Matrix metalloproteinase 2 (MMP-2) and tissue transglutaminase (TG 2) are expressed in periglandular fibrosis in horse mares with endometrosis

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Summary. Periglandular arrangement of myofibroblasts, associated with the deposition of extracellular matrix (ECM), is a cardinal feature of endometrosis in mares. We hypothesized that a disturbance in the expression of matrix degrading enzymes such as matrix metalloproteinases (MMP's) and matrix cross-linking proteins might lead to an imbalance in deposition and degradation of extracellular matrix components and thereby accentuate degeneration. Therefore, distributions of MMP-2, capable of collagen IV and laminin degradation, and tissue transglutaminase (TG2), a cross-linker of extracellular matrix proteins, were investigated by means of immunohistochemistry on uterine biopsies of healthy mares and animals with endometrosis. It was illustrated that both proteins were present in fibrotic regions of affected endometria, and that they were in most cases colocalized. Periglandular MMP-2 expression was significantly associated with dilated and fibrotic uterine glands. Furthermore, MMP-2 and TG 2 were demonstrated in the stratum compactum of healthy and endometrotic endometria. Gelatin zymography proved that active and inactive pro-form of MMP-2 were present in all examined samples with significantly higher amounts of total and active MMP-2 in affected endometria. TG 2-activity, determined by an *in situ* assay, was found in cases of severe periglandular fibrosis. We suggest that both enzymes play a major role in changes that occur in ECM homeostasis in endometrial fibrotic regions.

Key words: Endometrium, Endometrosis, MMP's, Transglutaminase, Horse

Introduction

Chronic endometrial degeneration in horses is characterised by pathological alterations of the endometrial glands (cystic dilatation, atrophy or hypertrophy of the epithelium), the surrounding connective tissue (periglandular fibrosis), and blood vessels. In addition, signs of chronic inflammation are frequently observed (Kenney and Doig, 1986). To clearly distinguish acute inflammatory reactions from chronic degenerative alterations the term "endometrosis" has been introduced for the latter (Allen, 1993). The diagnosis of the disease based on biopsy samples is an important factor in the breeding soundness examination of broodmares. The system of Kenney and Doig (1986) is widely used to classify the grade of alterations in equine endometrosis. Preliminary results for a further differentiation of the alterations in destructive and non destructive, as well as active and inactive type have recently been reported by Hoffmann et al. (2003).

We have previously demonstrated that periglandular arranged stromal cells in fibrotic regions differentiate to myofibroblast-like cells (Walter et al., 2001), a fact that has also been reported in liver and lung fibrosis (Phan, 2002; Schmitt-Graf et al., 1993). Besides expression of cytoskeletal elements of smooth muscle cells (α-smooth muscle actin SMA, partly tropomyosin and desmin), these myofibroblast-like cells in the altered horse endometrium have further been characterised by deposition of extracellular matrix (ECM) proteins such as collagen type IV, laminin, and fibronectin. The mechanisms leading to the endometrial periglandular fibrosis in the endometrium are still unknown. In general, progressive fibrosis takes place if the rate of matrix synthesis exceeds matrix degradation. It is supposed that either the regulatory mechanisms are overwhelmed by collagen production or the normal response to increasing collagen secretion is altered such

that the excessive matrix is not degraded (Alcolado et al., 1997). Enzymes capable of ECM degradation are the matrix metalloproteinases (MMP's). They are synthesized intracellularly and secreted as inactive pro-forms, which are activated in the extracellular space by other enzymes (including MMP's) or membrane type metalloproteinases (MT-MMP's). Downregulation is executed by tissue inhibitors of matrix metalloproteinases (TIMP's). A balance between zymogens, activators, and inhibitory agents is essential for physiological processes. Therefore, we hypothesized an imbalance of matrix production and degradation in periglandular endometrial fibrosis in the horse. Other fibrotic alterations, such as liver fibrosis, are accompanied by a significant upregulation in mRNA transcripts of MMP-2 (Benyon et al., 1996). For this reason, we examined uterine periglandular fibrotic regions for the presence of MMP-2 (gelatinase A, 72kDa collagenase) by means of immunohistochemistry. MMP-2 is capable of degrading gelatin, native collagen type IV, V, VII, elastin, fibronectin and laminin (Murphy et al., 1989; Woessner, 1991; Marbaix et al., 1992; Zhang et al., 2000). ProMMP-2 is readily activated by 4-aminophenylmercuric acetate (APMA) to a 68 kDa active form in vitro (Stetler-Stevenson et al., 1989) but is resistant to most endopeptidases. Other MMP's such as MMP-1 and MMP-7 are not very efficient activators of proMMP-2 (Woessner and Nagase, 2000), cell surface activation of proMMP-2 by membrane type matrix metalloproteinases (MT-MMP's) is considered to be physiologically more significant (Zhang et al., 2000). MT1-MMP on the cell surface serves as a receptor of TIMP-2 (complex formation) which in turn binds to proMMP-2 (Butler et al., 1998). However, soluble forms of MT1-MMP were also shown to activate proMMP-2 directly (Will et al., 1996).

