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Collagenolytic Activity and its Sensitivity to Doxycycline Inhibition in Tracheal Aspirates of Horses with Chronic Obstructive Pulmonary Disease

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Koivunen, A.-L., P. Maisi, Y.T. Konttinen, K. Prikk and M. Sandholm: Collagenolytic activity and its sensitivity to doxicycline inhibition in tracheal aspirates of horses with chronic obstructive pulmonary disease. Acta vet. scand. 1997, 38, 9-16. – The collagenolytic activity and its sensitivity to doxycycline inhibition in tracheal aspirates (TA) of horses with chronic obstructive pulmonary disease (COPD) was analyzed with SDS-PA gel electrophoresis (SDS-PAGE), using Type I collagen as the substrate. Both autoactive and total collagenase activities were significantly higher in TAs of horses with symptomatic COPD than in TAs of healthy horses. Doxycycline inhibition studies suggest that most of the TA collagenase is of the neutrophil type (MMP-8), but some is derived from other cells such as fibroblasts and monocyte/macrophages (MMP-1) and bacteria (bacterial collagenases). Drugs inhibiting collagenases in the respiratory tract might be worth a trial in the treatment of COPD in horses.

proteinase; proteolysis; metalloproteinase; MMP; activity; tetracycline; equine; lung; COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is common in horses exposed to dust from hay and bedding. Respiratory tract diseases have been reported to be the second most common cause of poor racing performance and premature retirement among racehorses *(Rossdale et al.* 1985) and excess mucus in the trachea has been shown to correlate with poor racing performance *(MacNamara et al.* 1990).

Horses with COPD have elevated levels of proteolytic activity in their respiratory tract secretions *(Grünig et al.* 1985, *Koivunen et al.* 1996). The serine proteinases, such as elastase, do not seem to have an important role in the respiratory secretions of the horse *(Grünig et al.* 1985, *Koivunen et al.* 1996). Increased levels of metalloproteinases, such as interstitial collagenases, have been detected in human lung diseases *(Campbell et al.* 1987, *Sepper et al.* 1995) and may be involved in the pathogenesis of equine respiratory diseases as well.

The interstitial type I collagen, together with other interstitial collagen types II, III and V, provides the structural basis of the extracellular matrix in the lung. The interstitial collagens are highly susceptible to the specific cleavage action of collagenases belonging to the matrix metalloproteinase (MMP) family. There are 2 genetically distinct types of interstitial collagenases, namely fibroblast collagenase MMP-1 and neutrophil collagenase MMP-8. Both cleave the native type I, II and III collagens to ¾

and ¼ cleavage products *(Campbell et al.* 1987). Fibroblasts *(Goldberg et al.* 1986), monocytes and macrophages *(Shapiro et al.* 1991, *Werb & Gordon* 1975), as well as endothelial cells *(Moscatelli & Rifkin* 1988) are cellular sources of pro-MMP-1. A latent proenzyme form of MMP-8 is released from neutrophils *(Woessner* 1991). Activation of cells enhances the synthesis and/or the release of collagenases *(Galis et al.* 1995). Both pro-MMP-1 and pro-MMP-8 can be activated in vitro by various proteolytic enzymes, reactive oxygen species, organomercurials and gold compounds *(Saari 1990, Woessner* 1991, *Grant et al.* 1992). In vivo activation probably takes place by proteolysis or by oxidation *(Mainardi et al.* 1991, *Sorsa et al.* 1992). The interstitial collagens can also be cleaved by bacterial collagenolytic proteinases, which act at multiple cleavage sites in a non-specific manner *(Sorsa et al.* 1992).

Tetracyclines, independently of their antimicrobial efficacy, directly inhibit human interstitial collagenases and other MMPs *(Golub et al.* 1992) as well as macrophage elastases *(Golub et al.* 1991). Tetracyclines can also prevent the oxidative activation of the latent procollagenases *(Lauhio et al.* 1992). The mammalian neutrophil derived MMP-8 is significantly more susceptible to tetracycline inhibition than MMP-1 *(Suomalainen et al.* 1992) (MMP-8: IC_{50} for doxycycline is 26 μM; and MMP-1: IC_{50} for doxycycline is 280 μM). On the other hand, different tetracycline compounds differ in their capacity to inhibit MMPs *(Golub et al.* 1992, *Suomalainen et al.* 1992).

The present study was undertaken to find out whether the increased proteolytic activity in the respiratory secretions from horses with COPD could be partly explained by elevated levels of interstitial collagenases. The sensitivity of equine interstitial collagenases to tetracycline inhibition in vitro was explored in order to determine the cellular origin of interstitial collagenases in the respiratory tract, as well as to test the potential for pharmacological intervention to decrease the proteolytic activity within the inflamed respiratory tract.

