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Role for Cathepsin K in emphysema in smoke-exposed guinea pigs

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Abstract

The protease-antiprotease imbalance in the lung plays an important role in the pathogenesis of smoke-induced emphysema. The aim of this study was to characterize the proteolytic responses leading to emphysema formation in the guinea pig smoke exposure model. Guinea pigs were exposed to cigarette smoke for 1, 2, 4, 8 and 12 weeks. Age-matched guinea pigs exposed to room air served as controls. Cigarette smoke induced inflammation after 4 weeks and generated emphysematous changes in the guinea pigs after 12 weeks of smoke exposure. Increased phosphorylation of ERK and JNK MAP kinases was demonstrated post-cigarette smoke exposure. A decrease in elastin and collagen, and the loss of type III collagen were observed in the alveolar wall of smoke-exposed guinea pigs. Interestingly, no change was seen in the expression of collagenolytic matrix metalloproteinases. Furthermore, we observed a three-fold increase in cathepsin K activity in the lungs of smoke-exposed guinea pigs. The significance of this finding was supported by human studies that demonstrate increased expression of cathepsin K in the lungs of patients with emphysema. Elevation of cathepsin K in guinea pig lungs after smoke exposure likely constitutes a critical event leading to the disruption of lung extracellular matrix in this model.

Keywords

emphysema; proteases; inflammation

Introduction

Cigarette smoking is the major and most preventable cause of emphysema, a disease characterized by the abnormal enlargement of airspaces and the destruction of alveolar walls. Over 4 million people in the United States are affected by this condition [1]. To investigate the pathology of smoke-induced emphysema, the use of an adequate animal model is essential. Mice have been shown to develop emphysematous changes after exposure to cigarette smoke. The mechanism for the abnormal development of the structural changes in the lung extracellular matrix implicates members of serine- and cysteine-protease families and matrix metalloproteases [2,3,4]. Their dysregulated activity likely causes the pathological disruption of pulmonary collagen and elastin leading to emphysema [5]. Genetically modified mouse models overexpressing these proteases spontaneously developed emphysema [6,7]. Transgenic mice expressing human MMP-1 developed alterations in the lung architecture, as well as emphysematous changes due to the digestion of type III collagen [8]. Induced overexpression of the inflammatory cytokine IL-13 in murine lungs stimulated the expression of MMPs and

cathepsins, which resulted in emphysematous changes in the lung [9]. In addition, mice with a loss of MMP-12 were resistant to smoke-induced emphysema, and did not demonstrate any increase in lung inflammation and in the destruction of alveolar walls after smoke exposure [10]. These studies support the role of proteases in the development of emphysema and emphasize that multiple proteases are involved in this process.

While mouse models have been used extensively to study emphysema, the difference in enzyme repertoire and immune response in rodents compared to higher species constitutes a major limitation in our understanding of the complex pathology of human emphysema. For instance, the murine homologue for MMP-1, a protease that is believed to contribute significantly to human emphysema, is not present in the mouse lung and its activity is not equivalent to the activity of human MMP-1 [11]. It is therefore important to characterize other animal models that may provide valuable insight into the human disease.

The contribution of the guinea pig model of smoke-induced emphysema to the understanding of human disease pathology is valuable since the development of emphysema in guinea pigs exhibits several similarities to that of the human disease [12]. The goal of this study was to characterize the guinea pig as a model of smoke-induced emphysema, in particular, to understand the effect of cigarette smoke on the lung extracellular matrix, cellular signaling pathways, and proteases expression.

Materials and Methods

Animal experiments

Forty male guinea pigs (Hartley strain) weighing 480 to 600g were obtained from Charles River (Wilmington, MA). Animals were exposed to cigarette smoke (Research Cigarettes Type 2RF, University of Kentucky) for 4 hours a day, 5 days per week, for 1 week, 2 weeks, 4 weeks, 8 weeks and 12 weeks at a total particulate matter (TPM) concentration of 250 mg/m³ in a specially designed smoking chamber (Teague Enterprises, CA). For each time point, 4 animals were exposed to smoke and 4 animals were exposed to room air as controls. The smoking machine generated two 70 ml puffs per minute from the cigarettes and delivered them to whole body exposure chambers. The concentration of TPM within the chambers was at the same level (250 mg/m³) at all times. Gravimetric analysis of filter samples taken during the exposure periods determined total particulate matter concentration in the chambers. Experiments were approved by the Institute for Animal Care and Use Committee of Columbia University.

Human lung tissue

Human lung samples were obtained at Columbia Presbyterian Medical Center (New York, NY) (Institutional Review Board protocol no. 9956) as previously described [4].