We were also looking for a possible antagonist to the matrix degrading MMP enzymes. TG 2 is a multi-functional, Ca^{2+}-dependent enzyme, which is known to act as a cross-linker of extracellular matrix proteins. It catalyses post-translational modifications of proteins by formation of isopeptide bonds within or between polypeptide chains making the tissue resistant to degradation (Greenberg et al., 1991). Enzymatic substrates of TG 2 include collagen, fibronectin, and several basement membrane constituents (Aeschlimann and Paulsson, 1991; Martinez et al., 1994). Belkin et al. (2004) have shown that the two proteases MMP-2 and TG 2 are functionally closely connected. Therefore, besides MMP-2 expression we studied the distribution of TG 2 in the normal horse endometrium and in specimens affected by endometrosis. The association of MMP-2 and TG 2 to pathological categories (according to Kenney and Doig, 1986) and to the oestrous cycle stage was analysed and statistically evaluated. Double labeling experiments (MMP-2 and TG 2) with fluorescein-conjugated secondary antibodies and evaluation by confocal laser scanning microscopy were performed on exemplary samples to test for a possible co-localization

of these two enzymes. Activity of TG 2 was determined in frozen sections by an *in situ* transglutaminase activity assay, amounts of active and latent forms of MMP-2 were identified by gelatin zymography.

Materials and methods

Animals

Uterine biopsy samples of 39 mares (3 to 23 years old) in different stages of the oestrous cycle were used for histological examinations. All animals were patients referred to the Centre for Artifical Insemination and Embryo Transfer for a breeding soundness examination. Tissue samples (one biopsy per animal) were taken transcervically from the basis of the right uterine horn. The stage of the oestrous cycle was determined by a combination of clinical examination (transrectal palpation, ultrasound, control of oestrous behaviour with a stallion) and determination of plasma progesterone concentrations. Reproductive stages in the examined animals were as follows: seasonal anoestrus n=11, oestrus n=12, dioestrus n=16.

Histology

Biopsies were fixed in 4% neutral buffered formaldehyde for 24 h. After fixation, specimens were dehydrated and embedded in Histo-Comp® (Vogel, Giessen, Germany) using an automated embedding equipment (Tissue Tek V.I.P. 2000, Miles Scientific, Mishawaka; IN). Sections of 5 µm were cut and placed on poly-L-lysine (Sigma, Vienna, Austria) or TESPA (3-aminopropyldiethoxysilane, Sigma) coated glass slides. H&E stained sections of biopsy samples were classified based on histopathological alterations according to Kenney and Doig (1986). Out of 39 mares, 19 were grouped as grade I, and I-IIA, showing no or only mild alterations, 7 were classified as IIA-IIB with mild to moderate alterations, 13 were classified as IIB, or IIB-III, having moderate to severe alterations.

Immunohistochemistry

Paraffin sections were rehydrated and peroxidase activity was removed by 0.3% v/v H_2O_2 in methanol. After that, slides were washed in tap water, mounted with PBS in coverplates® and put into an immunostaining center (Sequenza, Shandon). For immunodetection of TG 2, sections were boiled for 3x5 min in citrate buffer (pH 6.0) in a microwave oven. After 5 min washing, slides were treated with 1.5% v/v normal goat serum for 20 min at room temperature to minimize non-specific binding of the first antibody. Incubation with the first antibody was done overnight at 4°C. Antibodies used were monoclonal anti-MMP-2 (Ab4, Labvision, Neomarkers, USA), dilution 1:400, and monoclonal anti-tissue transglutaminase, dilution 1:500 (CUB 7402, Labvision, Neomarkers). The next day,

slides were washed in PBS and incubated with anti-mouse immunoglobulins conjugated to peroxidase labeled dextran polymer (EnVision+™, DAKO, Glostrup, DK) for 30min at room temperature. Finally, sections were washed with PBS and peroxidase activity detected with DAB substratum (10 mg 3,3'-diamino-benzidine in 50 ml 0.1M Tris buffer, pH 7.4) and 0.03% v/v H_2O_2 for 10 min at room temperature. After that, sections were counterstained with hemalumn, dehydrated, and mounted with DPX (Fluka, Buchs, CH). Control stains were performed by replacing the primary antibody or the secondary antibody with PBS.

