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TISSUE INFLAMMATION IN CANINE PULMONARY EOSINOPHILIA WITH SPECIAL REFERENCE TO MATRIX METALLOPROTEINASES

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ACADEMIC DISSERTATION

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Dedicated to my family
and
to the memory of my sister Eija

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1. ABSTRACT

Canine pulmonary eosinophilia (PE) is a lower respiratory disease involving the airways and lung parenchyma and causing bronchial and alveolar epithelial cell sloughing, resulting in basement membrane exposure. We characterized clinical and clinicopathological features and tissue inflammation in 20 PE dogs by assessing cellular changes as well as levels of matrix metalloproteinases (MMPs) -2, -8, -9, and -13 and laminin-5 γ2-chain degradation products in bronchoalveolar lavage fluid (BALF). Sixteen healthy dogs were used as controls, providing reference values for BALF. The effect of repeated lavage was evaluated by lavaging the control dogs six times at 5- to 7-week intervals.

Analysis indicated that repeated BAL was a safe and a reliable procedure, having an equal impact on PE and healthy dogs' arterial blood gas values. Laboratory processing caused a significant total cell loss in BALF. In blood, eosinophilia was detected in 50% of PE dogs, and in their BALF, cell count, the number and percentage of eosinophils and the numbers of macrophages, lymphocytes, neutrophils, mast cells, and epithelial cells were significantly elevated. Bronchointerstitial densities were detected in PE dogs' radiographs.

The levels of gelatinolytic MMPs (-2, -9) and collagenolytic MMPs (-8, -13) in BALF were higher in PE dogs than in healthy controls. Immunocytochemical methods showed that BALF macrophages and epithelial cells were the principal sources of MMPs -8 and -9; MMP-13 was found mainly in macrophages. A significant positive correlation was noted between the percentage of degraded type I collagen and the counts of BALF eosinophils, macrophages, lymphocytes, and mast cells.

We observed that inflammation caused epithelial cell sloughing in association with the degradation of the laminin-5 γ2-chain into small molecular weight fragments. The subsidence of inflammation after treatment was found to downregulate this degradation.

Overall, these findings suggest that upregulation of collagenolytic and gelatinolytic MMPs eventually contributes to pulmonary tissue destruction in canine PE. The laminin-5 γ2-chain degradation products may be linked to epithelial cell sloughing and basement membrane exposure or healing.

2. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by Roman numerals **I-IV**:

I Rajamäki, MM, Järvinen A-K, Saari SAM, Maisi PS (2001). Effect of repetive bronchoalveolar lavages on cytological findings in healthy dogs. American Journal of Veterinary Research 62: 13-16.

II Rajamäki MM, Järvinen A-K, Sorsa T, and Maisi P (2002). Clinical findings, bronchoalveolar lavage fluid cytology and matrix metalloproteinase-2 and -9 in canine pulmonary eosinophilia. The Veterinary Journal 163: 168-181.

III Rajamäki MM, Järvinen A-K, Sorsa T, Maisi P (2002). Collagenolytic activity in bronchoalveolar lavage fluid in canine pulmonary eosinophilia. Journal of Veterinary Internal Medicine 16: 658-664.

IV Rajamäki MM, Järvinen A-K, Sorsa TA, Tervahartiala TI, Maisi PS. Elevated levels of fragmented laminin-5 γ2-chain in BALF from dogs with pulmonary eosinophilia. The Veterinary Journal (Accepted for publication).

3. ABBREVIATIONS

$A\text{-}aDO_2$	arterio alveolar oxygen gradient
APMA	aminophenylmercuric acetate
ARDS	adult respiratory distress syndrome
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BM	basement membrane
CI	confidence interval
COPD	chronic obstructive pulmonary disease
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminotetra-acetic acid
ELF	epithelial lining fluid
HE	hematoxylin-eosin
kDa	kilodalton
Ln-5	laminin-5
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
MT-MMP	membrane-type matrix metalloproteinase
MW	molecular weight
$PaCO_2$	partial pressure of carbon dioxide in arterial blood
PaO_2	partial pressure of oxygen in arterial blood
PBS	phosphate-buffered saline
PE	pulmonary eosinophilia
PIE	pulmonary infiltrates with eosinophilia
PMN	polymorphonuclear leukocyte
r	correlation coefficient
SD	standard deviation
SDS	sodium dodecyl sulfate
TIMP	tissue inhibitor of matrix metalloproteinases
TNFα	tumor necrosis factor alpha

4. INTRODUCTION

Canine pulmonary eosinophilia (PE), also known as pulmonary infiltrates with eosinophilia (PIE), eosinophilic pneumonia, pulmonary hypersensitivity, and eosinophilic bronchopneumopathy is a syndrome characterized by eosinophil accumulation in the lung tissue and airways (Gibbs and Anderson 1990, Corcoran et al. 1991, Taboada, 1991, Clercx et al. 2000). PE causes variable respiratory signs, including cough and dyspnea, as well as such nonrespiratory symptoms as weight loss, lethargy, and fever (Hawkins 1998).

Lung inflammation can to some extent be studied by nondirect methods, e.g. blood tests and radiographs, but often changes, as in PE, are limited to the lung. In PE cases when blood eosinophilia is not present, special sample collection methods, such as bronchoalveolar lavage (BAL), are required. BAL enables characterization of the cellular profile and the inflammatory mediators collected directly from the site of inflammation. Bronchoalveolar lavage fluid (BALF) can be collected by bronchoscopic or unguided catheter or tube techniques. The technical protocols can vary greatly, for example, the recommended amount of lavage fluid or the laboratory processes (Saltini et al. 1984, Klech and Pohl 1989, Hawkins et al. 1990). This has led to a wide range of reference values, and thus, each laboratory needs to either set its own reference values or tightly adhere to established protocols.

Distal lung parenchyma and the bronchial epithelium are targets of inflammatory changes in PE. Proteolytic enzymes, including matrix metalloproteinases (MMPs), are believed to be the key pivotal mediators of structural changes both in pathological and physiological conditions because of their broad and efficient capacity to cleave various extracellular matrix and basement membrane (BM) proteins, including collagens, elastin, and proteoglycan, as well as connective tissue and BM glycoproteins (Corbel et al. 2002, Atkinson and Senior 2003). In addition, MMPs can process growth factors, cytokines, cell adhesion molecules, chemokines, cell adhesion molecules, cell surface receptors and apoptotic signals to modulate immune responses (Parks and Shapiro 2001, Visse and Nagase 2003). Certain MMPs may even exert anti-inflammatory or defensive characteristics (Owen et al. 2004). In human pulmonology, the involvement of MMPs has already been studied for a couple of decades, but in veterinary medicine this research field is relatively new. In this field, equine chronic obstructive pulmonary disease (COPD) is probably the best described animal disease. However, to our knowledge, no studies on canine pulmonary diseases prior to ours have been published.

Here, pathophysiologic changes in PE dogs, with special reference to MMPs, are compared with findings in healthy control dogs.

5. REVIEW OF THE LITERATURE

5.1. Anatomy of a healthy canine lung

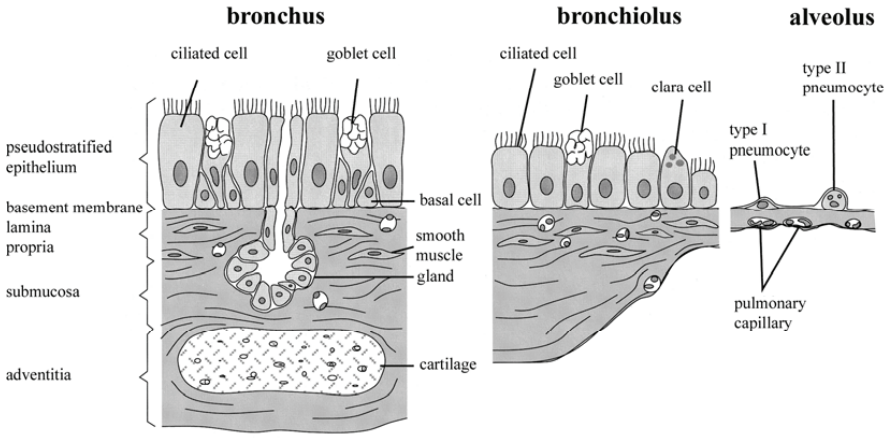
The lower respiratory tract includes the trachea (*trachea)*, the main bronchi (*bronchi principales)*, and the lungs (*pulmo*). The right lung (*pulmo dexter*) is larger than the left (*pulmo sinister*) and divided into four lobes: cranial (*lobus cranialis*), medial (*lobus medialis*), caudal (*lobus caudalis*), and accessory (*lobus accessorius*). The left lung consists of cranial and caudal lobes. The main bronchi divide into lobar bronchi (*bronchi lobares*), and thereafter, into segmental bronchi (*bronchi segmentales*). Each segmental bronchus and a portion of the lung behind it form an independent, coniform bronchopulmonary segment. In dogs, these segments are connected to each other, which enables ventilation also during the blockade to smaller bronchi (collateral ventilation).

Segmental bronchi divide into subsegmental bronchi (*bronchi subsegmentales),* which divide into bronchioles (*bronchuli*), then into terminal bronchioles (*bronchuli terminales*), respiratory bronchioles (*bronchioles respiratorii)*, and finally, into the alveolar duct (*ductus alveolares*), which extends inside the alveolar sacs (*sacculi alveolares*) (Nickel et al. 1960).

Alveolar sacs are surrounded by the lung interstitium, which represents an anatomic space between the BMs under the alveolar epithelial cells and the capillary endothelial cells. The boundaries of the interstitium extend to include the perivascular, perilymphatic, and peribronchiolar connective tissues, and the alveolar lumen. The interstitium contains the extracellular matrix, which is made up of macromolecules, including collagen, proteoglycans, and glycoproteins, as well as the noncollagenous proteins fibronectin and laminin. Low numbers of interstitial macrophages, fibroblasts, and myofibroblasts are also present.

The lung interfaces with the environment across a heterogeneous and continuous layer of epithelium attached to the BM. Bronchi are lined with ciliated, pseudostratified columnar epithelial cells that diminish in height to a simple cuboidal ciliated and gradually to a nonciliated epithelium layer in more distal airways. Numerous cells, including basal, goblet, and Clara cells, are present along the airways. Epithelial cells are attached to the thin BM by hemidesmosomes and to basal cells by desmosomes. Tissue below the epithelium is histologically divided into different layers called the lamina propria, submucosa, and adventitia. Submucosal glands lay in the deeper layers of the lamina propria and within the submucosa. A layer of obliquely arranged smooth muscle surrounds the lamina propria. The cartilage rings of the trachea transform into irregularly shaped cartilage plates in bronchi. Bronchioles lack cartilage and glands, but the amount of smooth muscle is proportional to the size of the bronchiolus. Alveolar ducts are surrounded entirely by alveoli. Ducts branch into three or more alveolar sacs. No smooth muscle is present at this level. The alveolar epithelium is composed mainly (95%) of remarkably thin squamous epithelial cells (type I pneumocytes), with the remainder being cuboidal epithelial cells (type II pneumocytes). Alveolar sacs enclose in their lumen macrophages, the primary function of which is defensive. (Figure 1).

Figure 1. A drawing of respiratory histology at three different levels (Reprinted and modified with the permission of Kustannus Oy Duodecim; originally published in Keuhkosairaudet; eds. V.Kinnula, P.Tukiainen, L.Laitinen 2. edition 2000).



5.2. Pathogenesis of canine pulmonary eosinophilia (PE)

Canine PE is a bronchointerstitial inflammatory pulmonary disease characterized by eosinophilic infiltration into the lung interstitium and the bronchial epithelium. PE is often associated with peripheral blood eosinophilia (Gibbs and Anderson 1990, Corcoran et al. 1991, Taboada 1991). Several terms, including pulmonary infiltrates with eosinophilia (PIE), eosinophilic pneumonia, pulmonary hypersensitivity, and eosinophilic bronchopneumopathy, have been used to describe the same syndrome (Gibbs and Anderson 1990, Corcoran et al. 1991, Taboada, 1991, Clercx et al. 2000). The etiology of PE is unclear, remaining in many cases unknown. Parasitic, bacterial, allergic, and fungal agents as well as a reaction to drug exposure have been described as potential etiologic agents (Noone 1986, Bauer 1989, Taboada, 1991). Recently, hypersensitivity mechanism I has been suggested to be involved in the etiology (Clercx et al. 2002).

Eosinophilic inflammation can involve primarily the airways or the interstitium, or both simultaneously (Dungworth 1993, Dail 1994, Hawkins 1998, Clercx et al. 2000). Histopathological changes at the bronchial level include an edematous and hyperemic lamina propria and infiltration of eosinophils accompanied by lymphocytes, macrophages, and plasma cells. The epithelium is highly susceptible to sloughing (Dungworth 1993, Clercx et al. 2000). When inflammation also occurs at the alveolar level, it causes type I alveolar epithelial cell necrosis and sloughing, leading to a temporary denuding of the alveolar BM during acute inflammation. Eosinophils, as a major inflammatory cell type, gather into alveolar spaces and the interstitium. Elevated numbers of macrophages, lymphocytes, mast cells, neutrophils,

and epithelial cells have also been found in PE dogs' BALF, and inflammatory cells also in the broadened interstitium (Dungworth 1993, Dail 1994). Proteinaceous exudates accumulate in the alveolar spaces, but the lung architecture is preserved. Cuboidal type II cells begin to line the denuded alveolar walls. Type II cells transform into type I cells, returning the normal structure after the inflammation, with the exception of chronic cases in which changes can proceed to collagen deposition and fibrosis (Dungworth 1993).

Typical clinical findings in canine PE include lower respiratory tract symptoms, eosinophilia in BALF and tracheal fluid, densities in thoracic radiographs, occasional blood eosinophilia and eosinophilic infiltration of the bronchial mucosa. The increase of absolute white blood cell count has been described to be 3- to 700-fold that of healthy dogs, and neutrophilia may also occur (Bauer 1989, Corcoran et al. 1991, Taboada 1991, Clercx et al. 2000, 2002).

The treatment of choice for canine PE is corticosteroid medication at a decreasing immunosuppressive dose unless a cause for the syndrome can be found and removed. Response to treatment is usually good and prognosis favorable; however, relapses are not uncommon (Hawkins 1995b, Johnson and Padrid 2000, Clercx et al. 2000, Norris and Mellema 2004).

5.3. Bronchoalveolar lavage (BAL)

The epithelial lining fluid (ELF) is a thin layer of fluid on the luminal surface of the respiratory tract. Lavage fluid contains both lavage fluid and ELF, and the dilution of ELF occuring during BAL could be determined by measuring a dilute present in ELF but not in lavage fluid. Additionally, the solute in ELF should also be the same or a constant fraction than in the plasma. Endogenous ELF solutes e.g. urea, albumin and protein have been utilized allthough there are major defects in their use. The urea method is likely to overestimate ELF because of diffusion from the circulation, which occurs to varying degrees in different patient groups. BAL albumin and protein levels are elevated in patients with a variety of interstitial diseases, reflecting the epithelium/endothelium barrier disturbance. Development of a practical method for the ELF assessment is still elusive and the emperical expression of BALF solute results as per milliliter of aspirate remains the most useful method (Ward et al., 1999).

Bronchoscopy is indicated when thoracic radiographs reveal either diffuse or localized abnormal findings and a diagnosis cannot be set by other methods. Depending on the lesion, BAL or biopsies are performed. BAL has been established to be a clinically useful procedure. Hawkins et al. (1995) found BAL to correlate definitely or supportively with clinical diagnosis in 75% dogs with alveolar or bronchial radiographic patterns, or the presence of pulmonary mass.

BAL can be obtained either with a bronchoscope or with a blind method using a tube or catheter (Hawkins et al. 1995, McCauley et al. 1998, Andreasen 2003). However, bronchoscopic method is considered superior because it allows a direct view of the airways for estimation of pathological changes on the epithelial surface, and specimens can be collected directly from the desired site.

