Association of endometriosis in horses with differentiation of periglandular myofibroblasts and changes of extracellular matrix proteins

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Periglandular fibrosis and cystic dilation of uterine glands are associated with equine endometriosis. The presence of extracellular matrix proteins (collagen type I, III and IV, laminin and fibronectin) in healthy and endometrotic specimens was demonstrated by immunohistochemistry. The distribution of collagen I, but not collagen III, was dependent on the stage of the oestrous cycle. The arrangement of collagen I and collagen III in endometrotic specimens was similar to that in normal endometrium. In periglandular fibrosis, collagen IV, laminin and fibronectin deposition outside the basement membrane was observed. In these regions, stromal cells were characterized immuno-

histochemically as myofibroblasts because of their expression of α -smooth muscle actin, and occasionally tropomyosin and desmin. Periglandular differentiation of contractile cells could be interpreted as a reaction to support the extrusion of secretions in cystic dilated glands. Moreover, the changes of extracellular matrix proteins are characteristic for neoplastic lesions, although further development of endometriosis to benign or malignant tumours is not known in horses. Knowledge of the factors responsible for these fibroblastic modulations may be the key to explaining the pathogenesis of endometriosis.

Introduction

Endometriosis, one of the most frequent reasons for infertility in horses, is a chronic degenerative endometrial disease involving severe alterations in the uterine connective tissue and glands. Kenney and Doig (1986) introduced a classification system to discern four groups, from animals with normal endometrium to animals with severe pathological alterations (I, IIA, IIB and III), which is still in use. Prognosis for successful breeding is detrimental for horses classified as categories IIB and III. However, endometriosis can only be diagnosed by histological examination of a uterine biopsy sample. Histological changes associated with endometriosis include formation of fibrotic and dilated glands or glandular nests. The affected glands are reported to differentiate irregularly during the oestrous cycle (Schoon *et al.*, 1997) and it has been shown by lectin histochemistry that uterine secretions are also altered (Walter *et al.*, in press).

Although the pathogenesis of this disease is still unknown, it has been established that the extent of these alterations is associated with age of the affected animal (Schoon *et al.*, 1997). It is likely that the equine endometrium undergoes cyclic remodelling of the connective tissue

extracellular matrix (ECM) as observed in cows (Kaidi *et al.*, 1991; Boos, 1994) and other species, including humans (Aplin *et al.*, 1988; Stenback, 1989; Goranova *et al.*, 1993). There are no data on the presence and distribution of different types of collagen in the uterus of healthy mares and in those affected by endometriosis. However, various studies on cell–ECM interactions have revealed that the ECM is a source for signals that are capable of influencing growth and differentiation of different types of cell (Bissell *et al.*, 1982). Fibroblastic cells play an essential role in the synthesis and regulation of extracellular connective tissue components and are involved in the pathogenesis of fibrotic processes. During pathogenesis, fibroblastic cells express a range of muscle differentiation factors, such as α -smooth muscle actin, tropomyosin and desmin, and were, therefore, identified as myofibroblasts (Sappino *et al.*, 1990; Schmitt-Gräf *et al.*, 1994). Myofibroblasts express various ECM proteins such as fibronectin, laminin, collagen type IV and tenascin (Berndt *et al.*, 1994; Hanamura *et al.*, 1997). It has been suggested that these ECM proteins could be important for the remodelling of neoplastic tissues, as many studies have demonstrated overexpression of fibronectin in carcinomas (Koukoulis *et al.*, 1993; Pujuguet *et al.*, 1996).

The aim of the present study was to elucidate the presence and distribution of ECM proteins (collagen type I,

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Table 1. Primary antibodies used in immunohistochemistry

Antibody	Type of antibody	Dilution	Total concentration of protein ($\mu\text{g ml}^{-1}$)	Source
Anti-collagen I	Monoclonal	1:500	NI	Sigma
Anti-collagen III	Monoclonal	1:25	40.0	Quartett
Anti-collagen IV	Monoclonal	1:25	NI	Quartett
Anti-laminin	Polyclonal	1:500	8.4	Dako
Anti-fibronectin	Affinity isolated	1:1000	0.5	Sigma
Anti- α -smooth muscle actin	Monoclonal, clone 1A4	1:200	39.0	Dako
Anti-tropomyosin	Monoclonal, clone TM311	1:4000	6.0	Sigma
Anti-desmin	Monoclonal, clone DE-U10	1:100	233.0	Sigma

NI: not indicated by manufacturer.