Double labeling

To determine if MMP-2 and TG 2 are colocalized, double labeling experiments were performed on exemplary serial sections found positive for MMP-2 and TG 2 after single immunolabeling. These sections were dehydrated and incubated with 1.5% normal goat serum to minimize unspecific binding. After that, the first primary antibody was applied for 2h at room temperature in a twofold concentration compared to single labeling (MMP-2, 1:200; TG 2, 1:250). Subsequently, the slides were washed and incubated with a fluorescent anti-mouse secondary antibody (Alexa488, Molecular Probes, 1:50) for 45 min at room temperature. Next, the proteinblock and the second primary antibody were used as described above. This time the secondary antibody was conjugated to Texas Red (Vector Laboratories, Burlingame, CA, USA, dilution 1:50). After washing in PBS, slides were mounted with Mowiol (Polysciences, USA)/n-propylgallate (Sigma) anti-fading medium. Evaluation was performed in a Leica TCS-NT confocal laser scanning microscope with an argon/krypton laser.

Statistical analysis

Enzyme expression in serial sections (single immunostaining) was scored semiquantitatively. Staining intensity was classified from negative to weak, moderate and strongly positive. Numbers of fibrosed glands surrounded by immunoreactive cells per section (few: 1, moderate: 2-5, many: more than 5) and number of layers in periglandular fibrotic cells (few: 1, moderate: 2 to 5, many: more than 5) were scored. Statistical comparisons were made with the SPSS/PC+ statistics package (Norusis, 1988). The influence of the reproductive stage and endometrosis category on the different parameters evaluated was tested by Kruskal-Wallis-Test. Correlations between different parameters were tested with the statistics procedure "correlations". A p value <0.05 was considered to be significant.

Detection of transglutaminase activity in situ

The procedure was performed as described in Schittny et al. (1997) on exemplary samples with and without severe periglandular fibrosis. Uterine biopsy samples were embedded in OCT compound (Sakura Finetek, Zoeterwoude, NL), shock frozen in liquid nitrogen and serial sections were cut. Unfixed cryosections were rehydrated in PBS and preincubated with 1% BSA in 0.1M Tris/HCl (pH8.3) for 30min at room temperature. Subsequently, sections were incubated in a solution of 0.1M Tris/HCl (pH8.3), 5 mM $CaCl_2$ and 1 mM monodansylcadaverine (Sigma) for 1h. Incorporated dansyl was detected by anti-dansyl antibody (dilution 1:100; Molecular probes, Leiden, NL) for 2 h at room temperature. After washing the sections in PBS, bound antibody was detected with anti-rabbit antibody (Powervision™, Immunovision Technologies, CA, USA). Controls included inhibition with EDTA, and omitting of anti-dansyl or anti-rabbit antibody. All controls gave negative results.

SDS-PAGE - Immunoblotting

Immunoblotting was performed to verify cross-reactivity of the used antibodies with horse tissue. One (for MMP-2) and two (for TG 2) freshly obtained uterine biopsies were processed for electrophoresis. The samples were divided and one part was processed for histology and classified as IIB. A sample of a human placenta was used as positive control for MMP-2, MCF7 cells (a breast cancer cell line) were used as positive control for TG 2. The tissue samples were cut into small pieces, homogenized in low salt extraction buffer supplemented with 1% protease inhibitor solution PMSF (174 mg phenylmethylsulfonylfluorid in 10 ml methanol, Merck, Darmstadt, Germany) and subjected to SDS-gelelectrophoresis under reducing conditions on 12% polyacrylamide gels. SDS-PAGE was performed according to Laemmli (1970) using a Hoefer Mighty Small II electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA). Kaleidoscope prestained standard (Bio-Rad, Hercules, CA, USA) or broad range molecular weight marker (Bio-Rad) was used to identify molecular weights. After separation by SDS-PAGE, the proteins were transferred onto a Hybond™-P membrane (Amersham Life Science, Buckinghamshire, UK) in a semi-dry blotting device (Semi-Phor, Hoefer Scientific Instruments). Immunolabeling was performed with the same concentration of primary antibody and secondary antibody as used for immunohistochemistry. Goat serum (3% v/v in PBS) was used as blocking solution to minimize non-specific binding of primary antibody, DAB was used as substrate (see immunohistochemistry).