The volume of the infused sterile isotonic saline varies considerably between different studies. Small-volume fluid washes utilizing weight adjusted 1-2 ml/5 kg (Cowell et al. 1999) or total amounts of 50-140 ml (Cohen and Batra 1980, Hawkins 1990) are more commonly recommended than large-volume (500 ml) washes (Pinsker et al. 1980). Small-volume washes are reported to yield a greater total cell count per milliliter and higher polymorphonuclear leukocyte concentration than large-volume washes, which contain higher bronchial cell concentrations (Pinsker et al. 1980). Pooling and combined analysis of collected aliquots are generally used. However, first aliquots have been reported to contain greater contribution from larger airways, reflected in higher neutrophil and epithelial cells propotions, than later boluses (Pinsker et al. 1980, Hawkins and Berry 1999). The recovered propotion of instilled fluid ranges between 50% and 90 % in healthy dogs; however, this propotion is clearly lower in dogs with diseased airways (Hawkins 1990, Clercx et al. 2002).

Although therapeutical lavages with large volumes of saline are rarely used in dogs; a recent report describes treatment of a dog with alveolar proteinosis using lung lavage (Silverstein et al. 2000).

5.3.1. Laboratory processing of bronchoalveolar lavage fluid (BALF)

When stored on ice, BALF must be analysed immediately. For later analyses, BALF can be stored in an ethylenediaminotetra-acetic acid (EDTA) tube (Hawkins 1990, Andreasen 2003). Mucus filtration, speed, duration, and number of centrifugations, as well as washing of cells, cytocentrifugation, and cell counting method all affect cellular results and can therefore cause distortion in the BALF findings (Saltini et al. 1984, Klech and Pohl 1989, Andreasen 2003). For example, performing centrifugation before cytocentrifugation causes an 18-34% loss of human BALF cells (Saltini et al. 1984, Mordelet-Dambrini et al. 1984).

Differential cell counts can be assessed from stained slides obtained by cytocentrifugation, manual preparation by smears, settling on slides, or by the use of Millipore filters. Each method may affect the results (Rennard et al. 1998).

A quantitative bacterial culture helps in differentiating pathogens from contaminants, as shown by Peeters et al. (2000), who compared quantitative culture with gram-stain intracellular bacteria counts from BALF specimens. A minimum of 1.7×10^3 colony-forming units (CFU) per milliliter was considered the threshold for defining clinically relevant bacterial growth.

5.3.2. Cellular content in BALF

There are some reports of the cellular content in healthy dogs' BALF (Rebar et al. 1980, Pinsker et al. 1980, Hawkins 1990, Vail et al. 1995, Hawkins 1998, Norris and Mellema 2004). Cellular content is expressed more often in relative values than in absolute values. Relative values seem to be more constant than the absolute values of

nucleated cells, and the maximum is often referenced as 500 cells per microliter (Hawkins 1998, Andreasen 2003, Norris and Mellema 2004).

Mean relative counts for macrophages are 65-80%, for lymphocytes 6-14%, for neutrophils 1-5%, for eosinophils 3-6%, for mast cells 1-2%, and for epithelial cells 0.8-1% (Rebar et al. 1980, Vail et al. 1995, Hawkins 1998). Results may deviate from these values if only leukocytes are included. In addition to differential counting, cells should be analyzed qualitatively; macrophages for activation, phagocytized material, lymphocytes, and plasma cells for reactivity, neutrophils for intracellular bacteria and degenerative changes, and noninflammatory cells for malignancies or infectious agents (Hawkins 1990).

The repetition of lavages at short intervals (24 hours) causes transient influx of neutrophils in BALF (Cohen and Batra 1980), but at longer intervals (3 weeks) no such changes have been noticed (Pinsker et al. 1980).

Few studies have reported on the cellular content of BALF in PE dogs. Relative eosinophilic inflammation predominates, but other cells are also present (Hawkins 1995b, Johnson and Padrid 2000, Norris and Mellema 2004).

5.3.3. Safety of BAL procedure

Patients for the BAL procedure must be selected carefully. BAL cannot be performed without risks in patients with respiratory distress mainly because of the requirement for general anesthesia and the transient hypoxemia (Hawkins 1999). However, in certain situations, the diagnosis or the cure cannot be achieved with less invasive techniques, and in these cases BAL is an alternative to thoracotomy and may be chosen after all pros and cons are weighed.

The potential acute hazard of bronchoscopy and BAL to dogs is decrease in arterial blood oxygen concentration caused primarily by decreased compliance and ventilation-perfusion mismatching (Hawkins 1999). This can usually be avoided by providing oxygen supplementation. Chronic effects of the large volume fluid lung lavage procedure have been investigated by Muggenburg et al. 1972, but minimal long-term risks were observed based on postmortem examinations and cardiopulmonary and lung function tests. Thus, in general, this procedure is considered safe for the selected patients population.

5.4. Matrix metalloproteinases (MMPs)

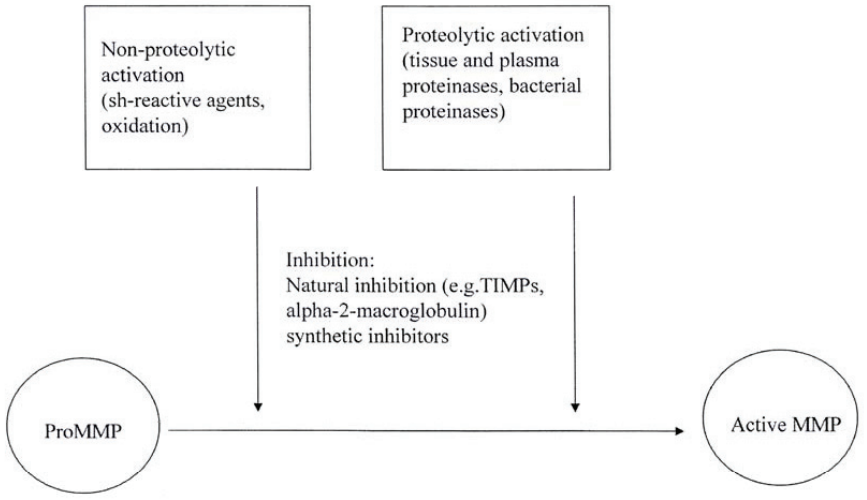
The lung extracellular matrix (ECM) is made up of several macromolecules including collagens, proteoglycans, matrix glycoproteins, fibronectin, laminins, elastin, and connective tissue glycoproteins (Schwarz 1998). The main collagen types in the lung interstitium are types I and III, and to lesser degree types IV, and V (Davidsson 1990, O'Connor and FitzGerald 1994, Fukuda et al. 1995). The BM, located between epithelial cells and the ECM, consists mainly of collagen IV, proteoglycans, and glycoproteins including laminins and nidogen. MMPs are a family of zinc-dependant extracellular and cell surface associated proteinases capable of cleaving almost all ECM and BM protein constituents. At present, at least 24 MMPs have been identified in vertebrates and grouped into subclasses based on substrate specificity, sequence similarity, and domain organization. These include three collagenases (MMP-1, MMP-8, MMP-13), two gelatinases (MMP-2, MMP-9), four stromelysins (MMP-3, MMP-10, MMP-11, MMP-12), seven membrane-type MMPs (MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP, MT5-MMP, MT6-MMP, MMP-23), two matrilysins (MMP-7, MMP-26), and others not classified in the previous categories (Visse and Nagase 2003) (Table 1). MMPs share to a large extent common structural and functional elements but are produced by different genes. They are composed of a signal peptide, a prodomain, a catalytic domain with a zinc-binding site, and a hemopexin- or vitronectin-like domain linked to the catalytic domain by a hinge region. Gelatinases MMP-2 and -9 also contain fibronectin type II inserts within the catalytic domain, and MT-MMPs a transmembrane domain (Kähäri and Saarialho-Kere 1999, Parks and Shapiro 2001, Ohbayashi 2002, Atkinson and Senior 2003). MMPs are distinguished from other classes of proteinases by their dependence on metal ions and neutral pH for activity as well as by their common structural homology including the sequence homology, the cysteine switch motif in the propeptide, and the zinc-binding motif in the catalytic domain (Visse and Nagase 2003). MMPs are secreted to extracellular milieu in a latent form, zymogens, induced by a response to exogenous signal and are proteolytically activated thereafter by cleavage to a truncated form with about 10 kilodalton (kDa) lower molecular size (Birkedal-Hansen 1993, Shapiro 1998, Kähäri and Saarialho-Kere 1999, Ohbayashi 2002). Majority of MMPs are *de-novo* expressed only after induction, and only presynthetized MMP-8 and MMP-9 are stored in secretory granules of neutrophils ready for rapid release and activation upon neutrophil degranulation (Kähäri and Saarialho-Kere 1999, Shapiro and Senior 1999).

Table 1. Classification of matrix metalloproteinases (Sternlicht and Werb 2001, Acharya et al. 2004).

Subgroup	MMP	Nomenclature
Interstitial collagenases	MMP-1	Collagenase-1
	MMP-8	Collagenase-2
	MMP-13	Collagenase-3
Gelatinases	MMP-2	Gelatinase-A
	MMP-9	Gelatinase-B
Stromelysins	MMP-3	Stromelysin-1
	MMP-10	Stromelysin-2
	MMP-11	Stromelysin-3
	MMP-12	Metalloelastase
Matrilysins	MMP-7	Matrilysin-1
	MMP-26	Matrilysin-2, Endometase
Membrane type MMP	MMP-14	MT1-MMP
	MMP-15	MT2-MMP
	MMP-16	MT3-MMP
	MMP-17	MT4-MMP
	MMP-24	MT5-MMP
	MMP-25	MT6-MMP
	MMP-23	CA-MMP
Others	MMP-18	Collagenase-4 (*Xenopus*)
	MMP-19	RASI-I
	MMP-20	Enamelysin
	MMP-21	CMMP (chicken)
	MMP-22	
	MMP-27	
	MMP-28	Epilysin

The proteolytic activity is delicately regulated by transcriptional regulation, proenzyme activation, and inhibition of proMMP activation and MMP activity (Corbel et al. 2002). In addition, chemical agents (e.g. organomercurials), oxidative stress, serine proteinases such as plasmin and trypsin, and other MMPs such as MT-MMP activate each other in a complex network (Sorsa et al. 1997, Brenneisen et al. 1997, Kähäri and Saarialho-Kere 1999, Moilanen et al. 2003, Holopainen et al. 2003). Microenvironmental changes including low pH and heat treatment, can also cause activation (Visse and Nagase 2003). Activation of MMPs is stepwise in many cases and can be initiated by other already activated MMPs or by several serine proteinases that can cleave peptide bonds within the MMP prodomain (Sternlict and Werb 2001). In addition, bacterial proteinases can activate proMMPs (Sorsa et al. 1992). Figure 2.

Figure 2. Activation of matrix metalloproteinases (MMPs) (Sorsa et al. 2004).



The function of MMPs is very complicated and subtle. They are involved in normal physiological processes as well as in pathological processes. Physiological processes include the normal turnover and maintenance of ECM proteins in connective tissue and BM and also in tissue remodeling and embryogenesis (Ohbayashi 2001, Parks and Shapiro 2001). Moreover, MMPs are engaged in the cell-cell and cell-matrix signaling. MMPs are able to modulate the activity of a variety of nonmatrix proteins; MMP-7 for instance is responsible for the activation of prodefensin in the small intestine, thereby participating in the bacterial defense process (Wilson 1999). Several MMPs, including MMP-1, -2, -3, -11, and -7 can modulate the activity of many growth factors, cytokines, chemokines, and adhesion receptors through proteolytic cleavage. Fragments of matrix proteins can act as chemoattractants (Levi et al. 1996, Haro et al. 2000). MMPs can also process pro-inflammatory mediators, defensins, complement components, cell adhesion molecules, and cell surface receptors participating in cell signaling and innate immunity (Parks and Shapiro 2000, Owen et al. 2004). Certain MMPs, such as MMP-8, MMP-9, and MMP-7, can exert anti-inflammatory or defensive characteristics (Parks and Shapiro 2000, Owen et al. 2004).

The cellular sources of different MMPs have been investigated widely. MMPs are usually derived from different pulmonary inflammatory cells, such as alveolar macrophages, neutrophils, and eosinophils, in addition to resident cells including bronchial epithelial cells, Clara cells, alveolar type II cells, fibroblasts, smooth muscle cells, and endothelial cells (Perez-Ramos et al. 1999, DiGirolamo et al. 2000, Segura-Valdes et al. 2000, Wakefield 2000, Imai et al. 2001, Prikk et al. 2001b, Wahlgren et al. 2001). The expression rates of MMPs differ between stimulated and nonstimulated conditions (Lee at al 2003, Woo et al. 2004).

5.4.1. The role of MMPs in diseases

Excessive expression of MMPs contributes to the pathogenesis of different pulmonary and nonpulmonary diseases. They are believed to play a role in the pathogenesis through degradation of ECM and BM. BM protein degradation might cause influx of inflammatory cells and perturbation of the epithelial/endothelial structure, whereas degradation of elastin and collagen could predispose to airspace enlargement (Senior and Shapiro 1999). Aberrant expression of MMPs has been associated with many destructive diseases including lung diseases, arthritis, periodontal diseases, atherosclerotic rupture, aortic aneurysm, and tumor progression (Shapiro and Senior 1999). Lung diseases, with excessive amounts of MMPs include equine and human COPD (Segura-Valdez et al. 2000, Parks and Shapiro 2001, Raulo et al. 2001a, 2001b), human asthma (Prikk et al. 2002), bronchiectasis (Sepper et al. 1994, 1995, Prikk et al. 2001b), and various interstitial diseases (Hayashi et al. 1996, Suga et al. 2000, Henry et al. 2002).

5.4.2. MMP-2 and MMP-9 (gelatinases)

MMP-2 and MMP-9 belong to the group of gelatinases. Their key feature is that they readily digest the denaturated collagens, gelatins. These MMPs have some differences in their capacity to cleave different types of collagens; MMP-2, but not MMP-9, can degrade native type I and II collagens (Aimes et al. 1998, Konttinen et al. 1998).

MMP-9, also known as gelatinase-B, is secreted from cells in a glycosylated 92-kDa form and activated thereafter to 77- to 82-kDa and 68-kDa forms by a protease cascade (Birkedahl-Hansen et al. 1993, Sorsa et al. 1997). The most efficient activators of MMP-9 are probably human trypsin-2 together with MMP-3 (Sorsa et al. 1997, Cuzner and Opdenakker 1999). Dimeric forms of the 220-kDa proMMP-9 are commonly found (Triebel et al. 1992). The protein sequence of canine MMP-9 displays similarities with human (79.6%), rat (72.0%), rabbit (80.6%) and bovine (82.3%) sequencies (Yokota et al. 2001). MMP-9 has been located in many different inflammatory cells such as macrophages (Lemjabbar et al. 1999, Raulo et al. 2001b), neutrophils (Dahlen et al. 1999, Raulo et al. 2001b), lymphocytes (Kumagai et al. 1999, Raulo et al. 2001b), mast cells (Kanbe et al. 1999), and eosinophils (Kumagai et al. 1999). Pulmonary resident cells do not produce MMP-9 in the normal lung but can express various MMP-9 species under stimulation (Atkinson and Senior 2003). In these resident cells, MMP-9 is expressed in bronchial epithelial cells, Clara cells (Zuo et al. 2002), alveolar type II cells (Pardo et al. 1998), fibroblasts (Wilhelm et al. 1989, Warner et al. 2004), smooth muscle cells (Kenagy and Clowes 1994), endothelial cells (Schnaper et al. 1993), and submucosal cells (Fukuda et al. 1998, Hoshino et al. 1999, Raulo et al. 2001b).