Materials and methods

Horses and sampling of the tracheal mucus

Tracheal aspirates (TA) (n = 30) were obtained from 3 categories: Horses (age 11.8 ± 4.3) with a healthy respiratory tract (10 samples), horses (age 10.4 ± 3.8) with intermittent signs of COPD in symptomatic disease stage (10 samples) and horses (age 12.4 ± 3.1) with permanent signs of COPD (10 samples). The clinical evaluation and sampling of the tracheal mucus was undertaken as reported previously *(Koivunen et al.* 1997). The tracheal aspirates were stored at –20 °C for further studies.

Correction for the dilution effect of the tracheal aspirate

To analyze the dilution effect due to tracheal wash, the urea concentration was analyzed in parallel from the blood serum and the tracheal wash using the method of *Guttmann & Bergmeyer* (1974). The dilution effect was calculated according to *Rennard et al.* (1986).

This dilution coefficient was used to adjust each TA sample with TNC-buffer (0.05 M Tris-HCl, 0.005 M $CaCl_2$, 0.2 M $NaCl_2$, pH 7.5) to an equal final dilution before analysis in SDS-PAGE.

Preparation of the samples for SDS-PAGE

The TA samples were centrifuged for 4 min at 170 G (Biofuge A, Heraeus, Sepatech, Germany) and the supernatant was collected.

Assay for interstitial collagenase activity

Type I collagen was purified from rat tail as described in detail elsewhere *(Miller & Rhodes* 1982, *Konttinen et al.* 1994). TA samples were

pretreated with or without 1 mM phenylmercuric chloride (PMC), an organomercurial activator of latent interstitial collagenases, for the measurement of the total and in vivo activated collagenases, respectively. Type I collagen, the plain TNC-buffer and the TNC with PMC were used as controls. Measurement of collagenase activity has been described in detail elsewhere *(Turto et al.* 1977). TA samples were incubated for 44 h with 1.5 μM triple helical Type I collagen monomers, the enzyme reaction was stopped and the reaction products were separated from collagen I by SDS-PAGE electrophoresis.

Susceptibility of interstitial collagenases to doxycycline-inhibition

The 7 TA samples (from periodically/permanently diseased horses) that showed at least 30% degradation of collagen after PMC-activation were selected for the doxycycline inhibition test. Before incubation with tetracyclin the original samples were first treated with 1 mM PMC to ensure the activation of the latent interstitial collagenases. After activation the samples were incubated with 100 μM and 600 μM doxycycline (Sigma D-9891) at +37 °C for one h after which 1.5 μM Type I collagen was added for 44 h. The assay for collagenase activity was performed using 10% SDS-PAGE as described above.

Analysis of the collagenolytic activity from the SDS-PA gels

Intact Type I collagen α_1 and α_2 chains and the ¾ α_{1A} and α_{2A} collagen degradation products were visualized as 4 blue bands against a clear background (Fig. 1). The 2 upper bands represent the undegraded Type I α_1 and α_2 collagen chains and the 2 lower bands their ¾ cleavage fragments, α_{1A} and α_{2A}; the ¼ cleavage fragments α_{1B} and α_{2B} have been run to the front of the gel for better separation *(Turto et al.* 1977; Fig. 1). The scanning of the bands was performed using a semi-automatic Kontron image analysis and processing system (Kontron Bildanalyse GMBH, Eching, Germany) equipped with a VIDAS 2.1 programme (Kontron Elektronik GMBH, Eching, Germany) as described by *Koivunen et al.* (1997). The background grey level of the gel was subtracted from the mean grey values of the electrophoretic bands, and the densitometric results were calculated using the area mode.

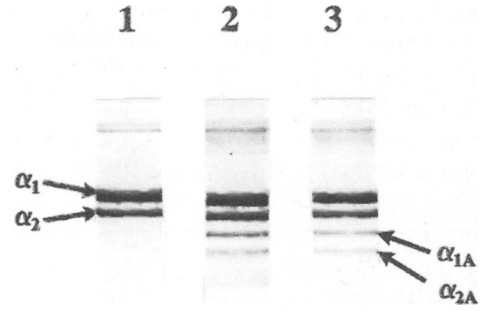

Figure 1. SDS-PAGE. Lane 1: Type I collagen treated with tracheal aspirate (TA) from a healthy horse. Lane 2: Type I collagen treated with TA from an intermittently diseased horse in its symptomatic stage. Lane 3: Type I collagen treated with TA from a horse with permanent COPD.

The value representing ¾-cleavage fragments (αA chains) was multiplied by ⅓ to obtain the total quantity of degradation products. The proportion of the degradation products of the total Type I collagen was calculated (in percentage) as a measure of collagenase activity in the sample.