Gelatin zymography

Gelatin zymography was performed using 10% zymogram gels containing 0.1% gelatin (Sigma) as substrate (Zhang et al., 2000). Uterine biopsy samples of 6 horses (3 horses diagnosed as group I-IIA and 3 horses diagnosed as IIB-III on earlier taken uterine biopsies)

were homogenized as described above. Protein content of the samples was determined by a Coomassie Brilliant Blue G-250 binding assay (Bradford, 1976), performed in microplate format (microplate reader Sunrise, Tecan, Groedig, Austria) with a BSA calibration curve. Human recombinant MMP-2 (Chemicon, Vienna, Austria) was used as positive control. Equal amounts of samples, normalized to 4 mg/ml total protein concentration, were mixed with sample buffer (Invitrogen, Vienna, Austria) containing 2% SDS, loaded to the gel and run under non-reducing conditions. The molecular weight of the enzymes was determined in relation to standard proteins (broad range marker, Bio-Rad, Vienna, Austria). After electrophoresis, the gels were incubated in renaturing buffer (Invitrogen) for 30 min, followed by 30 min incubation in developing buffer (Invitrogen). After that, the developing buffer was changed and the gels were incubated overnight at 37°C. The next day the gels were stained with 0.5% Coomassie Brilliant Blue R-250 in 15% acetic acid, 25% methanol in distilled water and destained in 15% acetic acid, 25% methanol in water. The proteolytic activity appeared as clear bands, visualizing both the proenzyme and the smaller active form, on a blue background. In order to determine the type of proteases observed on the zymograms, 10 mM EDTA was added to the buffer during the incubation period of a control gel.

Dried gels were scanned with a Sharp JX-330 flatbed scanner connected to a Sun SPARCstation 4 (Sun Microsystems, Mountain View, CA, USA). After inversion of the gel images and background subtraction, band volumes were determined using the software Quantity One version 2.7 (PDI, Huntington Station, NY, USA).

Results

MMP-2

In the normal horse endometrium, a weak to moderate MMP-2 intra- and extracellular expression in the stratum compactum was found in all specimens (Fig. 1). This positive subepithelial layer was variable in thickness. Changes in immunoreactivity of the positive stratum compactum were significantly influenced by the reproductive stage ($p<0.05$), the strongest staining was seen in dioestrus compared to oestrus and anoestrus. Individual uterine glands were surrounded by a thin layer of stromal cells immunoreactive to anti-MMP-2 (Fig. 1). The deeper lamina propria (stratum spongiosum) did not include any MMP-2 positive stromal cells in the normal endometrium. In samples affected by endometrosis stellate cells of the stratum compactum, part of the surface epithelial cells and glandular epithelial cells near the uterine lumen showed moderate to strong immunoreactivity for MMP-2 antibody (Fig. 2). On sites of infiltration with lymphocytes or plasma cells, MMP-2 expression in the lamina propria was strong as well. MMP-2 in the stratum

compactum of the affected endometria was not significantly related to the grade of endometrial alteration. MMP-2 was expressed in large quantities in periglandular fibrotic regions of non-dilated or dilated glands (Figs. 3, 4), also nests of uterine glands were often surrounded by MMP-2 positive cells (Fig. 5). This periglandular MMP-2 expression was significantly correlated with the abundance of dilated and fibrotic uterine glands in endometrotic specimens ($p<0.05$). Moreover, the number of layers of MMP-2-positive cells surrounding fibrotic uterine glands was significantly correlated to the grade of endometrial degeneration ($p<0.05$). Morphologically modest altered glands within degenerated endometria were sometimes surrounded by a single layer of MMP-2 positive stromal cells (Fig. 6).

Endothelia of blood vessels (arteries, veins, capillaries) and lymphatics showed a variable immunoreactivity for anti-MMP-2 in normal and affected endometria. In some of the specimens, all blood vessels showed MMP-2 expression in endothelial cells and in smooth muscle cells of the tunica media. In other specimens, immunoreactivity of blood vessels was restricted to the endothelial cells (Fig. 7). Additionally, small nerve fibres were demonstrated to contain MMP-2 (Fig. 7).

Tissue transglutaminase

Stromal cells in the stratum compactum of the normal endometrium and the endometrium affected by endometrosis were strongly immunoreactive for TG 2 (Fig. 8). Expression of TG 2 in the stratum compactum was neither associated with the grade of endometrial degeneration nor the oestrus cycle. Endothelia of blood vessels and lymphatics were immunoreactive for anti-TG 2 in healthy and affected endometria. Dilated and/or hypertrophic uterine glands, arranged in nests or single branches, were regularly encircled with TG 2-expressing stromal cells (Fig. 9). The grade of endometrial degeneration was significantly correlated with the number of periglandular layers positive for TG 2 ($p<0.05$).