MMP-9 plays a key and pivotal role in gelatin and type IV collagen degradation but also has many other substrates such as elastin, collagen V, VII, X, XI, XIV, and fibronectin. MMP-9 participates in basal cell migration (Legrand et al. 1999), ECM component digestion, and activity modulation of other proteases and cytokines (Shapiro 1998, Shapiro and Senior 1999).

MMP-2, or gelatinase-A, a 72-kDa gelatinase in its latent form, is converted into 59- to 62-kDa forms during activation (Birkedahl-Hansen et al. 1993). The canine N-terminal amino acid sequence has been found to be 87% identical to human MMP-2 (Coughlan et al. 1998). MMP-2 is not readily activated by serine proteinases including trypsin-2 and plasmin (Sorsa et al. 1997); instead, the main activation of proMMP-2 takes place on the cell surface mediated by MT-MMPs with the assistance of tissue inhibitor of matrix metalloproteinase 2 (TIMP-2) (Wang 2000, Visse and Nagase 2003). Macrophages as well as pulmonary structural cells, such as fibroblasts, pneumocytes, epithelial, and endothelial cells, synthesize MMP-2 (Ohnishi et al. 1998, Segura-Valdez et al. 2000, Corbel et al. 2002). MMP-2 shares mostly substrate specificities with MMP-9 but can also digest type I and II collagens similarly to the classical collagenases (MMP-1, -8, and -13) (Aimes and Quigley 1995, Konttinen et al. 1998).

Elevated levels of MMP-9 have been reported in such pulmonary diseases as asthma, adult respiratory distress syndrome (ARDS), human and equine COPD, and bronchiectasis (Torii et al. 1997, Raulo et al. 1998, Segura-Valdez et al. 2000, Suzuki et al. 2004). In normal, healthy resting tissues, the production of MMP-9 is at a low level (Parks and Shapiro 2001). MMP-9 has been proposed to merely modulate enzymes and cytokines to fine-tune both destruction and repair, and may even have a beneficial function in a subset of cells in the disease process, therefore not being heavily involved heavily in the ECM destructive processes (Atkinson and Senior 2003, McMillan et al. 2004). While few studies have focused on the role of MMP-2, its contribution has nevertheless been shown in inflammatory human lung diseases (Maisi et al. 2002). Increased levels of either MMP-2 or activated forms of MMP-2 have been found in lung diseases including experimental silicosis, COPD, bronchiectasis, asthma, and ARDS (Sepper et al. 1994, Torii et al. 1997, Perez-Ramos et al. 1999, Segura-Valdez et al. 2000, Maisi et al. 2002). Similar to MMP-9, MMP-2 has also been reported to act as an effector and downregulator in inflammation: McQuibban et al. (2001), for instance, demonstrated that MMP-2 can diminish inflammation.

5.4.3. MMP-1, MMP-8, and MMP-13 (collagenases)

Collagenases MMP-1, MMP-8, and MMP-13 make up a subgroup of MMPs. They readily cleave interstitial collagens I, II, and III at a specific site producing ¾ (αA)- and ¼ (αB)-degradation products (Visse and Nagase, 2003). They all digest fibrillar collagens, which then denature at body temperature and then are further degraded by other MMPs including MMP-2 and MMP-9. Collagenases can also digest a number of other ECM and nonECM molecules such as α_1-antitrypsin, α_2-macroglobulin, plasminogen, and tumor necrosis factor (TNF) -α (Birkedal-Hansen 1993, Shapiro and Senior 1998, Sternlict 2001).

MMP-8, collagenase-2, has in its latent form a molecular weight of 75-80 kDa and in its active form 64 kDa (Tschesche et al. 1992). However, cellular sources affect the molecular weight depending on the degree of glycosylation, activation, and fragmentation (Hanemaaijer et al. 1997, Palosaari et al. 2000). MMP-8 is primarily synthesized in polymorphonuclear leukocytes (PMNs), is matured in the bone marrow, and stored in intracellular-specific granules. MMP-8 is secreted by selective degranulation of these granules and thereafter activated by reactive oxygen species

trypsin-2, chymotrypsin, cathepsin G, MMP-3, MMP-10, MMP-7, and MMP-14 (Murphy et al. 1987, Sorsa et al. 1989, Goldberg et al. 1992, Knäuper et al. 1993, Ding et al. 1997, Moilanen et al. 2003, Holopainen et al. 2003). MMP-8 preferentially hydrolyzes type I collagen, the major ECM component of the lung (Crystal et al. 1991, Tschesche et al. 1992). In addition to neutrophils, MMP-8 has been identified not only in pulmonary monocytes/macrophages, bronchial and gingival/sulcular epithelial cells, keratinocytes, plasma cells, glandular cells and in oral cancer cells (Peres-Ramos et al. 1990, Tervahartiala et al. 2000, Wahlgren et al. 2001, Prikk et al. 2001b, Raulo et al. 2001a, Moilanen et al. 2002).

MMP-13, i.e. collagenase-3, is secreted in a latent 60-kDa form, transformed into an intermediate 50-kDa form, and finally processed into its active 48-kDa form (Knäuper et al. 1996). MMP-13 has a key role in the MMP activation cascade (Leeman et al. 2002).

MMP-13 has a wide substrate specificity. It effectively hydrolyzes type II collagen but is 5-6 times less efficient at cleaving type I or III collagen (Knäuper et al. 1996, Lindy at al 1997, Leeman et al. 2002). In addition, MMP-13 degrades BM type IV collagen and laminin-5 (Ln-5) γ2-chain, proteoglycans, fibronectin, fibrillin, versican, and tenascin C (Knäuper et al. 1996, Pirilä et al. 2003, Leeman et al. 2002).

MMP-13 is considerably restricted in its tissue expression due to its very efficient catalytic properties (Kähäri and Saarialho-Kere 1999). It has been found in pulmonary macrophages and in nonpulmonary cells including plasma cells and gingival epithelial cells (Perez-Ramos et al. 1999, Tervahartiala et al. 2000, Raulo et al. 2001a, Wahlgren et al. 2001, Kiili et al. 2002).

Increased collagenolytic activity has been shown in the pathological processes of equine COPD, murine experimental lung silicosis, and human bronchiectasis, COPD and emphysema (Sepper et al. 1995, Perez-Ramos 1999, Segura-Valdes 2000, Prikk et al. 2001b, Raulo et al. 2001a, Imai 2001). MMP-13 is expressed excessively in inflammatory diseases, such as equine COPD, and in experimental lung silicosis in rats (Perez-Ramos et al. 1999, Raulo et al. 2001a, Mariani et al. 2002). Additionally, MMP-13 appears to be critical especially in bone metabolism and homeostasis as well as in tumor metastasis and invasion (Leeman et al. 2002). Recently, MMP-8 has been found to exert unexpected anti-inflammatory characteristics in lipopolysaccharide (LPS) induced lung inflammation in mice (Owen et al. 2004).

5.4.4. Inhibition of MMPs

5.4.4.1. Physiological inhibition of MMPs

The activity of MMPs can be inhibited by endogenous or exogenous synthetic inhibitors. The endogenous inhibitors are known as TIMPs, and to date four TIMPs (TIMP-1, TIMP-2, TIMP-3, TIMP-4) have been identified in vertebrates. They play a key role in maintaining the delicate balance between ECM deposition and degradation in different physiological processes. TIMPs appear in most tissues and body fluids, but TIMP-3 is the only member of the TIMP family which is found exclusively in the ECM (Gomez et al. 1997) TIMPs inhibit the active enzyme as well as regulate the

activation process of MMPs. They form tight bonds with the activated MMPs, resulting relatively heat-, and proteolytic-resistant complexes (Sorsa et al. 1997, Brew et al. 2003, Lambert et al. 2004). TIMP-1 and -2 inhibit the activity of most MMPs, with the exception of MT1-MMP. TIMP-2 is a ten times more effective inhibitor of MMP-2 and MMP-9 than TIMP-1, and TIMP-1 is two times more effective against MMP-1 than against other MMPs. TIMP-1 forms complexes preferentially with proMMP-9, and TIMP-2 and -4 with proMMP-2 (Goldberg et al. 1989). TIMP-3 inhibits activity of MMP -1, -2, -3, -9, and -13. TIMP-4 inhibits the activity of MMP-2 and MMP-7 more potently than that of MMP -1, -3, and -9 (Kähäri and Saarialho-Kere 1999). Recent studies have revealed that these proteins can also exhibit biological activities that affect cell proliferation and survival. These activities are distinct from their interactions with MMPs or inhibition of MMPs (Crocker et al. 2004).

The predominant nonspecific serum inhibitor is α2-macroglobulin, which is also responsible for regulating various other proteinases, especially serine proteinases (Birkedal-Hansen et al. 1993).

5.4.4.2. Synthetic inhibitors of MMPs

Synthetic exogenous MMP inhibitors, such as Galardin™, Marimastat™, Batimastat™, and the gelatinase-selective CTTHWGFTLC-peptide (Belotti et al. 1999, Koivunen et al. 1999, Auge et al. 2004, Ramnath and Craeven 2004), are the target of much research activity, particularly in the pharmaceutical field. Extensive investigations have examined their biological activities in tumor growth, invasion, metastasis, and angiogenesis as well as in the diagnosis and treatment of inflammatory diseases. Doxycycline and nonantimicrobial tetracycline derivatives (chemically modified tetracyclines, CMT) possess anticollagenase activity (Golub et al. 1997, Golub et al. 1998). CMTs have been tested *in vitro* also in pulmonary epithelial lining fluid in equine COPD (Maisi et al. 1999). Despite tremendous efforts over the last decade with multiple inhibitor classes, simple, safe, and effective drugs for inhibiting individual MMPs have not yet emerged (Matter and Schudok 2004). The only inhibitor in clinical use is low-dose or subantimicrobial dose doxycycline which has been shown to be beneficial as adjunctive medication in human periodontal diseases (Golub et al. 1997, Golub et al. 1998, Emingil et al. 2004 a,b).

5.5. Laminin-5 (Ln-5)

BM is a thin structure separating epithelial or endothelial cells from the adjacent tissue. It provides a physiological support for tissues, acts as a barrier for cells of different origin, and is involved in cell differentiation and migration as well as tissue repair and remodeling. BM also acts as a reservoir of plasma proteins, enzymes, and growth factors (Yurchenco and O'Rear 1994). It consists mainly of collagen IV, proteoglycans, and glycoproteins, including Lns and nidogen (Ekblom et al. 1998). Lns are involved in cellular movement, growth, and differentiation and act as integrating elements between the extracellular matrix and the cytoskeleton of epithelial cells (Perez-Arellano et al. 1993, Yurchenco and O'Rear 1994, Baker et al. 1994, Sannes and Wang 1997, Michelson et al. 2000, Ghosh and Stack 2000). Ln-5 consists of α3-, β3-, and γ2-subunits (Miyazaki et al. 1993, Matsui et al. 1995, Ekblom et al.

1998, Michelson et al. 2000), and is one of more than ten different Ln isoforms with molecular weight of 400-440 kDa in its intact form (Mizushima et al. 1998, Elkhal et al. 2004). Ln-5 is a major component of the anchoring fibrils in the hemidesmosome, a specialized epithelial cell-ECM connection. When epithelial cells are detached from the BM, like alveolar epithelial cells in canine PE (Dungworth 1993) and bronchial epithelial cells in human asthma (Laitinen and Laitinen 1994), Ln degradation products, such as Ln fragment P1, can be released (Perez-Arellano et al. 1993, Behr et al. 1995, Lemjabbar et al. 1999). Their levels have been reported to be increased in BALF of human diffuse interstitial lung disease, fibrosing alveolitis, ARDS, and severe asthma (Perez-Arellano et al. 1993, Behr et al. 1995, Torii et al. 1997, Lemjabbar et al. 1999).

Various MMPs, including MMP-2, -3, -8, -12, -13, -14, and -20, can process the Ln-5 γ2-chain into lower molecular weight fragments, referred to as γ2x with molecular weight of 80 kDa or less (Koshikawa et al. 2000, Pirilä et al. 2003, Koshikawa et al. 2004). This fragment can stimulate epithelial cell migration and induction (Giannelli et al. 1997, Koshikawa et al. 1999, Koshikawa et al. 2000, Pirilä et al. 2003, Ogawa et al. 2004). MMP-8 also cleaves the Ln-5 γ2-chain, but the cleavage site is different from that of the other MMPs, and it does not induce epithelial cell migration (Pirilä et al. 2003). Using *in situ* hybridization, *in situ* zymography, and immunohistochemistry, the Ln-5 γ2-chain has been localized with MMP-2 and -13 in inflammatory odontogenic keratocysts, and also with MMP-2 in inflamed gingiva and inendogenously produced laminin-5 rich matrix (Mäkelä et al. 1999, Pirilä et al. 2001, Wahlgren et al. 2003). In addition, Kivelä-Rajamäki et al. (2003) and Emingil et al. (2004 a,b) have reported elevated levels of Ln-5 γ2-chain degradation products in oral inflammatory exudates in conjunction with increased levels of MMP-8.
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6. AIMS OF THE STUDY

This thesis examines the clinical and pathophysiologic changes present in the canine PE, with a special interest in MMPs and Ln-5 γ2-chain in BALF. These changes are compared to findings in healthy dogs. Specific aims were as follows:

1. to establish reference values for cytologic examination of BALF and to study the effects of repeated lavages on lung and on results of cytologic BALF analysis in healthy dogs.

2. to describe clinical changes, the effect of BAL on arterial blood gas values, and the effect of therapy on canine PE.

3. to study the expression of airway and lung inflammation in PE dogs' BALF, with special reference to MMP-2, -8, -9 and –13, to compare these findings with those in healthy dogs' BALF, to localize the cellular origin of the MMPs.

4. to describe changes encountered in the BM by measuring levels of the Ln-5 γ2-chain and its degradation products in BALF, comparing these in PE dogs and healthy dogs and in PE dogs before and after treatment.

7. MATERIALS AND METHODS

7.1. Dogs

7.1.1. Control dogs (I-IV)

Sixteen healthy Beagles (11 females, 5 males; mean age 5.5 years, range 3-10 years; mean weight 12 kg, range 8-18) were used for determination of BALF cytological reference values and for investigation of the effect of repetitive lavages on pulmonary health and on BALF cytological values **(I)**. All 16 dogs were lavaged once and 11 dogs a further six times at 5- to 7-week intervals **(I)**. The study protocol was approved by the Animal Care and Use Committee of the University of Helsinki.

7.1.2. PE-dogs (II-IV)

The study population of PE dogs consisted of 20 dogs (12 females, 8 males; mean age 3.8 years, range 7 months to 9 years; mean weight 17 kg, range 4-37 kg) presented to the Veterinary Teaching Hospital, University of Helsinki with symptoms and history of lower respiratory tract disease. All dogs had previously been treated with antibiotics. In addition five dogs had received corticosteroids, three bronchodilatators, three other symptomatic cough medications, four diuretics and one arterial dilatators for respiratory symptoms (**II**, Table II). All dogs had abnormal findings on clinical examination and eosinophilia in BALF (**II**, Table VI). Eosinophilia in BALF was diagnosed when the number of eosinophils exceeded the upper limit set in Study I. All 20 dogs were examined carefully for any clues of the etiology of pulmonary eosinophilia, but no other diseases, pulmonary or otherwise, were found.

Twelve dogs were re-evaluated by a similar examination protocol one to four weeks after the treatment, and five were also relavaged at the control visit and one during the treatment.

7.2. Examination protocol and sample collecting (I-II)

A similar examination protocol, including medical history, clinical examination, pulmonary radiographs, hematology and serum biochemistry panels, arterial blood gas analysis, fecal flotation tests, and bronchoscopy with BAL was performed on all dogs. Control dogs underwent the same protocol initially; with the exeption that fecal parasite tests (dogs routinely dewormed twice a year) and radiographs were taken only before the first lavage.