III and IV, laminin and fibronectin) in the equine endometrium during the oestrous cycle by immunohistochemical methods in both healthy and affected animals. In addition, morphological and histochemical characteristics of fibroblastic cells surrounding altered (fibrotic and dilated) uterine glands were examined. Further immunohistochemical characterization of periglandular fibroblastic differentiation was accomplished by using antibodies against α -smooth muscle actin, tropomyosin and desmin.

Materials and Methods

Animals

Uterine biopsy samples were collected from 50 mares (5–23 years of age) of various breeds (Haflinger: $n = 8$; Warmblood: $n = 20$; Trotters: $n = 4$; Thoroughbred: $n = 4$; Noriker: $n = 14$) at different stages of the oestrous cycle. Tissue (one biopsy per animal) was obtained transcervically from the proximal region of the right uterine horn using routine techniques. The stage of the oestrous cycle was determined clinically (transrectal palpation, ultrasonography, control of oestrous behaviour with a stallion) and by determination of plasma concentrations of oestrogen and progesterone according to Behrens *et al.* (1993). The reproductive stages in the animals examined were as follows: seasonal anoestrus, $n = 12$; oestrus, $n = 14$; and dioestrus $n = 24$.

Histology

Biopsy samples were either snap frozen in liquid nitrogen (cryosections) or fixed in 4% (v/v) neutral formaldehyde for 24 h at room temperature (paraffin wax sections). After fixation, specimens were dehydrated and embedded in Paraplast® using automated embedding equipment (Tissue Tek V.I.P. 2000, Miles Scientific, Mishawaka). Serial sections were cut at 4 μm , placed on poly-L-lysine-coated glass slides and dried at 53°C overnight. Cryosections were cut at 8 μm , dried onto glass

slides coated with TESPA (3-aminopropyltriethoxysilane, Sigma Chemicals, Vienna) and fixed in ice-cold acetone for 10 min before use.

Biopsy samples were categorized for the grade of endometriosis according to Kenney and Doig (1986) in haematoxylin and eosin stained sections. Out of 50 mares, $n = 13$ (26%) were classified as endometriosis grade I or I-IIA; $n = 17$ (34%) as IIA; $n = 10$ (20%) as IIA-IIB; and $n = 10$ (20%) as IIB-III.

Immunohistochemistry

Paraffin wax sections were rehydrated and peroxidase activity was inhibited by 0.3% (v/v) H_2O_2 in methanol. The sections were washed in tap water, mounted with PBS in coverplates® and put into an immunostaining centre (Sequenza, Shandon, Pittsburgh). After 5 min of washing, slides were treated with 1.5% (v/v) normal goat serum for 20 min at room temperature before incubation with the primary antibody overnight at 4°C. The antibodies used and their final concentrations are summarized (Table 1). On the subsequent day, slides were washed in PBS and incubated either with a fluorescein-conjugated secondary antibody (Alexa™488, Molecular Probes, Leiden) or EnVision+™ (anti-rabbit or anti-mouse immunoglobulins conjugated to peroxidase-labelled dextran polymer; DAKO, Glostrup) for 30 min at room temperature. In the case of fluorescence detection, slides were counterstained with propidium iodide (2 μl per 50 ml in distilled water for 5 min), washed in distilled water and mounted with Mowiol 4-88 (Hoechst, Frankfurt) *n*-propylgallate (Sigma). In the case of the EnVision™ method, sections were washed with PBS and peroxidase activity was detected using DAB substratum (10 mg 3'3' diaminobenzidine in 50 ml of 0.1 mol Tris buffer l^{-1} , pH 7.4) and 0.03% (v/v) H_2O_2 for 10 min at room temperature. Thereafter, sections were counterstained with haemalum, dehydrated and mounted with DPX (Fluka).