Tissue transglutaminase activity in situ

The TG 2 activity assay on frozen sections resulted in a strong reaction in severe periglandular fibrosis (Fig. 10a,b). Here, predominantly stromal cells were shown to contain active TG 2. In addition, active TG 2 was found in the stratum compactum. A control section treated with EDTA completely inhibited TG 2 reaction (Fig. 10c).

Double labeling

Immunohistochemical double labeling experiments showed that MMP-2 and TG 2 were colocalized in the stratum compactum and in the stroma surrounding altered uterine glands (Fig. 11a,b,c).

Immunoblotting

Western blotting experiments confirmed the cross-reactivity of the used antibodies with horse specimens. SDS-PAGE showed a double band after immunoblotting with MMP-2 (72kD and 62kD) in the human placenta
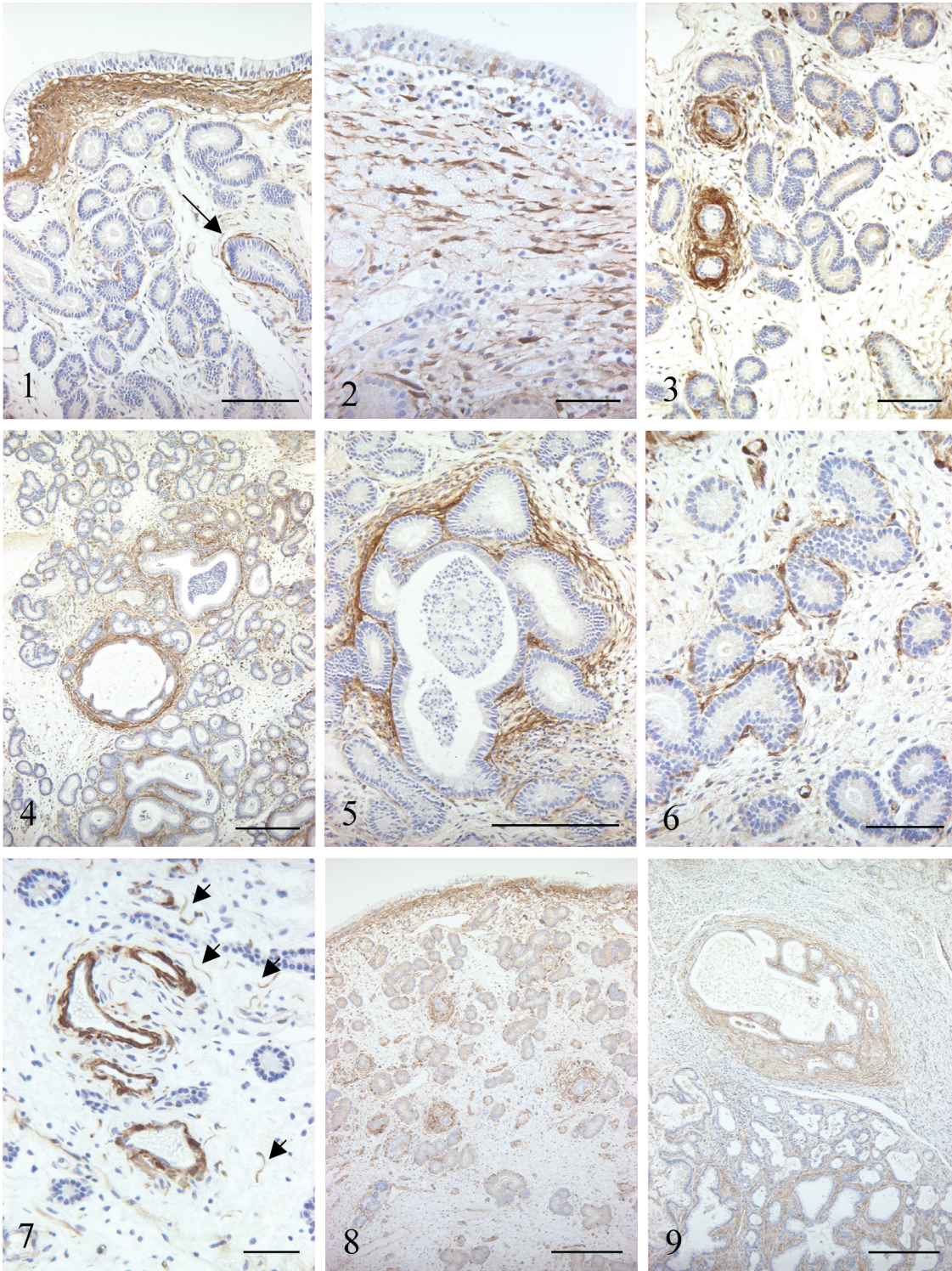
Figs. 1-9. Immunohistochemical demonstration of MMP-2. **Fig. 1.** Distribution of MMP-2 in the stratum compactum of a normal endometrium (dioestrus). Note the periglandular layer around a slightly altered gland, positive for MMP-2 (arrow). Bar: 50 µm. **Fig. 2.** MMP-2 in an endometrium affected by endometrosis. Bar: 25 µm. Periglandular fibrotic regions of undilated **(Fig. 3)**, extensively dilated glands **(Fig. 4)** as well as in glandular nests **(Fig 5)** were strongly marked by MMP-2. (3) Bar: 50 µm, (4) Bar: 200 µm), (5) Bar: 200 µm). **Fig. 6.** Inconspicuous glands in specimens with endometrosis were also encircled by a thin layer of MMP-2. Bar: 25 µm. **Fig. 7.** Blood vessels and small nerves (arrows) were immunostained for MMP-2. Bar: 25 µm. Immunohisto-chemical detection of TG 2. **Fig. 8.** The stratum compactum and periglandular fibroses were clearly marked by TG 2 immunostaining. **Fig. 9.** TG 2 was observed in periglandular fibrotic regions. Bars: 200 µm.