Statistical analysis

Mann-Whitney test (Statgraphics® (version 6.0), Manugistics, Rockville, Maryland, USA) was used to test the differences in the collagenolytic activity between healthy and diseased horses (Table 1). Differences were considered as significant if $p<0.05$.

Table 1. The total (PMC +) and auto-active (PMC –) collagenolytic activity (average ± SD / median, upper and lower quartile; in percentage) in tracheal aspirates of healthy, periodically diseased (in their symptomatic stage), and permanently diseased horses (COPD) and the proportion of endogenously active form from total collagenase activity in corresponding horses (PMC+ / PMC–; in percentage).

PMC	Healthy controls	Periodically diseased, (COPD) symptomatic stage	Permanently diseased (COPD)
		*	*
PMC +	8.2 ± 8.8 6.5 (0-16.2)	26.9 ± 19.9 28.2 (7.1-44.6)	26.2 ± 21.6 21.2 (12.9-31.5)
		*	n.s.
PMC –	4.3 ± 5.7 0 (0-11.5)	21.5 ± 18.8 16.3 (7.7-56.3)	9.3 ± 8.4 11.4 (0-16.6)
PMC+ / PMC –	65%	80%	35%

* = $p<0.05$, compared to values of the healthy controls.
PMC = phenylmercuric chloride, activator of latent interstitial collagenases.

Results

Horses with a healthy respiratory tract showed significantly lower values for autoactive (PMC –) collagenase activity as well as for total (PMC +) collagenase activity (Table 1, Fig. 1). Respiratory secretions from periodically diseased horses at their symptomatic stage and those from permanently diseased horses did not differ significantly as for total collagenase activity. Secretions from the respiratory tract of the periodically diseased horses at their symptomatic stage had significantly higher activity of autoactive collagenase compared to healthy and to permanently diseased horses. Autoactive collagenase of the permanently diseased horses did not differ significantly from the healthy controls.

Proportions of autoactive collagenase and total collagenase were compared. 65% of the total collagenase activity was in endogenously active form in the respiratory secretions of the healthy controls. Respiratory secretions from the periodically diseased horses at their symptomatic stage had the highest proportion of autoactive collagenase and permanently diseased horses the lowest (Table 1).

Doxycycline was able to inhibit total collagenase activity in the tracheal fluid in vitro (Fig. 2): 100 μM doxycycline had ability to inhibit 59% ± 26%, and 600 μM 87% ± 22% (average ± sd) of total collagenolytic activity found in the TAs of the COPD horses.

Discussion

In the present study, increased activities of the interstitial collagenases as well as increased total concentrations of collagenases were detected in the tracheal fluid of the COPD horses. This activity together with the increase of the activities of other MMPs (gelatinases) *(Koivunen et al.* 1997) seem to be partly responsible for the increased proteolytic activity found in respiratory secretions of COPD horses.

The degradation of the extracellular matrix by neutral proteinases, including metalloproteinases (MMPs), is believed to be an important component of physiologic processes such as

growth and tissue remodelling. In addition to the maintenance of normal tissue function, the process of uncontrolled matrix destruction has received attention in the study of pathologic states, including rheumatoid arthritis, blistering skin diseases, emphysema, tumour metastasis, cirrhosis, and pulmonary fibrosis *(Mainardi et al.* 1991). Collagen types I, II and III, which together with other interstitial collagens provide the structural basis of the extracellular matrix, can be degraded by only 2 mammalian enzymes, namely interstitial collagenases of the fibroblast and neutrophil type (MMP-1 and MMP-8) *(Mainardi et al.* 1991, *Woessner* 1991). The increased activity of these collagenases during inflammation evidently results from increased synthesis as well as activation of the latent procollagenases *(Weiss* 1989, *Mainardi et al.* 1991).

When respiratory secretions from the periodically diseased horses at their symptomatic stage were compared to those from the permanently diseased horses, the total collagenolytic activity (PMC +) was similar, but the periodically diseased horses had the collagenolytic enzymes in endogenously active (PMC –) form, while in the permanently diseased horses collagenase was in the latent procollagenase form (Table 1). This suggests that the disease process in chronically diseased horses is not as active as in periodically diseased horses. Although the proenzymes are present in high concentrations in the permanently diseased horses, activation is necessary before cleavage of the interstitial collagens is possible. The in vivo activation mechanism of MMPs within the respiratory tract remains to be resolved.

