ~24 kDa, and ~22 kDa, corresponding to the molecular masses reported for TIMP-1, -3, -4, and -2, respectively (Figure 1). TIMP-1 appeared to be the predominant TIMP present in the bovine CL.

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

Northern blotting demonstrated that a single TIMP-1 transcript (0.9 kb) was present in all three ages of CL (Figure 2A). 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 2B). The same pattern was observed when cyclophilin was used to normalize sample loading.

Two TIMP-2 mRNA species, a major one at 1 kb and a minor one at 3.5 kb, were present in all ages of CL (Figure 3A). 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 house-keeping gene cyclophilin, were low in the early stage, but increased sig-

nificantly (p < 0.05) in the mid and late cycle CL (Figure 3B). The same pattern was observed when 18S RNA was used to normalize sample loading.

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

Western blotting revealed an approximate 30 kDa immunoreactive TIMP-1 protein band in all ages of CL examined (Figure 2C). 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 2D). In addition, a 22 kDa TIMP-2 immunoreactive protein was also observed in all three ages of CL (Figure 3C). The TIMP-2 protein level in the mid and late cycle CL was significantly greater (p < 0.05) than in the early stage (Figure 3D).

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

Immunohistochemistry using TIMP-1 and TIMP-2 specific antibodies demonstrated that TIMP-1 was present in both large luteal cells and vascular smooth muscle cells (Figure 4A,4C, and 4E). The localization of TIMP-1 in the VSMC compartment was validated by staining for α-actin, a cellular marker for VSMC (Figure 4G). Although large luteal cells from all three ages of CL expressed TIMP-1, visual observations revealed that the highest level of expression was in cells of the mid-cycle CL (Figure 4C). TIMP-1 was expressed in VSMC of the early (Figure 4A) and late (Figure 4E), but not the mid cycle CL (Figure 4C). In all ages of CL, endothelial cells, as validated by staining with an endothelial cell marker VWF (Figure 4H), and large luteal cells showed positive staining for TIMP-2 protein (Figure 4B,4D and 4F). No positive signals were observed in the sections devoid of primary antibodies against TIMP-1 or TIMP-2 (data not shown).

Discussion
In order to eventually elucidate the physiological roles of TIMP-1 and TIMP-2 in the CL, we first determined the temporal and spatial expression patterns of these two inhibitors during the estrous cycle.