sample as well as in the horse uterine sample. According to the manufacturer's instructions the latent form as well as the activated form of MMP-2 are recognized by the used monoclonal antibody, suggesting that activated and inactivated forms of the enzyme were present in the examined horse endometrium sample (Fig. 12a). A band at about 77 kD was visualized after immunoblotting for

TG 2 in MCF7 cells and horse endometrial samples (Fig. 12b).

Gelatin zymography

Gelatin zymography (Fig. 12c) showed that latent MMP-2 (molecular weight 72kD) and the activated form
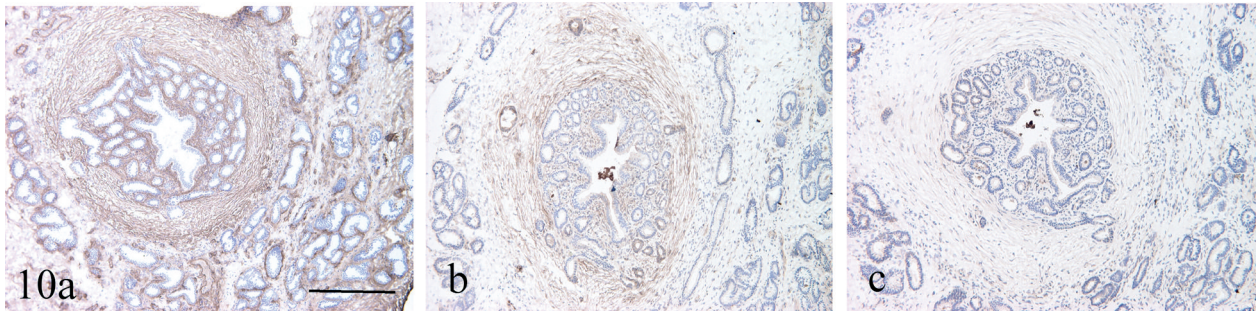

Fig. 10. Transglutaminase activity test on cryosections. Fibrotic regions showed TG 2 protein **(10a)** by immunohistochemistry, as well as TG 2-activity, verified by in situ TG 2 activity test **(10b)**. Inhibition test with EDTA prevented any positive reaction **(10c)**. Bar: 200μm.
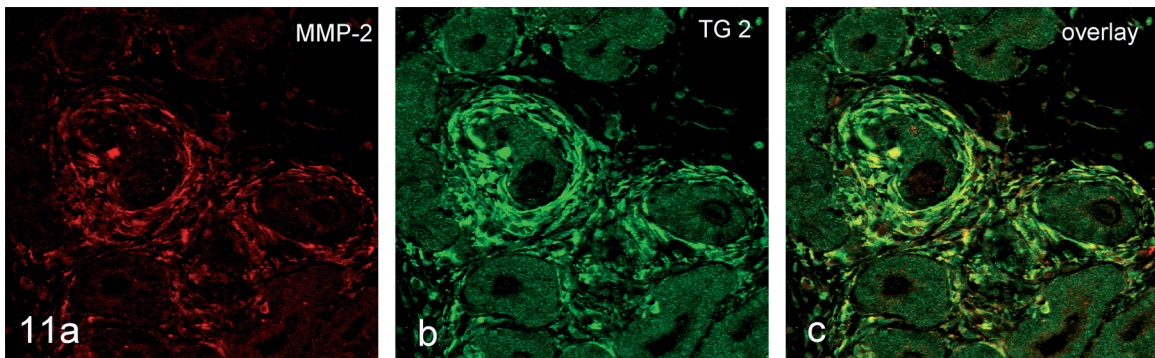