Protease pretreatment (protease from *Streptomyces griseus*, Sigma; 0.1% (w/v) in 0.05 mol Tris buffer l^{-1} , at pH 7.2, for 5 min at 37°C) was necessary for immuno-

histochemical demonstration of collagen IV, laminin and desmin. Pepsin digestion (Sigma; 0.1% (w/v) in 0.5 mol acetic acid I⁻¹) for 2 h at 37°C was accomplished before immunostaining of collagen I and III. Hyaluronidase pretreatment (Sigma; 0.016% (w/v) in PBS, pH 6.7, for 4 h at 37°C) was performed before immunostaining of fibronectin.

Cryosections were used for immunohistochemical demonstration of collagen I. Control sections were treated with PBS instead of primary antibody, or with unspecific mouse or rabbit IgG from normal serum applied in the same protein concentration as the primary antibody. Fluorescein labelled sections were evaluated using a confocal laser scanning microscope (Leica TCS-NT, Heidelberg).

Results

Immunohistochemical demonstration of collagen I, III and IV

The normal equine endometrium contains collagen type I fibres that are distributed uniformly in the lamina propria and the arrangement of these fibres is dependent on the stage of the oestrous cycle. In the proliferative phase, collagen type I fibres were thick and distributed loosely in the connective tissue (Fig. 1a). Fibres in the subepithelial stratum compactum were densely arranged. During the secretory phase, collagen type I fibres were tightly packed in all regions of the lamina propria (Fig. 1b). In contrast to collagen I, distribution of collagen type III fibres was not affected by the stage of the oestrous cycle and a dense network of fibres was constantly present in the endometrial connective tissue (Fig. 1c). The arrangement of collagen I and III in endometrotic tissue was similar to that in normal endometrium, but only single glandular nests with diminished collagen III content were observed (Fig. 1d).

In normal endometrium, collagen IV was restricted to the basement membrane of uterine glands and blood vessels (Fig. 1e). Severe fibrosis around the glandular nests was combined with divergent distribution of collagen IV. The multiple layers of fibrocytes surrounding these glands were observed to deposit collagen IV (Fig. 1f). In addition, cystic dilated glands were surrounded by collagen IV-producing fibrocytes in the case of fibrosis; however, this was not the case for non-fibrotic altered glands. Laminin, another component of the basement membrane, had a similar distribution pattern to that described for collagen IV in normal endometrial tissue (Fig. 1g). In cases of severe fibrosis (category IIB–III), laminin was found outside the basement membrane of uterine glands (Fig. 1h). Distribution of collagen IV and laminin was not dependent on the stage of the oestrous cycle.

Immunohistochemical characterization of stromal cells in endometrotic tissue

Fibroblasts surrounding fibrotic uterine glands frequently showed strong immunoreactivity for anti- α -smooth muscle actin (Fig. 2a–f). Distribution patterns of cells positive for α -smooth muscle actin in endometrotic tissue were extremely

Fig. 1. Immunohistochemical demonstration of collagen type I in the equine endometrium in proliferative (a) and secretory (b) stages of the oestrous cycle. (c) Distribution of collagen type III in normal endometrium. (d) Loss of collagen III fibres was observed in fibrotic glandular nests. (e) Collagen type IV in normal horse endometrium was restricted to the basement membrane of uterine glands and blood vessels. (f) In glands with fibrosis, collagen IV was also deposited by surrounding fibrocytes. (g) Immunohistochemical staining of laminin marked basement membranes in the normal horse endometrium. (h) In addition to the basement membranes, laminin was observed around fibrocytes that were close to altered glands in endometrotic specimens. All sections were counterstained with propidium iodide. Scale bars represent 50 μ m.