Five control dogs (aged 8-10 years) in the repetitive lavage group were euthanized 3 weeks after the last lavage.

7.2.1. Symptoms and clinical examination (I-II)

Same questions of general and respiratory symptoms were asked from all PE dogs' owners. A thorough clinical examination with inspection, auscultation, and palpation was carried out in all dogs prior to any other studies.

7.2.2. Pulmonary radiographs (I-II)

Ventrodorsal and left lateral thoracic radiographs of nonsedated dogs were taken after clinical examination at the initial visit and for 12 PE dogs at the control appointment after the treatment. For the 11 control dogs in the repetitive lavage group, radiography was repeated 2 weeks after the last BAL.

Two blinded examiners evaluated the radiographs independently for pulmonary and other diseases. Pathological changes at the alveolar, interstitial, and bronchial levels in the lungs were graded using a scale from 0 to 3+ (0 normal, 1+ mild, 2+ moderate, 3+ severe), and a radiographic score was calculated by summing all pattern scores.

7.2.3. Blood samples (I-II)

Venous blood was collected for hematological and biochemical analyses, and arterial blood samples were taken from the femoral artery for determination of arterial oxygen (PaO_2) and carbon dioxide pressures ($PaCO_2$) before sedation. The second arterial sample was taken within 5 min after BAL but before reversal of sedation. Arterial gas analysis was carried out (ABL 300, Radiometer, Holliston, MA, USA), and arteriao-alveolar oxygen gradients ($A\text{-}aDO_2$) were calculated.

7.2.4. Fecal samples (I-II)

The fecal flotation test (Dryden and Bolka 1992) was used for intestinal parasite determination.

7.2.5. BALF (I-II)

After sedation with medetomidine (40 µg/kg intramuscularly (im.) (Domitor, Orion Pharma, Turku, Finland) anesthesia was induced with propofol (1 mg/kg iv.)(Rapinovet, Schering-Plough A/S, Kenilworth, UK), and bronchoscopy was performed using a fiberoptic bronchoscope (Olympus GIF type N30, Olympus, Tokyo, Japan). Additional oxygen was not administered. Both caudal lobes were lavaged with 37ºC sterile saline (1 ml/kg twice/lobe), and the sample in a glass container was placed on ice and processed immediately. Sedation was reversed with atipametzole (200 µl/kg im) (Antisedan, Orion Pharma, Turku, Finland).

BALF was filtered through a one-layer cotton gauze, the volume measured and the recovered proportion of the total instilled fluid volume was determined. Four 500-

µl aliquots of native BALF were frozen at -70ºC. Cell count and viability in the original sample were assessed from the trypan blue stained sample (1:1) in the hemocytometer. The remaining sample was centrifuged at 100 X g for 10 min, and the was supernatant separated from the cell pellet. Four 500-µl aliquots of supernatant were frozen at -70ºC. The cell pellet was washed with 10 ml of phosphate-buffered saline (PBS), centrifugation was repeated, and the pellet was resuspended in 1 ml of PBS. Cell count and viability assessment were repeated and the result expressed in corresponding volumes. Aliquots containing 40 000 cells were cytocentrifuged (Cytospin 3, Shandon Scientific Ltd., Cheshire, UK) (250 X g for 10 min) on silicone-coated slides. For the differential cell count, slides were stained with May-Grünwald-Giemsa stain, and the rest of the slides were frozen at 70ºC for immunocytochemistry. Differential cell counts for macrophages, lymphocytes, neutrophils, eosinophils, plasma cells, mast cells, and epithelial cells were obtained by counting 300 cells. From the BALF cellular analysis results of all dogs, means, standard deviations (SDs), and 95% confidence intervals (CIs) were determined and the 95% CIs of control dogs were used in the later studies as reference values (**II, III, IV**). Quantitative bacterial culture (10 µl of BALF on a blood agar plate at 37°C for 48 h) was performed for 13 PE dogs; bacterial growth of 10^4 CFU/ml or more was set as the limit for infection. In addition, all specimens were inspected for intracellular bacteria.

7.3. Histologic evaluation (I)

Lung samples from cranial (nonlavaged) and caudal (lavaged) lobes (three samples from each lung lobe) were collected and fixed in 4% neutral-buffered formalin immediately after euthanasia. Formalin samples were stained with hematoxylin-eosin (HE) and Masson-Trichrome stains. Health of the dogs was verified by complete postmortem examinations.

7.4. Assays

7.4.1. Functional assays

7.4.1.1. Gelatin zymography (II)

For the incubation (2 h at 22°C), native BALF and cell-free BALF samples were mixed at a ratio of 2:1 with the sample buffer; containing 0.118M Tris (T-1378) (Sigma, St. Louis, MO, USA), 64 mM H_3PO_4 (Art. 573) (Merck, Darmstadt, Germany) 20% glycerol, 0.04 g/l bromphenol blue (Art. 8122) (BDH), and 6% sodium dodecyl sulfate (SDS, Prod. 44244) (BDH Chemicals Ltd., Poole, England), at pH 6.8, and then loaded onto a 10% SDS-polyacrylamide gelatin-containing gel (porcine skin gelatin 1 mg/ml, G-2625, Sigma Chemicals, St. Louis, MO, USA). A standard suspension of lysed equine neutrophils (Raulo and Maisi 1998) serving as an interassay control and a high-range molecular weight MW standard (Bio-Rad, Richmond, CA, USA) were also loaded. Standardization of this method has been described previously (Raulo and Maisi 1998). The zymograms were run at 4ºC, incubated for 17 h at 37°C, and after staining with Coomassie blue the gelatinolytic activity was visualized by colouring as clear bands against a blue background.

Zymograms were analyzed for total gelatinolytic activity and complex, proMMP-9, active MMP-9, and MMP-2 forms (Mellanen et al. 1998). The gels were scanned (Scanjet 4C/T, Hewlett-Packard) and analyzed using an image analysis and processing system (Cream, Kem-En-Tek, Copenhagen, Denmark). Briefly, densitometric analysis was performed as follows: a linear standard curve drawn from the molecular standard lane provided the reference to which every band was compared in molecular weight assessment. The analysis program then drew a curve of each band based on the densitometric properties expressed as arbitrary intensity units. Bands suggested by the program were compared with the gel's bands visible to the eye. The program compared each band with the equine neutrophil lysate standard, which was assigned a value of one hundred for total activity. Background intensity was subtracted from each band's curve, and densitometric results were gained from the program's calculations using the area mode. Total gelatinolytic activity was achieved by summarizing the results of different bands of a lane.

7.4.1.2. Type I collagen degradation (III)

Both cell-free BALF (20 PE dogs, 16 controls) and native BALF (9 PE dogs with the highest collagen activity in cell-free BALF, 9 randomly selected controls) samples at a 10-fold concentration with equal volume were used in the collagenolytic activity analysis (Raulo et al. 2001a). All samples were analyzed with and without activator (1 mM amino mercuric acetate (APMA)) treatment in parallel. Samples were incubated with 1.5 μM triple helical type I collagen for 96 h at 22ºC in the dark. Incubation was terminated by adding Laemmli's sample buffer and boiling, and degradation products were separated by the 10% SDS gel electrophoresis. Purified collagen I was used as a control. Gels were stained with Coomassie brilliant blue, destained, scanned, and analyzed with an image analysis and processing system in a similar manner as in zymography. Four bands were visible, with the two highest bands representing intact collagen I α1 and α2 chains and the two lowest ¾-(α1 and α2) degradation products whose analysis results were therefore multiplied by 4/3. The proportion of degradation products was calculated by comparing it with intact collagen I.

7.4.2. Immunological assays

7.4.2.1. Immunocytochemistry (II, III)

Immunocytochemistry analysis with Vectastain Rabbit/Mouse ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) was performed using either fresh (**II**) or frozen (**III**) samples on silicone-coated cytospin slides. Slides were fixed with acetone for 10 min at 4ºC, washed three times in PBS, and incubated in 0.6% methanol-perhydrol for 30 min at 22°C. Nonspecific binding was blocked with 2% normal goat (MMP-8, MMP-9) or horse (MMP-13) serum (1:50), primary antibody was applied, and samples were preincubated (**III**) for 1 h and then incubated at 4ºC overnight (**II, III**). Specific rabbit polyclonal anti-human MMP-8 (1:250) (**III**), MMP-9 (1:500) (**II**), and monoclonal anti-human MMP-13 (1:500) (**III**) antibodies were used (Oncogen, Merck, Darmstadt, Germany) (Lauhio et al. 1994, Sorsa et al. 1994, Westerlund et al. 1996, Sorsa et al. 1997, Lindy et al. 1997, Hanemaaijer et al. 1997, Mellanen et al.

1998, Kiili et al. 2002). For negative controls, primary antibodies were replaced with 1 μg/ml (MMP-8-, MMP-9) rabbit or 0.2 μg/ml mouse immunoglobulin G. The background was decreased by the use of normal goat/horse serum (2%) in both the negative control and primary antibody solutions (**III**). Secondary antibody (anti-rabbit/anti-mouse 1:250, 30 min, 22°C) and avidin-biotin solution (1:125, 30 min, 22°C) were added, and slides were stained with chromogen-AEC (Dako, Glostrup, Germany) and counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

7.4.2.2. Western immunoblotting (II-IV)

Western immunoblotting analysis was used to identify MMP-2, -8, -9, and -13 and to identify and quantify Ln-5 γ2-chain fragments. Samples were 20-fold (II, IV) or 10-/20-fold (III) concentrated cell-free BALF. Each gel was loaded with equal volumes of samples, molecular weight standard (Bio-Rad, Richmond, CA, USA), and positive controls, the latter being either purified human polymorphonuclear supernatant for MMP-8, or purified human MMP-2, -9, or -13 (21,23-25/III), or intact rat Ln-5 γ2-chain (Plopper et al. 1996, Koshikawa et al. 1999, Kivelä-Rajamäki et al. 2003, Pirilä et al. 2003).

Primary antibodies were the same as these used in immunocytochemistry, and for the detection of MMP-2, polyclonal anti-human MMP-2 (1:500) (Westerlund, Mellanen, 21, 23-24/III) (**II**, **III**), and a 1:800 solution of rabbit polyclonal anti-human Ln-5 γ2-chain were used (Pirilä et al. 2003) (**IV**). After electrophoresis on a 10% (**II, III**) or an 8 % (**IV**) SDS-polyacrylamide gel, samples were transferred to nitrocellulose membranes electrophoretically. Nonspecific binding was blocked by incubation with 3% gelatin (Sigma, St. Louis, MO, USA) (**II**) or low-fat dried 5% milk (Valio, Helsinki, Finland) (**III**). Following incubation with primary antibody (overnight at 22°C), secondary peroxidase-coated goat antirabbit antibodies (1:1000, 1 h at 22°C) (Sigma) (**II**) and horseradish peroxidase-labeled donkey anti-rabbit (NA 934) for MMP-8 (Amersham Pharmacia Biotech, Buckinghamshire, UK), sheep anti-mouse antibodies (NA 931) for MMP-13 (dilutions 1:800) (Amersham Pharmacia Biotech, Buckinghamshire, UK) (**III**), and goat anti-rabbit antibody (**IV**) were used. Detection was carried out by using nitroblue tetrazolium and 5-bromochloro-3 indolyl-phosphate stains in N-N-dimethylformamide (Sigma) (**II**) or with enhanced chemiluminescence (ECL) detection reagents on radiographic films according to the manufacturer's instructions (**IV**) (Amersham Pharmacia Biotech, Buckinghamshire, UK).

The membranes/films were scanned and analyzed qualitatively (**II, III**), or qualitatively and semiquantitatively (**IV**) in the manner described in section 7.4.1.1.

7.5. Statistical methods

Study I: Effects of repeated lavages on reference values were tested by repeated-measures ANOVA. In case of a significant effect, the Bonferroni *t*-test was used to evaluate difference between successive sessions.
Student's paired *t*-test was used to compare of arterial blood gas values, numbers of cells, and cell viability before and after lavage. Correlation coefficients were determined by using Pearson's correlation analysis.

Study II: A paired Student's *t*-test was used to detect differences between pre-BAL and PaO_2, $PaCO_2$, and A-aDO_2 values. Unpaired Student's *t*-test was used for comparisons between PE and healthy dogs' BALF and blood values. In case of unequal variance, Welch's correction was used. Pearson's correlation coefficients (r) were calculated to detect correlations between BALF and blood eosinophils as well as between BALF cell count and gelatinolytic activity. Spearman's correlation test was used for correlations between radiographic scoring and blood eosinophil count, BALF cytology, and BALF enzyme activities. The Wilcoxon signed-rank test was used to compare PE dogs' radiographic scores before and after treatment. The Mann-Whitney U-test was used for comparisons between PE and control dogs' radiographic scores.

Study III: The Mann-Whitney U-test was used to detect differences in collagenolytic activities between PE and control dogs as well as between cell-free BALF and native BALF. The Wilcoxon signed-rank test was used for comparisons between APMA-treated and nontreated samples. Spearman's correlation coefficients (r) were calculated to detect correlation between the percentage of degraded collagen I in cell-free BALF and the BALF cell count and differential cell count.

Study IV: The Mann-Whitney U-test was used to assess the difference in of Ln-5 γ2-chain immunoreactivities between PE and healthy dogs. Spearman's correlation test was used for calculations of correlation coefficients (r) between BALF cellular counts and Ln-5 γ2-chain levels. Wilcoxon signed rank test was used for comparison between pre and post-treatment Ln-5 γ-2-chain levels.

Statistical analyses were performed using commercial statistical software programs (Statgraphics version 6.0, Manugistics, Rockville, MD, USA; SAS version 6.12, SAS Institute Inc., Cary, NC, USA (I); Prism 3.0, Graph Pad Software Inc., San Diego, CA, USA (**II-IV**). A p-value <0.05 was considered significant.

8. RESULTS

8.1. Symptoms, clinical examination, radiographs, blood and faecal samples, and treatment response

8.1.1. Control dogs

Control dogs expressed no symptoms, and no signs of respiratory or other diseases were found. Pulmonary radiographs showed minimal or no abnormal findings

before the first and after the last lavage. Hematological and biochemical values, arterial blood gas analyses, and fecal flotation tests were not suggestive of any diseases. These results are presented in detail in Study I.

8.1.2. PE dogs

Continuous cough was present in 95% of dogs, and four of these dogs also had other symptoms such as sneezing, dyspnea, and exercise intolerance. Fever (>39.5°C) was noted in two dogs. The mean duration of symptoms was 20 weeks (range 3-80 weeks) (**II, T**able II**)**.

Thoracic radiographs revealed interstitial and bronchial densities in all PE dogs, with the changes being severe in four and six dogs, respectively. The radiographic score of healthy dogs differed significantly from that of PE dogs ($p<0.001$) (Table 2). PE dogs' radiographic score correlated significantly with their BALF cell count ($r=0.63$, $p<0.01$), BALF eosinophil percentage ($r=0.50$, $p<0.05$) and eosinophil count ($r=0.62$, $p<0.01$), but not with their blood eosinophil count. The radiographic score was significantly lower after treatment ($p<0.001$).

Table 2. Radiographic scores of pulmonary eosinophilia (PE) (n=20) and healthy (n=13) dogs (A), and radiographic scores of PE dogs' (n=12) before and after treatment (B). * p < 0.05, ** p < 0.01, *** p < 0.001

A.

Pattern	Healthy dogs, mean ± SD	PE dogs, mean ± SD
Alveolar	0 ± 0	0 ± 0
Interstitial	0.1 ± 0.3	1.5 ± 0.8 ***
Bronchial	0.2 ± 0.4	1.7 ± 0.9 ***
Radiographic score	0.3 ± 0.6	3.2 ± 1.6 ***

B.