Fig. 11. Confocal laser scanning of simultaneous fluorescent labeling of MMP-2 (**a**, TRITC-channel) and TG 2 (**b**, FITC-channel). **c.** Overlay of both channels. Bar: 50 μm.
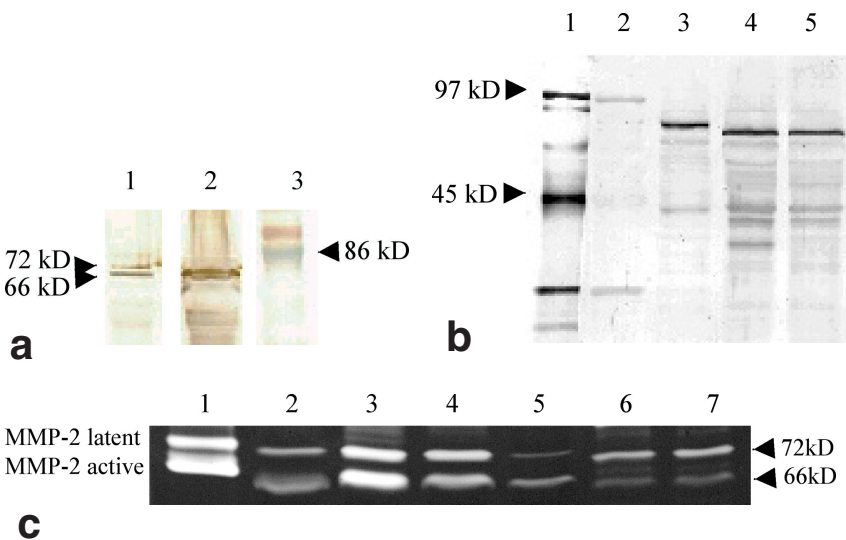

Fig. 12. a. SDS-PAGE and immunoblotting of equine endometrial sample (lane 1) and human placenta (lane 2) with MMP-2 antibody. Molecular weight marker (lane 3). **b.** SDS-PAGE and immunoblotting of equine endometrium samples (lanes 4 and 5) and MCF7-cells as positive control (lane 3). Biotinylated molecular weight standard broad range (lane 1), non-biotinylated molecular weight standard broad range (lane 2). **c.** Gelatinolytic MMP activity detected by zymography. The zymogram shows samples of three normal or mildly altered specimens (lanes 5,6,7) and three specimens severely affected by endometrosis (lanes 2,3,4). Human recombinant MMP-2 was used as marker and positive control (lane1). The band at 72kD represents the latent form of MMP-2, and that at 66kD the active form.

of MMP-2 (62kD) were present in equine uterine biopsy samples of both healthy or only mildly affected and moderate to severely affected endometria. Semiquantitative evaluation of zymography illustrated that overall MMP-2 content in normal or mildly affected endometria was one third compared to severely affected ones. Moreover, healthy specimens contained about 58% active MMP-2, whereas in endometrotic samples values rose to 76%. The presence of 10mM EDTA in the developing buffer completely inhibited enzyme activity on the gelatin gel (not shown).