In the present study, TIMP-1 mRNA was determined to be highly expressed in the early and mid cycle bovine CL, but decreased in the late stage. In the porcine CL, the TIMP-1 transcript was also highly expressed in the early stage, and is slightly reduced as the estrous cycle progresses before it decreases significantly in the regressing stages [35]. In contrast, TIMP-1 mRNA expression in the ovine [16] and human [29] CL does not change throughout the estrous or menstrual cycle, respectively. In the pseudopregnant rat, a different pattern emerges whereby the strongest TIMP-1 mRNA expression is observed during CL formation and regression [36]. Clearly, there are species differences with
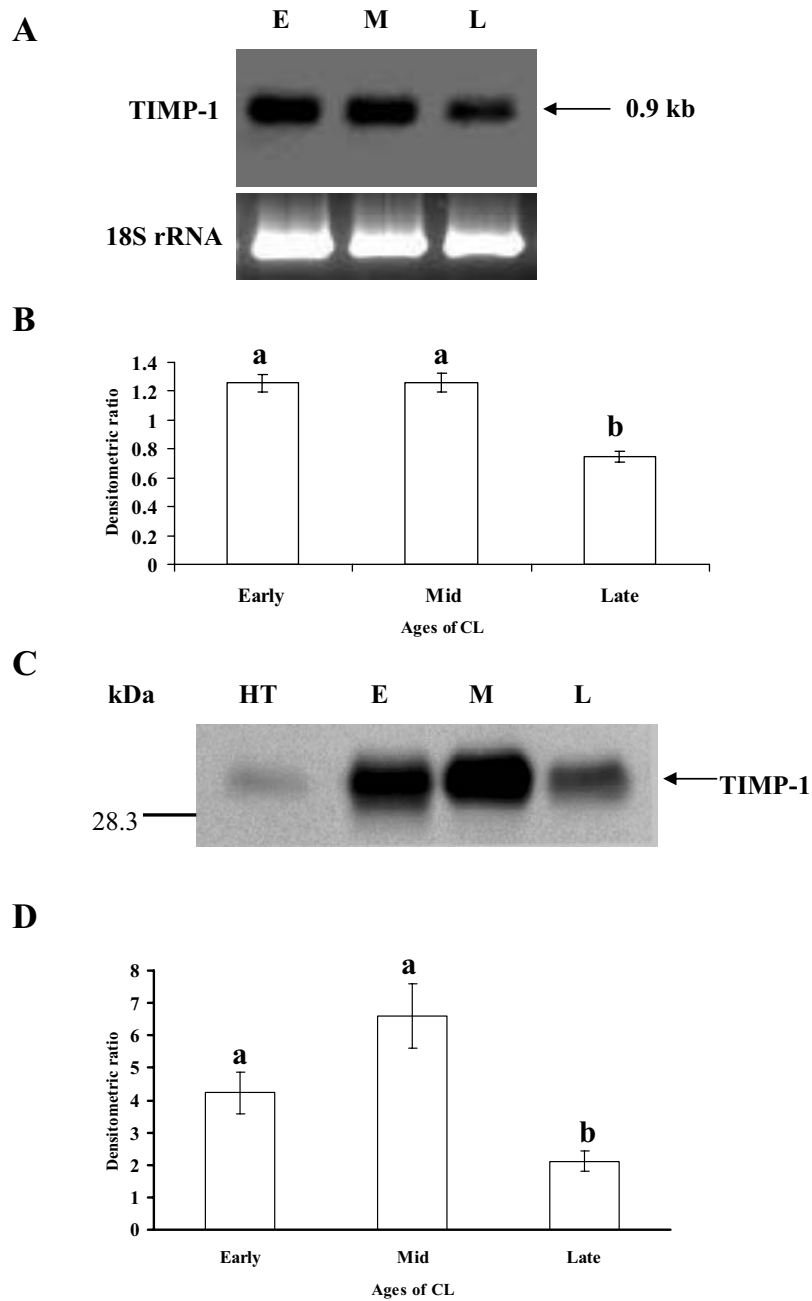
Figure 2
TIMP-1 mRNA and protein expression in the bovine CL. **A**) Northern blotting of TIMP-1 mRNA in early (E), mid (M), and late (L) stage CL is shown in the upper panel. The arrow indicates the 0.9 kb TIMP-1 transcript. The