Pattern	Pre-treatment, mean ± SD	Post-treatment, mean ± SD
Alveolar	0 ± 0	0 ± 0
Interstitial	1.7 ± 0.8 ***	0.5 ± 0.5
Bronchial	1.9 ± 1.0 ***	0.5 ± 0.5
Radiographic score	3.6 ± 1.8 ***	1 ± 0.9

Significantly higher values were noted in leukocytes, eosinophils and basophils (leukocytosis in 30%, eosinophilia in 50%, and basophilia in 55% of the PE dogs) ($p<0.05$, $p<0.01$, $p<0.05$) in blood differential count; other values did not differ from those of healthy dogs (Table 3). Biochemical blood values were within the normal range, and fecal samples were negative for parasites.

Table 3. Blood cytology in healthy (n=16) and pulmonary eosinophilia (PE) (n=20) dogs. * p < 0.05, ** p < 0.01.

	Healthy dogs		PE dogs	
.	Mean±SD	95% CI	Mean±SD	95% CI
Packed cell volume (%)	46.6 ± 5.1	43.9 – 49.3	49.5 ± 5.0	47.1 – 51.8
Leukocytes (cells x $10^3/\mu l$)	10.2 ± 4.1	8.1 – 12.4	17.8 ± 12.3 *	12.1 – 23.6
Segmented neutrophils (cells x $10^3/\mu l$)	7.3 ± 3.5	5.3 – 9.3	8.0 ± 4.1	6.0 – 10.0
Band neutrophils (cells x $10^3/\mu l$)	0.03 ± 0.1	0.0 – 0.1	0.03 ± 0.1	0.0 – 0.1
Lymphocytes (cells x $10^3/\mu l$)	2.2 ± 0.7	1.8 – 2.6	2.5 ± 1.8	1.6 – 3.3
Monocytes (cells x $10^3/\mu l$)	0.5 ± 0.2	0.3 – 0.6	0.6 ± 0.4	0.5 – 0.8
Eosinophils (cells x $10^3/\mu l$)	0.4 ± 0.3	0.2 – 0.5	6.7 ± 9.1 **	2.1 – 11.2
Basophils (cells x $10^3/\mu l$)	0.0 ± 0.0	0.0 – 0.0	0.5 ± 0.8 *	0.1 – 0.8

Clinical criteria (anamnesis, clinical examination, blood leukocyte and differential cell counts, thoracic radiographs, BALF cytology) were used for the evaluation of the treatment response. Ten of 12 dogs re-evaluated had responded well to therapy. Two dogs still coughed, although radiographic changes and blood eosinophilia had decreased. Owners reported relapses in six dogs, which were then remedicated. (**II**, Table II).

These results are presented in detail in Study II**,** Tables II, III, and V.

8.2. Arterial blood gas analysis

Mean ± SD of A-aDO_2, PaO_2, and $PaCO_2$ were determined (Table 4). Arterial pre-BAL PaO_2 values measured at the first visit were significantly lower and A-aDO_2 values higher in PE dogs than in healthy dogs ($p<0.01$ and $p<0.001$, respectively), while no difference was noted in $PaCO_2$ levels. However, A-aDO_2 and PaO_2 values were within reference values, except in three PE dogs showing mild hypoxemia (<85 mmHg i.e. < 11.3 kPa). PaO_2 values decreased and $PaCO_2$ and A-aDO_2 values increased due to BAL procedure equally in both control ($p<0.001$, $p<0.05$, $p<0.001$, respectively) and PE dogs ($p<0.001$, $p<0.01$, $p<0.001$).

Repeated lavages in control dogs did not significantly affect A-aDO_2, PaO_2, or $PaCO_2$ before BAL. Medication of PE caused no clear trend of an increase or decrease in PaO_2, $PaCO_2$, and A-aDO_2 values in the repeated lavage group.

Table 4. Results of arterial blood gas analysis in healthy (n=15) and pulmonary eosinophilia (PE) (n=18) dogs. Significant differences between healthy and PE dogs are indicated. * p < 0.05, ** p < 0.01, *** p < 0.001.

	Healthy dogs		PE dogs	
.	Mean±1 SD	95% CI	Mean±1 SD	95% CI
PaO_2, pre-BAL(mmHg)	105.2 ± 5.1	102.4 – 108.0	94.8 ± 10.4 **	89.3 – 100.4
postBAL(mmHg)	79.8 ± 10.3	74.1 - 85.5	74.7 ± 12.2	68.2 – 81.2
$PaCO_2$, preBAL(mmHg)	36.1 ± 2.0	35.0 - 37.3	36.9 ± 3.2	35.3 – 38.5
post-BAL(mmHg)	38.6 ± 1.5	37.8 – 39.5	39.8 ± 4.7	37.3 – 42.3
A-aDO_2, pre-BAL (mmHg)	2.5 ± 5.1	-0.3 – 5.3	16.1 ± 9.1 ***	11.2 - 20.9
post-BAL (mmHg)	27.2 ± 9.6	21.9 – 32.5	35.9 ± 10.8 **	30.1 – 41.6
Differences due to BAL:				
Pre-BAL PaO_2 – post-BAL PaO_2	25.5 ±16.7	15.8 – 35.1	18.6 ± 8.8	13.2 – 23.9
Post-BAL A-aDO_2 – pre-BAL A-aDO_2	22.4 ± 15.5	13.5 – 31.4	22.5 ± 15.2	13.4 – 31.7

8.3. Bronchoscopic findings and cytological examination of BALF

8.3.1. Control dogs

Bronchoscopy revealed no abnormal findings in the airways.

Mean (± SD) recovery volume of instilled fluid was 63 ± 8%. No correlation was present between percentage of fluid recovered and A-aDO_2 after BAL.
The recovered BALF (mean ± SD) 103.9 ± 68.5 cells/µl, and the resuspended cell pellet contained 61.8 ± 46.3 cells/µl. Alveolar macrophages (76.4 ± 48.2 cells/µl, 75.3 ± 6.9%) were the dominant cells detected. In order of decreasing frequency, the remaining cells consisted of lymphocytes (15.0 ± 13.7 cells/µl, 13.3 ± 5.8%), neutrophils (6.4 ± 7.1 cells/µl, 4.9 ± 3.8%), eosinophils (3.5 ± 5.0 cells/µl, 3.6 ± 4.7%), mast cells (2.0 ± 1.8 cells/µl, 2.2 ± 1.7%), epithelial cells (0.6 ± 1.0 cells/µl, 0.6 ± 0.7%), and plasma cells (0.2 ± 0.3 cells/µl, 0.3 ± 0.4%). In addition, 95% CIs for total and differential cell counts were determined (Table 5).

Significant variations among repeated BAL sessions in control dogs were detected for percentages of macrophages, lymphocytes, and mast cells, whereas variations in cell counts and in percentages of neutrophils, eosinophils, plasma cells, and epithelial cells were not significant. Between two successive sessions, a significant difference in values was found only for mast cells (between BAL sessions 5 and 6), whereas all other differences were not associated with a specific dog or BAL session.

A significant ($p=0.001$) number of cells were lost during the two centrifugations and one washing (range, 2-88%; mean ± SD, 59 ± 13%). Cell loss was not dependent on a specific BAL session or dog. Correlation analysis indicated a significant strong positive correlation between the number of cells in the original BALF sample and the number of cells in the resuspended cell pellet ($r=0.87$, $p<0.001$). No correlation was observed between body weight of a dog and cell count in the original BALF sample.

Cell viability in the original BALF samples was 85 ± 17%. Cell viability decreased significantly ($p=0.01$) with the two centrifugations and 1 washing to 71 ± 11%.

8.3.2. PE dogs

The most typical bronchoscopic finding was increased airway secretion (13/17). Mucosal hyperemia (5/17) or nodulation (3/17), blood-tinged secretions (2/17), and dynamic collapse of main-stem bronchi (2/17) were also noted (**II**, Table II). In the control bronchoscopy after the treatment, secretions had decreased markedly in four dogs. Mean (± SD) recovery volume of instilled fluid was 46±11 %.

Bacterial culture of BALF was negative in PE dogs, and no intracellular bacteria were found in PE dogs'or in healthy dogs' BALF samples. Airway cytology was also negative for parasites.

BALF cytology (mean ± SD) was 2272.9 ± 2353.0 cells/µl for total cells, 263.9 ± 242.3 cells/µl (20.9 ± 18.7%) for macrophages, 88.3 ± 63.5 cells/µl (7.1 ± 7.4%) for lymphocytes, 115.2 ± 163.6 cells/µl (8.2 ± 11.1%) for neutrophils, 1784.2 ± 2088.5 cells/µl (62.3 ± 24.4%) for eosinophils, 17.6 ± 25.6 cells/µl (1.0 ± 0.9%) for mast cells, 6.2 ± 10.9 cells/µl (0.5 ± 0.8%) for epithelial cells, and 0.6 ± 1.7 cells/µl (0.1 ± 0.3%) for plasma cells (Table 5). The cell count, the number and percentage of eosinophils, and the number of macrophages, lymphocytes, mast cells, neutrophils, and epithelial cells were significantly elevated in PE dogs' BALF. Both the number and the percentage of BALF eosinophils correlated positively with the number of eosinophils in blood ($r=0.60$, $p<0.01$) ($r=0.74$, $p<0.01$) (Study **II**, Table VI). The BALF cell count, eosinophil count, and percentage of eosinophils decreased after treatment (Figure 3).

Figure 3. BALF total cell count (A), BALF eosinophil count (B), blood eosinophil count (C), and BALF eosinophil percentage (D) before (week 0) and after treatment.

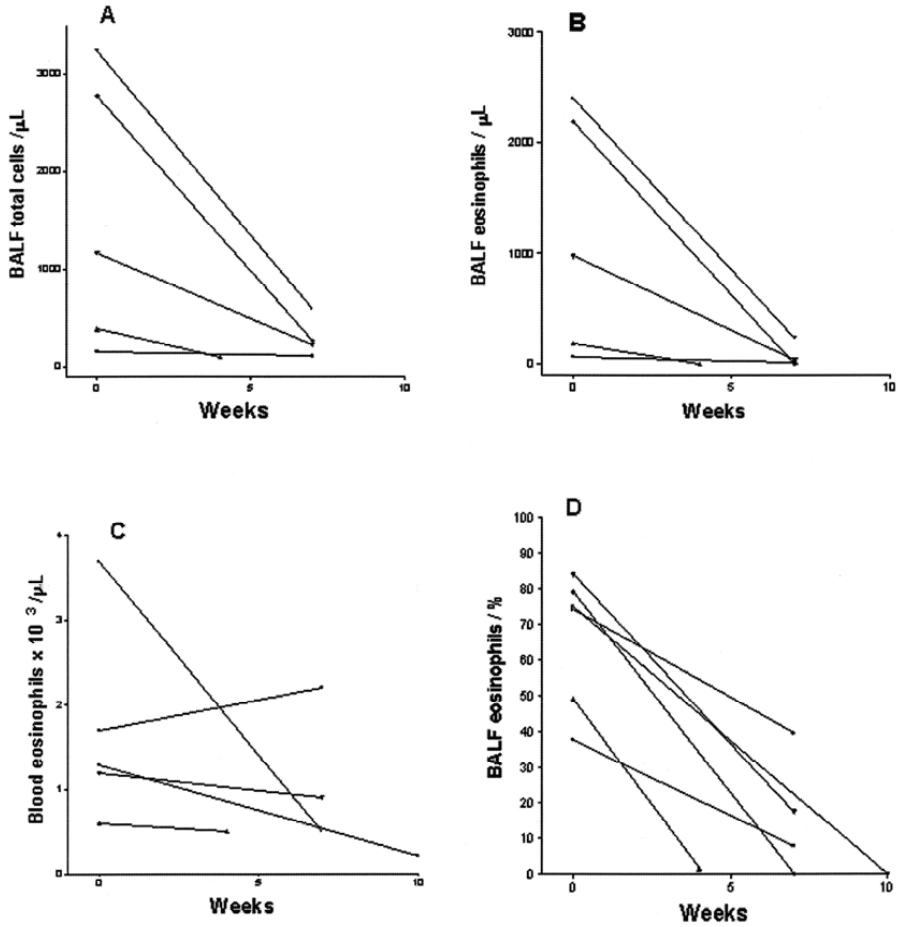


Table 5. Cytological findings in bronchoalveolar lavage fluid of healthy (n=16) and pulmonary eosinophilia (PE) (n=20) dogs. * p < 0.05, ** p < 0.01, *** p < 0.001.

	Healthy dogs		PE dogs	
	Mean ± SD	95% CI	Mean ± SD	95% CI
Viability (%)	84.8 ± 17.3	76.1 – 93.4	90.9 ± 5.4	88.2 – 93.5
Cell count (cells/µl)	103.9 ± 68.5	67.4 – 140.5	2272.9 ± 2353.0 **	1102.7 – 3443.0
Macrophages (%)	75.3 ± 6.9	71.8 – 78.7	20.9 ± 18.7 ***	12.1 – 29.6
(cells/µl)	76.4 ± 48.2	50.7 – 102.1	263.9 ± 242.3 **	143.4 – 384.4
Lymphocytes (%)	13.2 ± 5.8	10.3 – 16.1	7.1 ± 7.4 *	3.7 – 10.6
(cells/µl)	15.0 ± 13.7	7.7 – 22.3	88.3 ± 63.5 ***	56.7 – 119.9
Neutrophils (%)	4.9 ± 3.8	3.0 – 6.8	8.2 ± 11.1	3.0 – 13.4
(cells/µl)	6.7 ± 7.1	2.6 – 10.2	115.2 ± 163.6 *	33.8 – 196.5
Eosinophils (%)	3.6 ± 4.7	1.2 – 6.0	62.3 ± 24.4 ***	50.9 – 73.7
(cells/µl)	3.5 ± 5.0	0.8 – 6.2	1784.2 ± 2088.5 **	745.5 – 2822.8
Mast cells (%)	2.2 ± 1.7	1.3 – 3.0	1.0 ± 0.9 *	0.5 – 1.4
(cells/µl)	2.0 ± 1.8	1.0 – 3.0	17.6 ± 25.6 *	4.9 – 30.3
Epithelial cells (%)	0.6 ± 0.7	0.2 – 0.9	0.5 ± 0.8	0.1 – 0.9
(cells/µl)	0.6 ± 1.0	0.1 – 1.1	6.2 ± 10.9 *	0.8 – 11.6
Plasma cells (%)	0.3 ± 0.4	0.1 – 0.5	0.1 ± 0.3	0.0 – 0.2
(cells/µl)	0.2 ± 0.3	0.1 – 0.4	0.6 ± 1.7	0.0 – 1.4

8.4. Histological samples

Histological samples taken from the control dogs were typical of the lungs of aging dogs (Robinson and Gillespie 1973) with enlarged distal air spaces and atrophic interalveolar septa in all samples, and anthracotic pigment and some foci of interstitial fibrosis, in the cranial lung lobes in particular. Three dogs had minimal amounts of pulmonary mineralization. Numbers of inflammatory cells were normal. All the changes identified in the nonlavaged cranial lobes were similar to, or more prominent than, changes in the lavaged caudal lobes (**II**).

8.5. Assays

8.5.1. Functional assays

8.5.1.1. Gelatin zymography (II)

PE and healthy dogs' native BALF and cell-free BALF revealed gelatinolytic activity in zymography. Five clear gelatinolytic bands were detected; two high molecular weight bands (>100 kDa) representing complex forms of gelatinase, one band proMMP-9 (range 84-92 kDa), one active MMP-9 (range 70-82 kDa), and one proMMP-2 (range 61-72 kDa) were identified. In addition, low molecular weight bands (<50 kDa) were detected. All, except one, of the PE and healthy dogs' samples contained complex forms, proMMP-9, and proMMP-2. (Figure 4). Total gelatinolytic activity, complex forms, proMMP-9, and active MMP-9 were significantly elevated in PE dogs' cell-free BALF ($p<0.001$, $p<0.05$, $p<0.01$, $p<0.05$, respectively) and native BALF (prior centrifugation) ($p<0.01$, $p<0.01$, $p<0.01$, $p<0.05$) as compared with healthy dogs' BALF (Figure 5). Total gelatinolytic activity and MMP-9 level in cell-free BALF correlated with BALF neutrophil count ($r=0.83$, $p<0.0001$) ($r=0.80$, $p<0.0001$), but not with any other cell counts in BALF.