Discussion

Periglandular differentiation of stromal cells into myofibroblast-like cells associated with the expression of muscle-like cytoskeletal elements (α-SMA, desmin) and deposition of matrix components, is a characteristic feature in horses with endometrosis (Walter et al., 2001). It is known that myofibroblast-like cells are a main source of fibrosis components (Blazejewski et al., 1995). Moreover, these cells have been shown to be the major source of the interstitial collagenases (Iredale et al., 1997) and to express increased amounts of MMP´s (MMP-2, 9) in pulmonary fibrosis (Hayashi et al., 1996) and liver fibrosis (Takahara et al., 1995). In the present study, the fibrotic periglandular regions in horse endometria were found to regularly express the enzymes MMP-2 and TG 2. MMP's are secreted as latent forms and have to be activated extracellularly. Thus, we examined the factual degrading activity of the MMP-2 enzyme. The anti-MMP-2 antibody used in the present study recognizes both the latent and the activated MMP-2 and, therefore, makes it impossible to distinguish the active enzyme from the latent one on tissue sections by immunohistochemistry. By using Western blotting and gelatin zymography techniques we could clearly demonstrate that both forms were present, and moreover, that the active form of MMP-2 was increased in endometria affected by endometrosis. The disadvantage of these electrophoretic methods is that they do not give information on tissue-specific distribution of the enzyme forms and require the use of fresh tissue. A combination of several methods is strongly recommended. However, the size of a diagnostic biopsy sample taken from a patient mare is limited, therefore, it is impossible to provide the complete spectrum of methods on one sample. Further studies to determine MMP-2 and TG 2 quantitatively on a larger sample number are desirable.

Immunohistochemical staining for MMP-2 was already seen on modestly altered glands and might therefore reflect the very beginning of endometrotic changes. Areas showing anti-MMP-2 immunoreactivity in altered equine endometria were characterized by co-expression of TG 2, which is known to act as a stabilizer of the ECM. TG 2 can cross-link all major ECM-proteins to form stable ECM. These cross-links (isopeptide bonds, γ-glutamyl-ε-lysine cross-links) are resistant to proteolytic and mechanical damage

(Greenberg et al., 1991). Moreover, transglutaminase cross-linking of collagen is able to increase both the rate and level of its deposition (collagen III, IV and fibronectin) as well as reduce its rate of degradation in vitro thus tipping the balance towards collagen accumulation (Skill et al., 2004). MMP-2 is capable of degrading collagen IV, fibronectin and laminin, which are abundant in endometrial periglandular fibrosis. Recently, it has been shown that a functional collaboration between TG 2 and MMP-2 exists in a way that MMP-2 together with MT1-MMP-2 hydrolyzes cell surface associated TG 2. TG 2 in return associates with the activation intermediate of MMP-2 and decreases the rate of MMP-2 maturation. This leads to a protection of TG 2 against proteolysis by MMP-2 (Belkin et al., 2004). However, in the case of endometrosis the temporal pattern of development is still unknown, therefore, several possibilities of fibrosis progression are conceivable: TG 2-mediated cross-linking in fibrotic areas might be a reaction to prevent ECM degradation by present high amounts of activated MMP-2. However, it might also be true that the MMP-2 expression in the fibrotic areas is a futile attempt to break down the overwhelming matrix deposits. Moreover, the question of which cells are responsible for MMP-2 and TG 2 production and the ways of their activation or inhibition warrant further investigations.

The abundance of TG 2 in the stratum compactum indicates a highly cross-linked matrix in this endometrial region throughout the oestrous cycle. This might prevent oedematization of the connective tissue as seen in the stratum spongiosum during oestrus. MMP-2 expression in the stratum compactum was most abundant in dioestrus, leading to the assumption of a cycle-dependent regulation of this protein. This hypothesis is supported by reports demonstrating the regulation of MMP's by steroid hormones (Rodgers et al., 1994; Osteen et al., 1999; Park et al., 2001). *In vitro* experiments with human endometrial cells and endometrial cancer cells showed that progesterone inhibits the activation of proMMP-2 via the action of the hormone on expression of MT-MMP's (Marbaix et al., 1992; Zhang et al., 2000; Di Nezza et al., 2003). Salamonsen et al. (1997) found increased production of MMP-1, -2, -3, and -9 of human endometrial stromal cells after withdrawal of progestin *in vitro*. However, they suggested that progestin withdrawal is unlikely to be the only factor responsible for *in vivo* induction of MMP's. The control and regulation mechanisms of MMP-2 in the horse endometrium remain to be elucidated.

The presence of MMP-2 in endothelia of blood vessels in horse specimens was highly variable. Variable MMP-2 expression in blood vessels has also been reported for the human endometrium (Freitas et al., 1999; Zhang et al., 2000). The function of MMP-2 in vascular structures is poorly understood, a relationship to vascular growth and angiogenesis has been suggested. Moreover, a further investigation of MMP-distribution in

degenerative angiopathies which are common in equine endometria (Grüninger et al., 1998; Schoon et al., 1999; Aupperle et al., 2003) would be desirable.

In conclusion, our results show significant MMP-2 and TG 2 abundance in endometrotic specimens of the mare which are supposed to reflect multiple complex changes that occur in ECM homeostasis in periglandular fibrotic regions.

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