ethidium bromide stained 18S rRNA in corresponding luteal samples is shown in the lower panel. **B**) Changes in TIMP-1 mRNA, expressed as a densitometric ratio of TIMP-1 mRNA to 18S rRNA, are shown in the lower panel. Dissimilar letters denote significant difference at p < 0.05. **C**) A representative Western blot of TIMP-1 in early (E), mid (M), and late (L) stage CL. Conditioned medium of HT1080 cells (HT) was loaded in the first lane and was used as a positive control. **D**) 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.
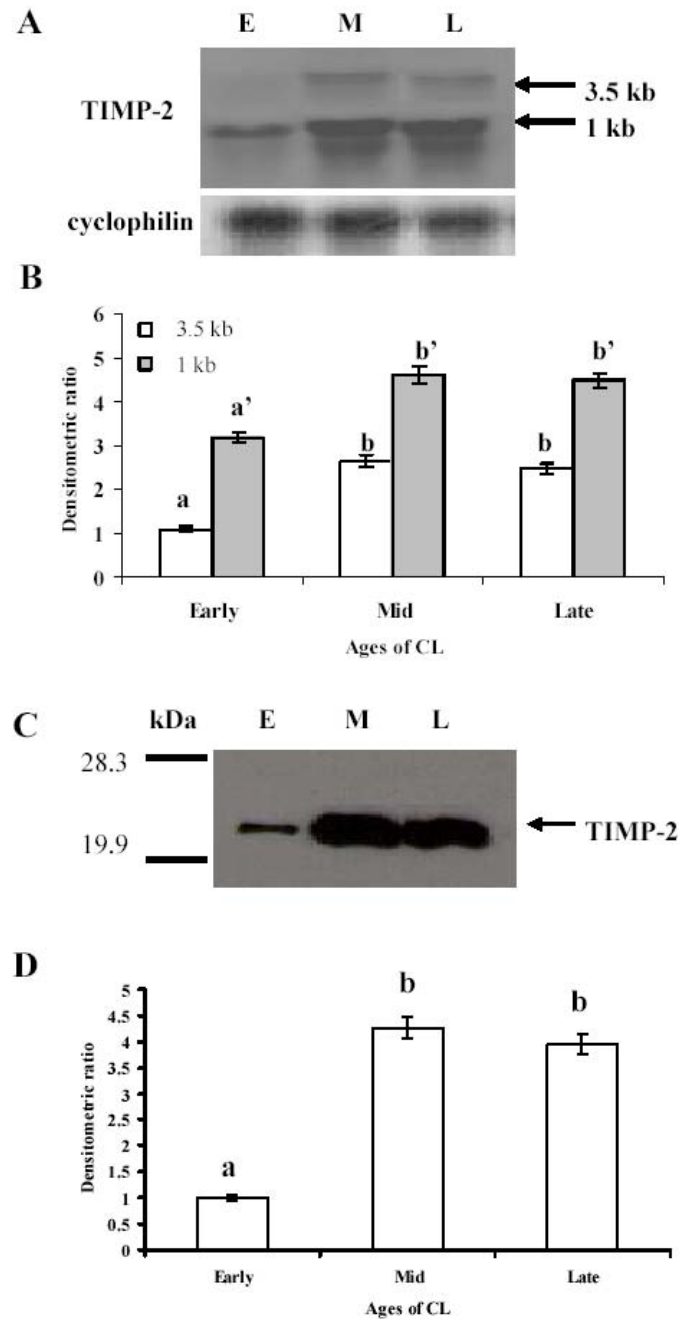
Figure 3
TIMP-2 mRNA and protein expression in the bovine CL. **A**) In the upper panel, Northern blotting of TIMP-2 mRNA in early (E), mid (M), and late (L) stage bovine CL. The arrows indicate the 3.5 and 1 kb species. The lower panel is the same membrane hybridized with a human cyclophilin probe. **B**) Densitometric ratios of each TIMP-2 mRNA band to cyclophilin in corresponding