Figure 4. Gelatin zymogram of cell-free BALF from a dog with pulmonary eosinophilia (PE) and from a healthy dog.

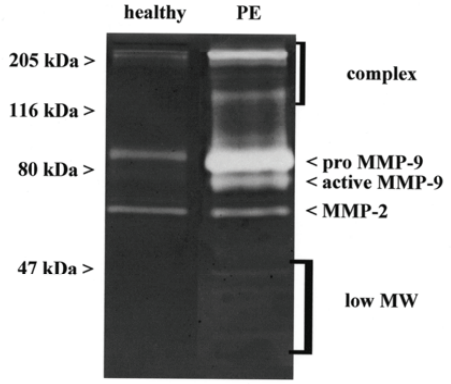
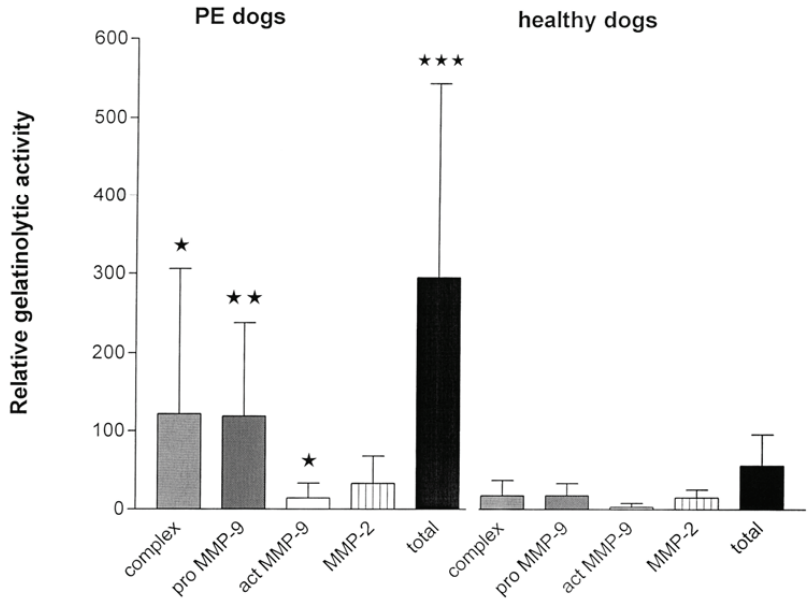


Figure 5. Relative gelatinolytic activities in cell-free BALF of dogs with pulmonary eosinophilia (PE) (n=20) and healthy (n=16) dogs (mean ± SD). Analyzed as total activity, complex forms, proMMP-9, active MMP-9, and MMP-2. * p < 0.05, ** p < 0.01, *** p < 0.001.



8.5.1.2. Type I collagen degradation (III)

Collagenolytic activity was detected in both PE and healthy dogs' native BALF and cell-free BALF samples with and without APMA treatment. PE dogs' collagenolytic activity was significantly higher than healthy dogs' activity in cell-free BALF ($p<0.001$) and native BALF ($p<0.05$), and APMA treatment had no significant effect on the amount of collagen I degradation. Neither PE dogs' nor healthy dogs' collagenolytic activity in cell-free BALF differed significantly from that in native BALF ($p=0.07$, $p=0.25$; respectively) (Figure 6, Table 6).

Figure 6. Collagen I degradation in PE and healthy dogs' BALF with and without APMA treatment.

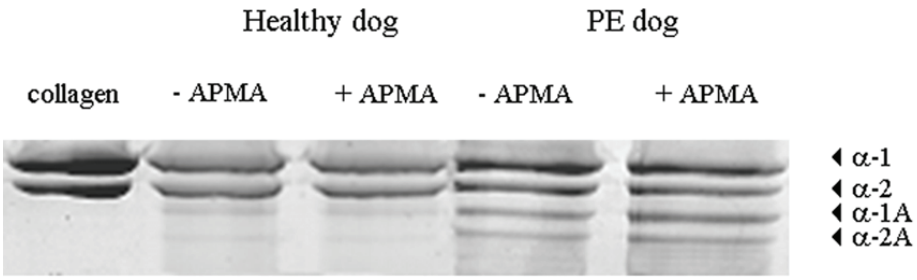


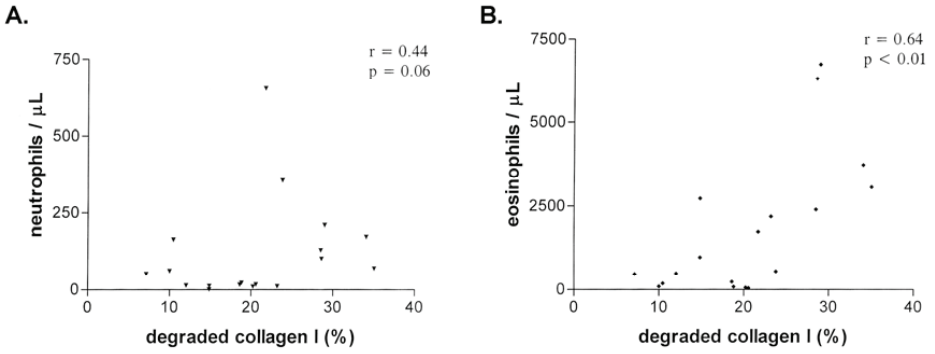
Table 6. Collagenolytic activity before and after APMA treatment in healthy and PE dogs' cell-free and native BALF, expressed as percentage of collagen I degraded. Values reported are medians (ranges).

	PE dogs		Healthy dogs	
	Cell free BALF (n = 20)	Native BALF (n = 9)	Cell free BALF (n = 16)	Native BALF (n = 9)
+ APMA	21.8 (5.8-44.8)***	44.9 (0.0-95.8)	6.8 (0.0-31.2)	12.1 (0.0-34.3)
- APMA	20.4 (7.1-39.9)***	35.6 (0.0-100.0)*	7.3 (0.0-67.5)	18.2 (0.0-21.8)

n, number of dogs
APMA, aminophenyl mercuric acetate
* $p < 0.05$ as compared with healthy dogs
*** $p < 0.001$ as compared with healthy dogs

A significant positive correlation was noted between the percentage of degraded collagen I in the PE dogs' cell-free BALF and the cell counts of BALF eosinophils ($r=0.64$, $p<0.01$), macrophages ($r=0.64$, $p<0.01$), lymphocytes ($r=0.57$, $p<0.05$), and mast cells ($r= 0.70$, $p< 0.01$) as well as the cell count of BALF ($r=0.68$, $p<0.01$). However, there was no significant correlation with the BALF neutrophil ($r=0.44$, $p=0.06$) or epithelial cell ($r=0.12$, $p=0.62$) counts and the correlation with BALF plasma cell count was negative ($r=-0.41$, $p=0.09$). The peripheral eosinophil and leukocyte counts did not correlate with the collagenolytic activity in cell-free BALF ($r=0.22$, $p=0.39$; $r=0.17$, $p=0.47$ respectively) (Figure 7).

Figure 7. Correlation between the percentage of degraded collagen I and bronchoalveolar lavage fluid (BALF) neutrophil (A) and eosinophil (B) counts.



8.5.2. Immunological assays

8.5.2.1. Immunocytochemistry (II, III)

Major immunoreactivity for MMP-8 and MMP-9 was detected in BALF macrophages and epithelial cells, while no expression of MMP-9 was noted in BALF eosinophils. MMP-13 was found in macrophages (Figure 8A, 8B).

Figure 8A. Immunocytochemical stainings (A-D) and May-Grunwald-Giemsa staining of PE dogs' BALF cells (E). Negative control (A), MMP-13 immunopositive macrophages (B), MMP-8 immunopositive macrophages (C), MMP-8 immunopositive epithelial cells (D), and eosinophils in PE dog's BALF cytospin slide (E). Bar = 20 μm.

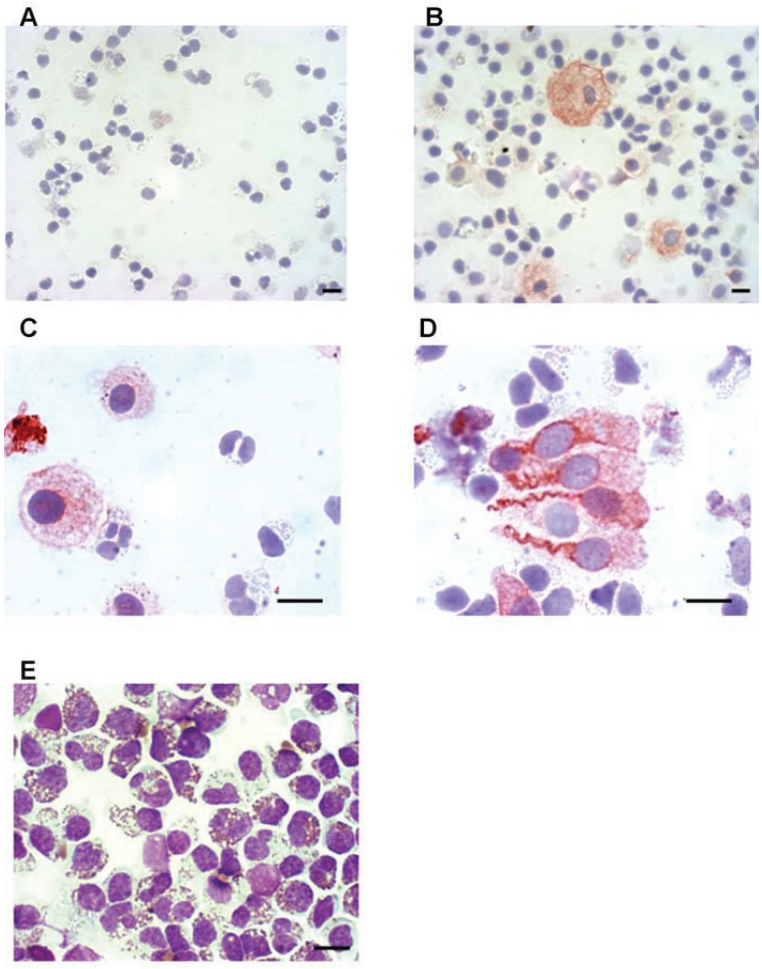
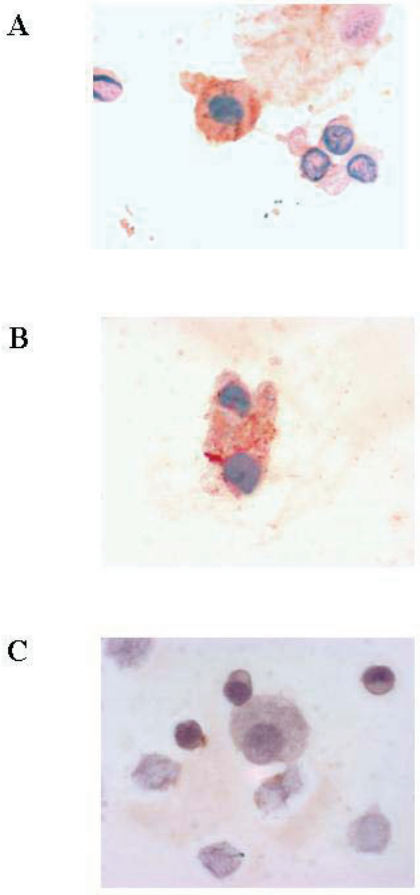


Figure 8B. Immunocytochemistry staining of BALF cells with an antibody against MMP-9. Immunopositive macrophage (A), immunopositive epithelial cell (B), and negative control (C).

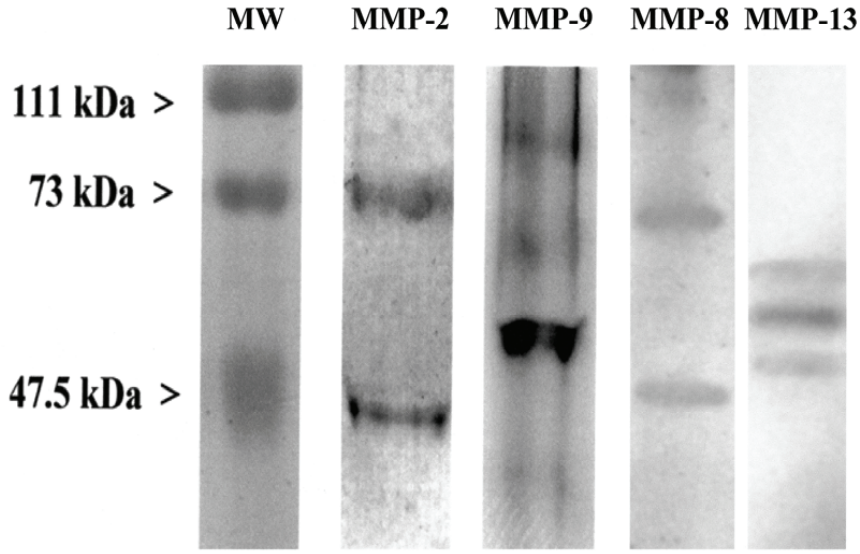


8.5.2.2. Western immunoblotting (II-IV)

The main gelatinolytic proteinases were further identified by Western immunoblotting to be MMP-2 and MMP-9 (**II**). Three different MMP-9 species with molecular weights of approximately 90-94 kDa, 69-75 kDa, and 54 kDa were identified. MMP-2 Western immunoblotting revealed two species with approximate molecular weights of 72 kDa and 41 kDa (Figure 9 A).

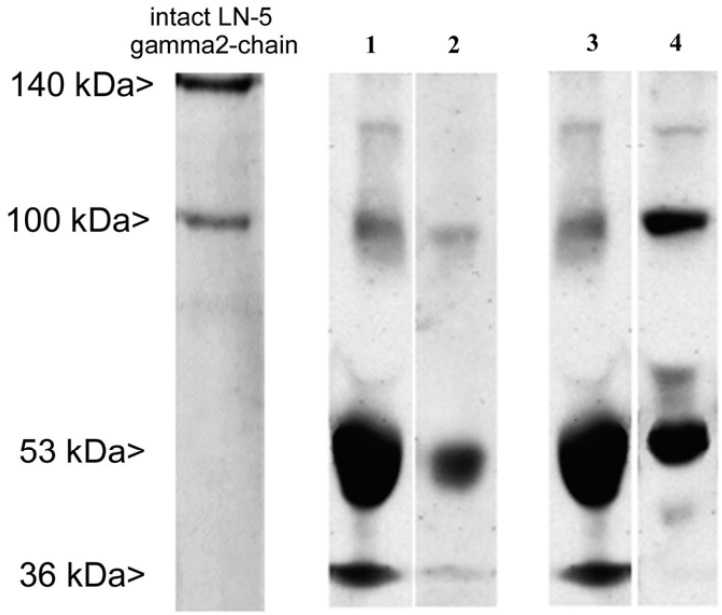
MMP-8 species of four different molecular weights were found: >100 kDa, 75-80 kDa, 40-50 kDa, and <30 kDa. MMP-13 species showed two different bands representing molecular weights of 60 kDa and 50-55 kDa (Figure 9 A).

Figure 9 A. MMP-9, MMP-2, MMP-8, and MMP-13 Western blot of BALF from a PE dog. Molecular weights indicated on the left.



Western immunoblotting of the Ln-5 γ2-chain (**IV**) revealed multiple immunoreactive species ranging from 31 kDa to 200 kDa, with mean sizes of >200, 159,126, 94, 73, 53, 42, and 36 kDa (Figure 9B).