Fig. 2. Immunohistochemical staining for α -smooth muscle actin α -SMA in the equine endometrium. Note the variable distribution of α -SMA positive cells: (a) in thick periglandular sheaths around fibrotic glands, (b) limited to the inner layer of fibrosis, (c) in the stratum compactum, (d,e) distinct immunolabelling of fibrotic, cystic glands, or (f) a single layer of cells around histologically inconspicuous uterine glands. Fibronectin expression was demonstrated immunohistochemically around fibrotic uterine glands in endometrotic specimens (g) and in the normal equine endometrium (h). Tropomyosin (i) and desmin (j) were observed in some cases in fibrotic regions. Scale bars represent (a,c,e,f,g,h,i) 100 μ m, (b,j) 50 μ m, (d) 200 μ m.

variable: single uterine glands or gland branches that were highly fibrotic, but not dilated, were cuffed by a thick layer of circularly arranged fibroblasts expressing α -smooth muscle actin (Fig. 2a). In addition, cystic dilated glands were coated by a distinct layer of cells positive for α -smooth muscle actin (Fig. 2b,d,e). In some cases of thick fibrotic periglandular sheath, only the layer next to the glandular epithelium was positive for α -smooth muscle actin (Fig. 2b). In mild cases of endometriosis (category IIA), single cells positive for α -smooth muscle actin were arranged closely around the glandular epithelium of histologically incon-

spicuous (non-fibrotic, not dilated) glands (Fig. 2f). In single specimens, fibrocytes of the stratum compactum were also shown to express α -smooth muscle actin (Fig. 2c), a characteristic not correlated with a particular stage of the oestrous cycle. The deeper connective tissue in the normal endometrium as well as that in endometrotic specimens was free of cells positive for α -smooth muscle actin. Smooth muscle cells of blood vessels and the myometrium were positive.

Fibronectin staining was weak although distributed evenly throughout the uterine stroma. Basement membranes

and endothelia of blood vessels showed intense immunostaining in normal endometrial specimens (Fig. 2h). Highly fibrotic uterine glands were encircled with layers of stromal cells positive for fibronectin (Fig. 2g).

In endometrotic tissue, periglandular fibroblasts expressing α -smooth muscle actin were often also immunoreactive for tropomyosin (Fig. 2i), but only a few of the fibroblasts showed co-expression of smooth muscle-specific desmin (Fig. 2j).

Discussion

In the present study, alterations of stromal cells in the uterine lamina propria were compared between horses with endometrosis and healthy horses. Distribution of ECM proteins (collagen type I, III and IV, fibronectin and laminin) was examined in uterine biopsy samples. There are no reports concerning the distribution of different types of collagen during the oestrous cycle in the normal horse endometrium. Therefore, the present study is the first to demonstrate physiological formation and distribution of collagen fibres in the normal equine endometrium. Immunohistochemical staining demonstrated that the distribution of collagen type I was dependent on the stage of the oestrous cycle, as also observed in cows (Boos, 1994). During the proliferative phase, collagen type I fibres were distributed loosely in the connective tissue due to the excessive oedema present at this stage of the oestrous cycle. At the same time, collagen fibres remained densely arranged in the subepithelial stratum compactum, indicating smaller amounts of interstitial fluid in the subepithelial region. During the secretory phase, collagen type I fibres were densely packed in the whole lamina propria due to the diminished oedema during dioestrus. Fibrillar collagen types I and III, which are the major components of the stromal uterine tissue, did not increase in fibrotic areas in mares that had endometrosis. These findings are in contrast to those of Evans *et al.* (1998), who observed that endometrosis in horses was correlated with an increase in endometrial periglandular collagen volume fraction, as demonstrated by picrosirius red staining. These contradictory results may be due to the different staining methods used. Blanchard *et al.* (1987) referred to the limited usefulness of a collagen-specific stain (Masson's trichrome) to enhance visualization of endometrial stromal collagen.