stages are shown. Dissimilar letters denote significant difference at p < 0.05. **C**) A representative Western blot of TIMP-2 in the bovine CL. Molecular masses (kDa) of protein standards are shown on the left. A single 22 kDa TIMP-2 immunoreactive protein band, indicated by the arrow, is present in early (E), mid (M), and late (L) stage CL. **D**) Densitometric analysis of TIMP-2 protein expression among different stages of CL. Dissimilar letters denote significant difference at p < 0.05.
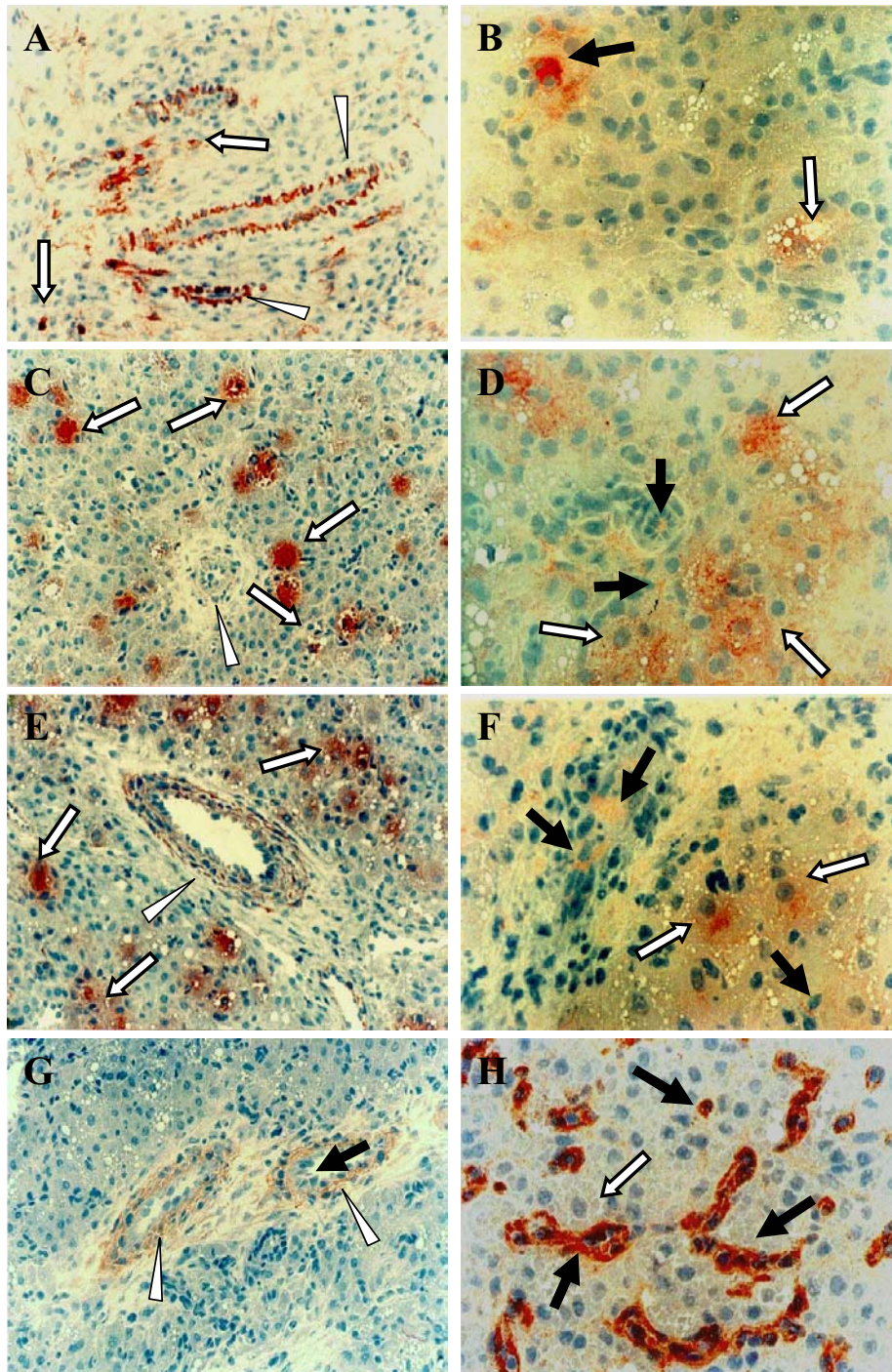
Figure 4
Immunohistochemistry of TIMP-1 (A, C, E; ×200) and TIMP-2 (B, D, F; ×400) in early (A and B), mid (C and D), and late (E and F) stage CL. Vascular smooth muscle cells and endothelial cells are identified by staining with α-actin (G; ×200) and VWF (H; ×400). Positive staining (red color) is observed in large luteal cells (white arrows), endothelial cells (black arrows), and vascular smooth muscle cells (white triangles).