Figure 9 B. Western immunoblot of laminin-5 γ2-chain immunoreactivities in pulmonary eosinophilia (PE) dog (1), healthy dog (2), PE dog before (3) and after (4) corticosteroid medication. Intact laminin-5 γ2-chain is demonstrated. Mobilities of molecular weight markers indicated on the left.

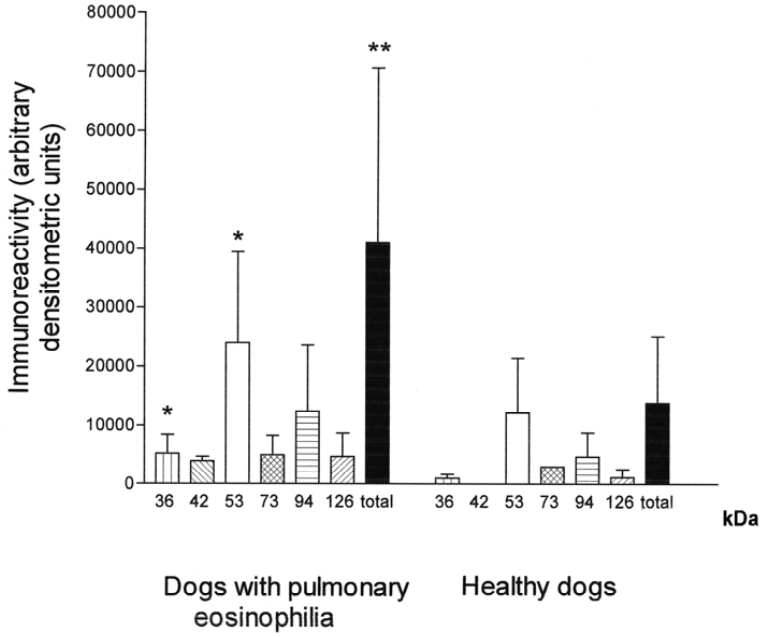


The most frequent species in PE dogs' BALF were 36, 53, and 94 kDa (in 65%, 100%, and 75% of samples, respectively), and in healthy dogs' BALF 53, 36, and >200 kDa (100%, 38%, and 31%, respectively). In healthy dogs' samples, bands >90 kDa were extremely weak. Levels of total immunoreactivity and 36-kDa and 53-kDa fragmented species were significantly elevated in PE dogs' samples ($p<0.01$, $p<0.01$, $p<0.05$, respectively) as compared with healthy dogs' samples. Corticosteroid treatment reduced the levels of fragmented Ln-5 γ2-chain 36-kDa ($p<0.05$) and 53-kDa species (not significantly) in PE dogs' BALF (Figure 10 A, B).

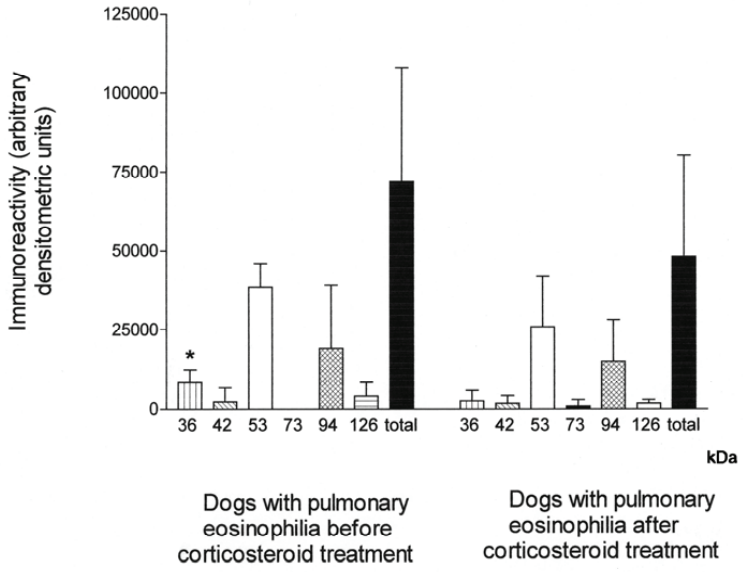
No correlation was present between total Ln-5 γ2-chain immunoreactivity or any of the Ln-5 γ2-chain immunoreactivities and BALF total or differential cell counts.

Figure 10. Multiple species of laminin-5 γ2-chain immunoreactivities in bronchoalveolar lavage fluid (BALF) from pulmonary eosinophilia (PE) and healthy dogs (A). and PE dogs before and after corticosteroid treatment (B). Data presented as mean ± SD.

A.



B.



9. DISCUSSION

9.1. Clinical findings, blood samples, and thoracic radiographs

The key clinical features of PE dogs were similar to those reported in earlier studies; the PE dogs were young although the age range was broad, and the most prominent clinical sign was persistent cough (Lord et al. 1975, Grauer and Riesendal 1977, Corcoran et al. 1991, Clercx et al. 2000). Peripheral blood eosinophilia was noted in half of the dogs, and thus, is only suggestive for this disease. Approximately equal percentage has been described by Corcoran et al. (1991) and Clercx et al. (2000). We found radiographic changes to mostly be interstitial and bronchial; the alveolar patterns noted in a previous study Clercx et al. (2000) (probably connected to bacterial pneumonia) were not seen here.

9.2. Arterial blood gas analysis

Our PE dogs' A-aDO_2 and PaO_2 values were within reference values, with only three dogs showing mild hypoxemia (King and Hendricks 1995). Had the lesions been more advanced at the alveolar level (detectable also in radiographs), changes in blood gases would likely have been detected more often, as has been noted in pneumonia (Wingfield et al. 1997). This may also explain why the BAL procedure decreased the PaO_2 values and increased the A-aDO_2 values equally in healthy dogs and PE dogs, and why the medication caused no clear trend of an increase or a decrease in PaO_2, $PaCO_2$, and A-aDO_2.

9.3. BAL and BALF

The recovery percentage of instilled fluid was lower in PE dogs than in healthy dogs, consistent with the earlier findings of canine and human BAL procedures in healthy and diseased patients (Hawkins 1990, Baughman and Rennard 1999, Clercx et al. 2002).

We were able to set reference values for healthy dogs' BALF cytology. Reference values for BALF are dependent on the methods used in sample collection and in laboratory processing. However, these techniques have not been rigorously standardized in veterinary or human pulmonology. BALF laboratory analysis methods, e.g.filtration of the sample to remove mucus prior to centrifugation, speed, duration, and number of centrifugations, washing of cells, cytocentrifugation, and cell counting, differ between laboratories, causing variations in the results and their interpretation in practice and in the research field (Saltini et al. 1984). We found that two centrifugations and one washing caused significant cell loss, and a decrease in cell viability, as well as a large variation in these two variables among dogs. However, total cell counts of the original BALF and the resuspended cell pellet correlated significantly, and thus, reflected the cellular status of lavaged lobes. This differs from the study of Lapointe et al. (1994) who found that centrifugation does not cause significant cell loss of equine BALF cells. However, their study used only one

centrifugation. Similar to us, they noticed marked variation between individual horses. In studies of human BALF cells, centrifugation and washing have caused cell loss of 18-34% (Saltini et al. 1984, Mordelet-Dambrine 1984). In view of these findings, we recommend that separate reference values should be used for processed and nonprocessed cell counts. More reliable estimates of cellular content can, however, be obtained by using counts from nonprocessed, native BALF samples.

We used weight-adjusted amount of lavage fluid; not used in all studies reporting reference values for canine BALF adjust for weight (Rebar et al. 1980, Pinsker et al. 1980, Mayer et al. 1990, Hawkins 1990, Vail et al. 1995). However, usually such studies have had dogs with a fairly narrow range of weight, in contrast to the range for entire canine species. Thus, the range of fluid per kilogram is also narrow. However, the amount of fluid per kilogram does affect cellular results; Pinsker et al. (1980) reported that a smaller volume (50 ml) lavage had a greater total cell count and concentration of neutrophils than a larger volume lavage (500-600 ml), which in turn had a higher concentration of bronchial cells. Our BALF cellular counts are comparable with those in most studies utilizing even somewhat higher fluid volumes and different laboratory and lavage procedures; the maximum cellular count reported for healthy dogs is four times higher than the cellular count here (Vail et al. 1995, Andreasen 2003). This difference is tolerable when values for comparison healthy and PE dogs are compared because of the great cytological (20 times, **II)** difference between these groups. However, differences with other respiratory diseases might be smaller.

BALF differential counts of healthy dogs are consistent with findings of previous studies (Pinsker et al. 1980, Rebar et al. 1980, Hawkins 1990, Vail et al. 1995). Relative values, in particular, remain constant when same cell types are included in the differential count. However, the use of relative values limits the interpretation of actual counts, especially when the total cell count changes because of a disease. Thus, our data suggest the concurrent use of both values. The most remarkable findings in PE dogs' BALF were the increased cell count and absolute and relative eosinophilia. Absolute eosinophil count together with increased cell count was considered better than relative count in distinguishing PE dogs from healthy dogs because no overlapping, noted in some relative eosinophil values, occurred. In addition, significantly elevated absolute numbers of macrophages, lymphocytes, neutrophils, mast cells, and epithelial cells as well as elevated relative numbers of macrophages, lymphocytes, and mast cells were found in PE dogs' BALF. The increase of cells other than eosinophils is also noteworthy. These cells may act as important inflammatory mediators as well, although eosinophils are the predominant cell in canine PE.

9.4. Effect of repeated lavages

The effect of repeated lavages on the health of the dogs was also studied by examining BALF cellular results, arterial blood oxygen concentrations prior to BAL, radiologic findings of the lungs, and results of histologic examination of pulmonary tissues. We found repeated lavages at 5- to 7-week intervals to be a safe procedure, not causing significant changes in cell counts, or permanent changes to the lavaged lung

based on the previous criteria. The incidental changes in relative counts of macrophages, lymphocytes, and mast cells were not associated with a specific dog or BAL session. Only once, a significant difference was noted in values between two successive sessions (mast cells in sessions 5 and 6) indicating that the repetition of lavage did not affect markedly on cell counts.

In previous studies, transient 48-h BALF neutrophilia has been described in dogs, horses, sheep, and monkeys after BAL, with changes in BALF cell types in sheep returning to baseline within 1-3 weeks after lavage (Cohen and Batra 1980, Woodside et al. 1983, Sweeney et al. 1984, Carre et al. 1985, Haley et al. 1989). The equal effect of BAL on healthy and PE dogs' PaO_2 and $A\text{-}aDO_2$ values enables the bronchoscopist to predict changes in oxygenation and thereby to evaluate safety of the procedure as well as the need for oxygen supplementation beforehand.

9.5. Etiology of PE

Dogs with PE represented 35% of all dogs undergoing BAL during the collection period. Attempts to find the primary etiology were made, investigating such factors as drug exposure, parasitic, bacterial, and fungal infections, and as a result 5 of 25 dogs were excluded from the study because a possible cause for eosinophilia (intestinal/nasal parasites in 3 dogs; bacterial infection of the lower respiratory tract in 2 dogs) was identified. In the remaining PE dogs, no nonrespiratory diseases capable of causing eosinophilia were found. In several previous studies, simultaneous dirofilariasis has been associated with PE (Lord et al. 1975, Calvert and Losonsky 1985, Halliwell and Gorman 1989). We did not, however, observe any signs of dirofilariasis. This was expected because all dogs were of Finnish origin, and Finland is not an endemic area for heartworm. Other parasites, (e.g. *Filaroides spp.*, *Crenosoma vulpi, Angiostrongylus vasorums*) possibly capable of causing PE, were also not found in these 20 remaining dogs in bronchoscopy, BALF cytology, or fecal flotation tests. None had a bacterial infection in the lower respiratory tract based on quantitative culture and the search for intracellular bacteria in BALF samples. However, all of our dogs had had previous antimicrobial therapy, which might have affected culture results but not BALF eosinophilia. In two dogs excluded from the study, bacterial infection may have been the cause of PE, as has been suggested in some earlier studies (Grauer and Riesendal 1977, Hawkins et al. 1995, Clercx et al. 2000). Aspergillosis and drug exposure as causes for PE were not very probable. Aspergillosis can cause a variety of pulmonary lesions, including PE, in humans, and drug exposure is also etiological factor for PE in humans, and perhaps in dogs as well (Noone 1986, Bauer 1989, Bain 1996, Clercx et al. 2000). However, proper control studies are needed to confirm these etiologies. Here, neither of these etiologies was suspected because Aspergillus was not found in any BALF cytology samples and none of the dogs needed antifungal medication. In addition, all of the dogs had had their symptoms prior to the administration of any medications, and there had also been medication-free periods during the disease. Based on the tests conducted, unknown or allergic etiologies were the only ones remaining. Allergy tests or hyposensitization treatments were not, however, performed. A potential role has been suggested for type I hypersensitivity (Suter 1984, Bauer 1989, Clercx 2002), but confirmation requires further studies. The remaining 20 dogs in our study were thus characterized as PE dogs with unknown or allergic etiology.

9.6. Recovery of PE

The recovery of dogs from PE was followed by clinical examinations, radiographs, hematology, arterial blood gas analysis, and BALF analysis. The BALF cellular and eosinophil absolute and relative counts were considered useful in the recovery evaluation. At least one of these markers of inflammation decreased during the medication period, although some abnormal values were still noted. A longer follow-up would reveal whether values return to normal levels or remain elevated despite the disappearance of clinical signs. Radiography was superior to hematology because hematological changes were lacking in half of the dogs and because the intensity of radiographic findings correlated with absolute and relative counts of BALF eosinophils. The overall response to therapy was good, allthough six dogs were reported to have relapsed later. These findings are consistent with previous reports of PE (Corcoran et al. 1991, Clercx et al. 2000).

9.7. MMPs

We detected significantly elevated gelatinolytic and collagenolytic activities in PE dogs' BALF compared with healthy dogs' BALF, suggesting a pivotal role of these MMPs in canine PE. BALF was utilized instead of pulmonary tissue due to extensiveness of the lung biopsy collection method. Gelatinolytic and collagenolytic MMPs can together create degradation of the pulmonary tissue. Other MMPs and proteinases, including such serine and cysteine proteinases, as trypsins, elastase, and plasmin, are likely involved in this process, acting in a cascade that activates and potentiates the catalytic effects of each component (Prikk et al. 2001a).

9.7.1. Gelatinolytic MMP-2 and MMP-9

The gelatinolytic proteinases, MMP-2 and -9, were present in both healthy dogs' and PE dogs' BALF samples, although the levels of total, complex, and pro- and active MMP-9 observed in PE dogs' cell-free BALF were significantly higher than in healthy dogs' specimens. MMP-2 levels were not elevated, and no active MMP-2 was detected. This suggests that the presence of MMP-9, particularly its activation, is one of the key molecular factors involved tissue destruction in PE. However, growing evidence also suggests that MMP-9 might be important in the migration of airway inflammatory cells *in vitro* (Okada et al. 1997, Kumagai et al. 1999). Thus, MMP-9 may play a dual role also in canine PE. Our findings of the contribution of MMP-9 are in accordance with earlier studies of equine and human pulmonary diseases showing the elevation of inductively expressed MMP-9 more frequently than constitutively expressed MMP-2 (Sepper et al. 1994, Torii et al. 1997, Raulo and Maisi 1998, Segura-Valdez et al. 2000). An increase in the levels and activation of MMP-9 has also been described in equine COPD, in human asthma, and COPD and in induced asthma (Raulo and Maisi 1998, Cataldo et al. 2003).

In some lung diseases, MMP-2 may also be involved in tissue remodeling. Elevated levels of MMP-2 have, for instance, been found in human emphysema and

COPD (Ohnishi et al. 1998, Segura-Valdes et al. 2000, Cataldo et al. 2000), but neither of these share common pathological features with PE.