In addition to the characteristic ¾ collagen-degradation products, also low molecular weight bands were seen in some of the samples. These bands could be due to bacterial collagenases in the samples *(Sorsa et al.* 1992). This could also explain, why TA collagenases in horses were

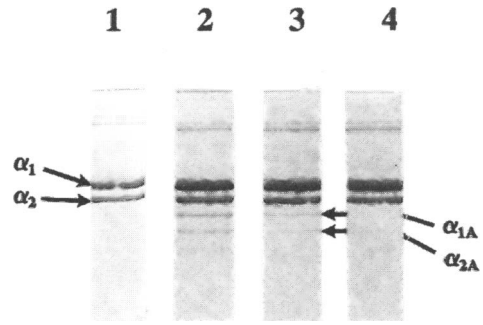

Figure 2. An example of doxycycline inhibition. Lane 1: Type I collagen. Lane 2: Type I collagen treated with a TA sample showing collagenolytic activity without doxycycline. Lane 3: Type I collagen treated with the same sample and 100 μM doxycycline. Lane 4: Type I collagen treated with the same sample and 600 μM doxycycline.

not inhibited to 100% as has been found to be the case in man, when purified MMP-1 and MMP-8 enzyme species, uncontaminated with bacterial collagenases, are used *(Suomalainen et al.* 1992).

If MMPs play a role in pathogenesis of COPD, there could be possibilities to pharmacologically inhibit these enzymes and to protect the lung from proteolytic attack. Tetracyclines have been shown to inhibit interstitial collagenases and other metalloproteinases, independently of the antimicrobial effect *(Golub et al.* 1992). If the dimethyl-amine group is removed from the carbon number 4 of the A ring of the tetracycline, the drug looses its antimicrobial potency, but retains its ability to inhibit collagenases *(Golub et al.* 1992, *Suomalainen et al.* 1992). In humans the proteinase inhibitory effect of tetracyclines has also been proven in vivo *(Lauhio et al.* 1994). Alpha$_1$-proteinase inhibitor is an endogenous inhibitor of serine proteinases and a substrate for interstitial collagenases and other MMPs. Since it can be inactivated, either oxidatively or proteolytically, through MMP action, it may be possible that tetracyclines can prevent

general proteolytic events – in addition to preventing specific collagenases – by maintaining the α_1-proteinase inhibitor shield *(Sorsa et al.* 1993).

The natural inhibitors of MMPs, α_2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs), inhibit fibroblast collagenase (MMP-1) more efficiently than neutrophil collagenase (MMP-8). This is interesting because the neutrophil derived collagenases are more sensitive to tetracycline inhibition than MMP-1. IC_{50} for doxycycline in the prevention of mammalian neutrophil derived collagenase (MMP-8) is 26 μM, but 280 μM for MMP-1 *(Suomalainen et al.* 1992).

In the present study 59% of the collagenase activity was suppressed by 100 μM doxycycline and 87% by 600 μM doxycycline respectively. This suggests, that most of the collagenase in TA samples in horses derives from the neutrophils, but part is from fibroblasts and macrophages/monocytes. Also *Grünig et al.* (1985) suggested that the proteinase activity in tracheal aspirates may not only derive from neutrophils, although the number of neutrophils correlated well with the total proteinase activity. Second, some of total collagenolytic activity in TA samples in horses may be of bacterial origin.

The normal microbial flora in the intestinal tract of the horse is sensitive to many antimicrobials, such as tetracyclines, which, however, can be used in short term. Chemically modified tetracyclines (CMTs), which have anticollagenolytic activity but no anti-microbial activity (vide supra), have been developed *(Golub et al.* 1987). These drugs could have a potential for long term use also in the COPD horse.

The findings that both activities of interstitial collagenases and gelatinases are higher in TAs of COPD horses compared to healthy horses suggest, that the MMPs could be of importance in the pathogenesis of equine COPD. They could serve as markers of an actively ongoing disease, but also probably mediate local tissue destruction in the lung. Doxycycline seems to be an effective inhibitor of equine MMPs, at least in vitro. The mode of action of the proteolytic enzymes and their inhibitors in vivo in the lung and their importance in the pathogenesis of COPD needs still further clarification.

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Sammanfattning

Kollagenolytisk aktivitet och dess känslighet för doxicyklin i trakealaspirat från hästar med kronisk obstruktiv lungsjukdom.

Den kollagenolytiska aktiviteten och dess känslighet för doxicyklin i trakealaspirat (TA) från hästar med kronisk obstruktiv lungsjukdom (COPD) undersöktes med SDS-PA gelelektrofores (SDS-PAGE), varvid kollagen Typ I användes som substrat. Såväl den autoaktiva som den totala kollagenasaktiviteten var signifikant högre i TA från hästar med symptomgivande COPD än i TA från friska hästar. Resultaten från doxicyklininhibitionsstudier antyder att kollagenaset i TA till största del hänför sig till neutrofiler (MMP-8), men att aktivitet återfinns även i andra celltyper, t.ex. fibroblaster och monocyter/makrofager (MMP-1) samt bakterier (bakteriella kollagenaser). Läkemedel som hämmar kollagenasaktiviteten i luftvägarna kunde tänkas vara värda att utprovas för behandling av COPD hos hästar.

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