regard to the temporal expression of TIMP-1 transcript in the CL.

The expression of TIMP-1 protein in three ages of bovine CL was also demonstrated in the present study. Reverse zymography demonstrated that TIMP-1 was the predominant TIMP in the bovine CL, similar to the finding in sheep [37]. In the present study, 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 participate in regulating 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 luteal regression in bovine [38], ovine [39,40], porcine [35], and primate [19] CL. Collectively, these data implicate TIMP-1 as an important player in the physiology of the CL.

The TIMP-1 protein was localized in large luteal cells of early, mid, and late stage bovine CL. In the ovine CL, TIMP-1 was co-localized with oxytocin in secretory granules of large luteal cells [41]. In addition, TIMP-1 expression was also detected in isolated ovine [16] and porcine [35] large luteal cells, and luteinized human granulosa cells [42]. The presence of TIMP-1 in steroidogenic cells may be associated with its ability to enhance steroid production [13]. This may be related in part 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 [43]. Additionally, although female mice lacking the TIMP-1 gene do not show detectable differences in serum estradiol-17β concentrations when compared to the wild type, the TIMP-1 deficient male mice have higher concentrations of total serum testosterone than the wild type [44,45]. Furthermore, cell culture studies demonstrated that TIMP-1 is able to increase estradiol-17β production by granulosa cells from both TIMP-1 deficient and wild-type mice [44], and estradiol-17β and progesterone production from porcine thecal cells [46]. 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 [47]. However, other cell types in the CL are positive for TIMP-1 expression as well. 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 [41] and rat [48] CL stain positively for TIMP-1. The localization of TIMP-1 in the vascular smooth muscle compartment supports its poten-

tial role during angiogenesis and vascular maintenance. Indeed, overexpression of TIMP-1 in VSMC by exogenous gene transfer reduced VSMC cell proliferation and migration [49,50]. In addition, stimulation of TIMP-1 expression impaired angiogenesis in a variety of tumor types [51–53]. These data collectively suggest that TIMP-1 may act as a negative regulator of blood vessel formation [32,54].

Extensive angiogenesis occurs during early CL development when 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 MMP activity. However, in the last step of angiogenesis, recruitment and maintenance of pericytes (VSMC) are critical for vascular maturation and survival [55,56]. Thus, the high level of TIMP-1 expression in VSMC of 4-day old bovine CL may provide an environment where MMP action was curbed as the vasculature matures. As the CL ages, its structure remains relatively stable. Although angiogenesis is ongoing, it is slowed during CL maintenance [57–59]. 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 hypothesis of Redmer et al. [60], who proposed that maintenance of the vasculature may be necessary for the transport of degraded products during luteal regression, 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 may be involved in the pro-MMP-2 activation process by binding an MT1-MMP to form a "co-receptor" for pro-MMP-2 on the cell surface [25]. This bound pro-MMP-2 would then be presented to an adjacent MT1-MMP for activation [23,25]. Although a transmembrane-deleted MT1-MMP is capable of activating pro-MMP-2 without the participation of TIMP-2 [61], TIMP-2 deficient mice have a dramatically reduced ability to activate pro-MMP-2 [62]. 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 that for active MT1-MMP and MMP-2 [31], 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 embryonic development [63], 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, the stoichiometrically correlated expression with MT1-MMP and pro-MMP-2 may facilitate the activation process of pro-MMP-2[31], which is critical for the angiogenesis process[64]. On the other hand, inhibition of angiogenesis by TIMP-2 cannot be excluded since TIMP-2 has now been demonstrated to be a potent inhibitor of both physiological and stimulated angiogenesis in vivo[54] and overexpression of TIMP-2 blocks vascular smooth muscle cell invasiveness [65] and reduces angiogenic ability [54,66]. The latter observation is due, in part, to down-regulation of vascular endothelial cell growth factor (VEGF) [67].

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 is then able to bind integrin $\alpha v \beta 3$ [68], 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. Although there is no direct evidence demonstrating involvement of TIMP-2 in steroidogenesis, the dynamic interactions between large luteal cells and their local ECM may induce biochemical changes related to the steroid biosynthetic process in this cell type [69]. For example, disruption of the links between ECM, integrins, and the cytoskeleton [70] may perturb the intracellular transport of substrates, such as cholesterol, for steroidogenesis [71]. Additional in vitro and in vivo studies are needed to elucidate the physiological roles of these TIMPs in this ovarian endocrine gland.

Conclusions
The present study demonstrates that TIMP-1 and TIMP-2 exhibit a coordinated expression pattern in the bovine CL throughout the estrous cycle. The distinct temporal and spatial expression patterns of TIMP-1 and TIMP-2 suggest that these two inhibitors may have multiple and complementary roles in luteal development and angiogenesis during the life span of the CL. Taken together, the expression of these multi-functional TIMPs and MMPs during the bovine estrous cycle suggests that they are key regulators of CL physiology.

Acknowledgements

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