In the present study, immunohistochemistry revealed that collagen IV, normally restricted to the basement membrane, was also present around stromal cells in periglandular fibrosis of single glands and glandular nests, a characteristic of endometrosis. A similar distribution pattern was observed after immunohistochemical staining for laminin, but only in severe cases of fibrosis. Periglandularly arranged fibroblasts in endometrotic specimens were further characterized so that these deposits of ECM could be interpreted. Immunohistochemical staining elucidated that these mesenchymal cells expressed α -smooth muscle actin indicating myofibroblastic differentiation. Evans *et al.*

(1998) showed myofibroblast features of periglandular cells in endometrotic tissue by transmission electron microscopy. Myofibroblasts are known to build up an incomplete layer of basal lamina on their cell surface, which explains the presence of collagen IV and laminin in these regions. These components of the basal lamina are known to maintain smooth muscle cells in a differentiated stage (Schmitt-Gräf *et al.*, 1994). In addition, these cells were frequently found to be positive for tropomyosin and occasionally they showed co-expression of desmin, indicating a smooth muscle cell-like differentiation and contractile potential of these periglandularly arranged cells. Differentiation of periglandular cells to myofibroblasts was also reported for fibrotic dilated glands of the human endometrium (Czernobilsky *et al.*, 1993) leading to a comparable histopathology. It is well known that local stimuli induce smooth muscle differentiation in resident fibroblasts. These stimuli can be produced by neighbouring epithelial or mesenchymal cells (Schmitt-Gräf *et al.*, 1994). However, in the case of equine endometrosis, further investigations are necessary to identify the signals and emitting cells that are responsible for myofibroblast differentiation (and for periglandular fibrosis). Czernobilsky *et al.* (1993) interpreted periglandular tissue remodelling as a reaction to support the hindered extrusion of secretions. In endometrotic specimens, cystic dilated glands are frequently filled with secretions and cellular debris; therefore, the hindered extrusion of secretions may be the primary reason for their dilation. However, glands that were not dilated, but were highly fibrotic, were frequently observed in endometrotic specimens. These glands were surrounded by cells positive for α -smooth muscle actin, although the function of these cells remains unclear. Immunostaining for α -smooth muscle actin was also positive around glands that did not appear to be fibrotic by histological examination. This finding could be helpful for the characterization of biopsy samples in a very early stage of endometrosis or to determine horses with a predisposition to the disease.

In highly fibrotic regions of the horse endometrium, fibronectin was present indicating that it has been deposited by stromal cells expressing α -smooth muscle actin. This finding supports observations in the present study of deposition of basement membrane components by periglandular stromal cells, as fibronectin is known to be an extrinsic component of the basement membrane produced by connective tissue cells (Flug and Köpf-Meier, 1995). Weak reactivity for anti-fibronectin was frequently present in the stroma and in the blood vessels of the normal equine endometrium which is comparable to data from baboons (Fazleabas *et al.*, 1997). α -Smooth muscle actin has also been found in the stroma of benign and malignant epithelial tumours and, therefore, is considered to be a marker for neoplastic lesions (Sappino *et al.*, 1988). Pujuguet *et al.* (1996) showed that fibronectin is deposited by cancer cells and tumour-associated myofibroblasts. Although further development of endometrosis to benign or malignant

tumours is not known in horses, it is assumed that periglandular stromal cells are highly active in cases of fibrosis.

In the present study, it was demonstrated that periglandular fibrosis in endometrotic tissue is not characterized by an increased incidence of collagen fibres, but by periglandularly arranged fibroblasts in one or more layers, which produce the ECM proteins collagen IV, laminin and fibronectin. In addition, these fibroblasts express α -smooth muscle actin, tropomyosin and occasionally desmin, which confirms their differentiation into smooth muscle-like cells. It remains to be elucidated whether the differentiation of fibroblasts into myofibroblasts has further consequences on the uterine gland epithelium or the stromal cells, as has been shown for various pathological processes. Myofibroblasts are able to produce cytokines, for example interleukins (Zhang *et al.*, 1996); therefore, these cells could be involved in the stimulation of ECM production and degradation which are known to be cytokine-controlled. The immunohistochemical identification of cells positive for α -smooth muscle actin might help to evaluate preneoplastic lesions. Further characterization of mechanisms responsible for the observed fibroblastic modulations may be the key to exploring the pathological mechanisms leading to equine endometrosis.

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