To confirm the zymographic results, Western blot analysis with specific antibodies was applied (Westerlund et al. 1996, Sorsa et al. 1997). Three different MMP-9 immunoreactive species were observed. The species with the highest molecular weight (90-94 kDa) represented proMMP-9, the second species (69-75 kDa) the active form, and the third species (54 kDa) the proteolytically cleaved form (Westerlund et al. 1996, Sorsa et al. 1997). The two MMP-2 species visible represented the complex (72 kDa) and the lower (41 kDa) proteolytically truncated forms (Westerlund et al. 1996, Sorsa et al. 1997). In addition to pro- and active MMP-9 and proMMP-2, low molecular weight bands (<50 kDa) were detected in zymography analysis evidently representing proteolytically processed MMPs (Lehti et al. 1998, Maisi et al. 2002). Gelatinolytic activity noted at 25-28 kDa may represent trypsin-like serine proteinases capable of participating in MMP activation cascades (Prikk et al. 2001a).

Our findings suggest a major role for pro- and active MMP-9, and consistent with other publications, demonstrate that although MMP-2 and -9 are very similar in terms of structure and substrate specificity they appear to be involved in distinct ways in different diseases and animal species. Overall, inductively expressed and activated MMP-9 is abundantly present in inflammatory lung diseases in both dogs and humans (Ohbayashi et al. 2002, Atkinson and Senior 2003).

9.7.2. Collagenolytic MMP-8 and MMP-13

Involvement of collagenolytic MMPs in canine PE was shown in a similar manner as involvement of gelatinolytic MMPs; PE dogs' BALF demonstrated a high capability of degrading collagen I (the optimal collagen substrate with the least variability in degradation capacity between collagenolytic MMPs) compared with healthy dogs' BALF, implicating a role for collagenases in the pathogenesis of canine PE. These findings support and extend previous studies reporting the contribution of collagenolytic MMPs, both MMP-8 and MMP-13, in the pathological processes of various pulmonary diseases including equine and human COPD and human bronchiectasis, emphysema, and asthma (Hoshino et al. 1999, Segura-Valdez et al. 2000, Raulo and Maisi 2001, Prikk et al. 2001b, Imai et al. 2001, Prikk et al. 2002).

Western immunoblotting was performed to identify subspecies of collagenolytic MMP-8 and MMP-13. The four bands in MMP-8 Western immunoblot evidently represented complex forms of MMP-8 bound to endogenous MMP inhibitors, such as α_2-macroglobulin and/or TIMPs, or MMP-8 dimers (>100 kDa), highly glycosylated PMN-type proenzyme colocalizing with the human PMN-type MMP-8 (75-80 kDa) isoform, nonPMN-type MMP-8 isoform (40-50 kDa) and fragmented isoforms of MMP-8 (< 30 kDa) (Nagase 1997, Hanemaaijer et al. 1997, Romanelli et al. 1999, Prikk et al. 2001). The expression of nonPMN-type MMP-8 species has been shown to be upregulated by proinflammatory mediators TNF-α and IL-1ß in mesenchymal cell lines including fibroblasts, endothelial cells, chondrocytes and plasma cells (Chubinskaja et al. 1996, Cole et al. 1996, Hanemaaijer et al. 1997, Wahlgren et al. 2001). Differences caused by the degree of activation, fragmentation,

or glycosylation between various species are thus possible (Hanemaaijer et al. 1997, Kiili et al. 2002). Similar patterns of MMP-8 species released by degranulating neutrophils (Ding et al. 1996, Ding et al.1997, Owen et al. 2004) have been shown, at least in part, to represent shedding of membrane-bound forms of MMP-8, some of which exert anti-inflammatory or defensive properties in acute lung injuries (Owen et al. 2004).

APMA induces stepwise activation of MMPs by generating a reduction of 10-15 kDa in molecular weight by cleavage of the propeptide domain (Leppert et al. 1995). In our Study (**III**), preincubation of the native or cell-free BALF with APMA had no significant effect on collagen I degradation capacity. This indicates that collagenases are mostly in autoactive form in canine PE BALF. Oxidative stress is associated with various lung diseases, such as human asthma and equine lower respiratory diseases (Bowler and Crapo 2002), and MMP-8 activation by reactive oxygen species may not necessarily be related to the molecular weight changes (Sorsa et al. 1989, Saari et al. 1990, Ding et al. 1996, Westerlund et al. 1996, Ding et al. 1997, Owen et al. 2004). Autoactive collagenolytic activity and activated collagenase species have both been previously noted in human and equine respiratory secretions, differing from guinea pig peritoneal lavage fluid eosinophil extract which requires chemical activation before showing collagenolytic activity (Hibbs et al. 1982, Davis et al. 1984, Sepper et al. 1995, Raulo 2001a, Prikk et al. 2001b).

The two bands with MMP-13 immunoreactivities at approximately 60 kDa represent proenzymes, and the band at 50-55 kDa proteolytically activated species. The lower of the two bands at 60 kDa correspond to the molecular weight the MW of human rheumatoid fibroblast proMMP-13 (Knauper et al. 1996, Kiili et al. 2002).

9.7.3. Cellular sources of MMPs

BALF represents the cells and their protein content in the final stage after transmigration from the blood capillaries through the BM and the ECM into the alveolar space. During acute tissue inflammation, inflammatory cells are recruited from the circulation to the inflammation site (Corbel et al. 2000). Different proteinases degrading the tissue along the way play an important role in this phenomenon (Okada et al. 1997), and it is possible that the protein content of a cell may change during the transmigration.

We examined expression of MMP-8, -9, and -13 on BALF cytospin slides by immunocytochemical staining. Major immunoreactivities were recorded, focusing on the most frequent and morphologically easily identifiable cell types (macrophages, epithelial cells, and eosinophils). The double-staining method for identifying groups of cells occuring less frequently (e.g. neutrophils) was not utilized. Therefore, in addition to the cells listed below, MMPs can probably be detected in other BALF cells and pulmonary resident cells not examined here.

We localized immunoreativity for MMP-9 in alveolar macrophages and epithelial cells. Earlier studies of human and equine BALF samples have also reported similar immunoreactivities for macrophages and bronchial epithelial cells (Fukuda et al. 1998, Lemjabbar et al. 1999, Raulo et al. 2001b). The proteases of eosinophils have

not been investigated extensively. Our findings of MMP-9-negative BALF eosinophils in dogs resemble those of Becky Kelly et al. (2000) in humans with allergic airway disease. Their findings correspond to ours, best when freshly prepared BALF cells are examined by immunocytochemistry and zymography (Becky Kelly et al. 2000). However, elevated levels of MMP-9 mRNA and protein in bronchial tissues have been detected in some earlier studies of human asthma (Ohno 1987). Shute et al. (1997) also reported MMP-9-positive eosinophils in bronchial epithelium in asthmatic humans by immunohistochemical analysis Shute et al. (1997) and Okada et al. (1997) MMP-9 activity in purified and isolated peripheral blood eosinophil supernatant and in the conditioned medium of a transmigration assay. Indirect support for eosinophil-derived MMP-9 in bronchial asthma is provided MMP-9 levels being correlated with the number eosinophil, in BALF/sputum/bronchial tissue of asthmatic patients (Hoshino et al. 1999, Lee et al. 2003, Warner et al. 2004). Thus, the lack of immunostaining may be due to rapid release of MMP-9 from eosinophils; this could also explain our finding of MMP-9-negative eosinophils. However, the highly significant correlation found between cell-free BALF total gelatinolytic activity and MMP-9 level and BALF neutrophil count, but not eosinophils or any other cell counts in BALF (**II**), strongly suggests that eosinophils are not the primary source of MMP-9 in canine PE.

MMP-8 was regarded mainly as a neutrophil-specific enzyme until recently (Chubinskaja et al. 1996, Cole et al. 1996, Hanemaaijer et al. 1997, Prikk et al. 2001b). We found MMP-8 immunoreactivity in canine epithelial cells and macrophages. These results support previous studies reporting MMP-8 protein and mRNA expression in pulmonary monocytes/macrophages, bronchial epithelial cells, and glandular cells (Prikk et al. 2001b, 2002). No MMP-8 or MMP-13 immunoreactivity was noted in eosinophils, which is also in accordance with previous studies (Schlopov and Hasty 1998). However, correlation analysis did show a significant correlation between the percentage of degraded collagen I in BALF and BALF eosinophil count, supporting indirectly the hypothesis that eosinophils can also be sources of collagenolytic activity. Shlopov and Hasty (1998) have demonstrated that human blood eosinophils, showing negative immunostaining with MMP-8, MMP-13 and MMP-1 antibodies, contain a collagenolytic enzymes capable of degrading type I and II collagens similarly to MMP-1, -8, and -13. This is supported by earlier studies reporting collagenolytic activity in eosinophils against collagen types I and III (Hibbs et al. 1982, Davis et al. 1984). It has therefore been suggested that eosinophils produce an unknown enzyme with collagenolytic activity, and this enzyme could also exist in PE dogs' BALF eosinophils (Shlopov and Hasty 1998). Alternatively, eosinophils could act as inflammatory mediators, secreting substances that induce collagenolytic activity-possessing cells, such as epithelial cells, mast cells, plasma cells, and macrophages, to the inflammation site. Furthermore, collagenolytic activity probably also originates from residential cellular sources including bronchial epithelial cells, fibroblasts, type II pneumocytes, endothelial cells, and glandular cells (DiGirolamo and Wakefield 2000, Segura-Valdes et al. 2000, Imai et al. 2001, Prikk et al. 2001b, Wahlgren et al. 2001). However, numbers of these cells do not increase in the same manner as the numbers of inflammatory cells during the inflammation. Finally, MMP-2 and the soluble form of MMP-14 are able to degrade type I collagen similarly to MMP-1, -8, and -13 and thus, may contribute collagen I degradation (Konttinen et al. 1998, Fosang et al. 1998).

Major MMP-13 immunoreactivity in PE dogs' BALF was localized to the macrophages. This finding is in agreement with previous results in experimental

pulmonary silicosis in rats and in equine COPD (Perez-Ramos et al. 1999, Raulo et al. 2001).

9.7.4. Ln-5 γ2-chain

Ln-5 is a major component of the anchoring fibril in the specialized epithelial cell-ECM connection known as hemidesmosome. The breakdown of this link by the sloughing of the epithelial cells evidently causes the release of Ln-5 γ2-chain degradation products into BALF, simultaneously leading to a temporarily naked BM. We detected elevated levels of Ln-5 γ2-chain degradation products in BALF of PE dogs compared with control dogs, suggesting that epithelial injury results the contact between the BM and the alveolar/bronchial lumen during inflammation. At the histopathological level, this phenomenon of epithelial sloughing has been described in canine interstitial and bronchial lung diseases (Dungworth 1993, Dail 1994, Clercx et al. 2000), and is reflected in the increased number of epithelial cells in BALF in Study **II**. Study IV is the first to utilize measurement of Ln-5 γ2-chain degradation products to indirectly assess epithelial sloughing. Perez-Arellano et al. (1993), Behr et al. (1995), and Lehmjabbar et al. (1999) have previously used measurement of a BM degradation product, laminin fragment P1, in BALF of humans with diffuse interstitial lung disease, fibrosing alveolitis, and severe asthma. In addition, increased amounts of intraepithelial cytoskeletal structures, namely cytokeratin 19, have been observed in BALF from patients with idiopathic pulmonary fibrosis (Inage et al. 2000). The improvement in PE dogs' clinical, radiological, and hematological values described in Study II is associated with decreases in total Ln-5 γ2- chain, 36-kDa, and 53-kDa fragmented Ln-5 γ2-chain species indicating a beneficial effect of corticosteroid treatment on PE dogs' epithelium. Based on the significant difference between healthy and PE dogs in these values, we considerer that the fragmented Ln-5 γ2-chain species seemingly reflect an active ongoing inflammation which affects the BM.

In studies **II** and **III**, we detected increased collagenolytic and gelatinolytic MMP activity in PE dogs' BALF. Various MMPs, including the collagenolytic MMP-8 and -13, and gelatinolytic MMP-2 but not MMP-9, can process the Ln-5 γ2-chain into lower molecular size species (Giannelli et al. 1997, Pirilä et al. 2003). MMP-2 and -13 have been shown to be localized with the Ln-5 γ2-chain in inflammatory odontogenic keratocysts, and MMP-2 also in the endogenously produced Ln-5-rich matrix (Mäkelä et al. 1999, Wahlgren et al. 2003). Kivelä-Rajamäki et al. (2003) and Emingil et al. (2004 a,b) reported elevated levels of Ln-5 γ2-chain degradation products in inflammatory oral fluid exudates collected around dental implantitis-, and periodontitis-affected teeth, and high MMP-8 levels in the exudates. The effects of MMPs on the laminin-5 γ2-chain are likely a consequence of collective release and expression by many different cells at the inflammatory site. This speculation is based on correlations not noted being present between BALF total or differential cell counts and total, fragmented 36-kDa, or 53-kDa Ln-5 γ2-chain immunoreactivities. Earlier findings of pulmonary resident cells, such as bronchial epithelial cells, Clara cells, alveolar type II cells, fibroblasts, smooth muscle cells, and endothelial cells, producing and releasing MMPs (Wilhelm et al. 1989, Kenagy and Clowes 1994, Pardo et al. 1998, Yao et al. 1999, Prikk et al. 2001b, Zuo et al. 2002) support this notion.

We showed that Ln-5 γ2-chain degradation products decreased after treatment with corticosteroids (**IV**). The mechanism of this downregulation of Ln-5 γ2-chain degradation might be regulated by MMPs. Corticosteroid treatment has previously been shown to downregulate MMPs in cell-culture conditions *in vitro*, as well as in clinical studies of human asthma (Shapiro et al. 1991, Bosse et al. 1999, Hoshino et al. 1999). Emingil et al. (2004 a,b) have recently shown that MMP-inhibitor medication (i.e. low-dose or subantimicrobial dose doxycycline) for periodontitis patients reduced pathologically elevated levels of both Ln-5 γ2-chain fragments and MMP-8 to healthy control level. We conclude that elevated levels of gelatinolytic and collagenolytic MMPs indicate their involvement in pulmonary tissue disruption and shedding of Ln-5 γ2-chain degradation products from the BM in canine PE.

9. CONCLUSIONS

1. Cytological reference values for BALF in healthy dogs were established. The lavage procedure repeated multiple times at 5- to 7-weeks intervals was shown to be safe, with no apparent effects on dogs' health or BALF cytology.

2. PE dogs' radiographs revealed bronchointerstitial densities. In BALF cell count, the number and percentage of eosinophils and the numbers of macrophages, lymphocytes, neutrophils, mast cells, and epithelial cells were significantly elevated. Fifty percent of PE dogs had blood eosinophilia. The BAL procedure had an equal effect on PE and healthy dogs' arterial blood gas values. Treatment of PE decreased BALF cell count, eosinophil count, and percentage and radiographic changes.

3. Gelatinolytic activity, associated primarily with proMMP-9 and active MMP-9, was elevated in PE dogs' BALF compared with healthy dogs' BALF. MMP-9 immunoreactivity was predominantly found in macrophages and epithelial cells.
PE dogs' BALF also showed elevated collagenolytic activity compared with healthy controls. Western immunoblotting identified the presence of MMP-8 and MMP-13. Major immunoreactivity for MMP-8 was observed in macrophages and epithelial cells, and for MMP-13 in macrophages. These findings suggest that upregulation of both gelatinolytic and collagenolytic cascades contributes to tissue inflammation in canine PE.

4. Fragmented Ln-5 γ2-chain species reflect an active ongoing inflammation affecting the BM. This claim rests on the significant differences found between healthy and PE dogs the levels of total Ln-5 γ2-chain immunoreactivity, and 36-kDa and 53-kDa fragmented species, as well as on the downregulating effect of corticosteroid treatment on these immunoreactivities. The Ln-5 γ2-chain fragmented species could be considered as an adjunctive diagnostic marker of respiratory inflammation extending to the BM.

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