Histology and immunohistochemistry

After exposure to smoke or room air, the animals were sacrificed by carbon dioxide inhalation. The trachea was cannulated with a 16g argon catheter secured with a silk suture. The lungs were lavaged first with PBS (10ml), to collect bronchoalveolar lavage (BAL) fluid, then with formalin for fixation. Tissues were embedded in paraffin and sectioned (6 µm). Sections were stained with H&E for histological analysis and quantification of macrophages. Serial sections were also stained with elastica van Gieson stain for elastic fibers. For collagen quantification, the lungs of control and smoke-exposed guinea pigs were stained with Masson's trichrome. Stained areas were quantified using video-microscopy and ImagePro 4.5 software. For each animal, 4 sections equally distant from each other were used. For immunohistochemical analysis, rabbit polyclonal antibodies for type III collagen (Rockwell) [8] and cathepsin K (Biovision) were used. Quantification of collagen staining was performed using the ImagePro

4.5 software. Morphometric analysis of the H&E (hematoxylin and eosin) stained lungs was performed as previously described [8]. We adhered to a rigorous protocol for our morphometric analysis and the lungs were pressure-perfused at 25 cm H₂O with 10% buffered formalin for 24 hours.

Western Blots

Freshly dissected guinea pig lungs (10 mg) were homogenized in 1ml of protein lysis buffer (PBS containing Triton X-100 0.1%), and centrifuged (14000g for 10 minutes). Fifty µg of the lung lysates of each group were subjected to Western Blot analysis. Rabbit polyclonal antibodies against p-ERK, p-JNK, and p-p38 (Cell Signaling) and mouse monoclonal antibody (Calbiochem) against cathepsin K were used, following the manufacturer's instructions.

Zymography

Zymography was performed to detect proteases having gelatinolytic activity, MMP-2 and MMP-9 as previously reported [13].

Cathepsin K, S and L activity assays using a fluorescent substrate

Cathepsin K, S and L activity in lung extracts was assayed using specific fluorescent substrates (Assay Kits from BioVision). This kit uses specific substrate sequence VVR labeled with AFS (amino-4-trifluoromethyl coumarin), which is released after proteolytic cleavage. Two µl of 10 mM Ac-VVR-AFS substrate were added to 50 µl of each sample. Samples were run in triplicate. Specific inhibitors of cathepsins were included in the assay, as negative controls.

Quantitative RT-PCR

Total RNA was extracted from two specimens of lung tissue 0.3cm³ in size with the use of a RNeasy kit (Qiagen). TaqMan gene expression assays were performed to assess gene-transcript levels with the use of an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems Corporation, Foster City, CA). Primer and probe sets were purchased from ABI and included the following: murine collagenase-A (Mcol-A; Mm00473485_m1), MMP1 (Hs00233958_m1), MMP8 (Mm00772335_m1), MMP9 (Hs00234579_m1 and Mm00442991_m1), MMP12 (Hs00159178_m1 and Mm00500554_m1), MMP13 (Mm00439491_m1), and MMP14 (Mm00485054_m1). Beta-actin was used as the housekeeping gene. As the complete guinea pig sequence is not published we tested assays that have been developed based on the human and mouse sequences. Quantitative RT-PCR analysis was performed on lung tissue from four animals in each group.

Statistical analysis

Data are shown as mean ±SD. Student's t-test analysis was performed to determine statistical significance (p<0.05).

Results

Inflammatory response to cigarette smoke in guinea pigs

To determine the influence of cigarette smoke on lung inflammation, macrophages and neutrophils were quantified in tissue sections of the smoke exposed guinea pigs and non-exposed controls. An increased number of macrophages was observed after 12 weeks of smoke exposure (18.64±3.31 macrophages per mm² for the non-exposed vs. 28.63±4.47 macrophages per mm² for the smoke-exposed, p<0.001) (Figure 1A). Both groups of animals had a low and similar number of neutrophils (2.39±3.03 vs. 1.81±1.43 neutrophils per mm²) (Figure 1A). This histological analysis of lung tissue sections reveals that cigarette smoke increases lung inflammation in guinea pigs, which consists primarily of macrophages and not neutrophils. At

all of the time points, total BAL cell count and macrophages were increased after smoke exposure. Gelatin zymography was performed on the BAL fluid of guinea pigs exposed to cigarette smoke for 2 and 4 weeks (Figure 1B). Increased activity of MMP-9 was observed, which is predictable since MMP-9 is expressed mainly in macrophages [14].

Activation of ERK and JNK in the lungs of guinea pigs exposed to cigarette smoke

Phosphorylation of mitogen activated protein (MAP) kinases leads to the activation of transcription factors that participate in the regulation of inflammatory genes. Our laboratory has observed elevated phospho-ERK in both in vitro and in vivo models treated with cigarette smoke extract and in human emphysema. To determine if cigarette smoke modulates the phosphorylation of major MAP kinases in the guinea pig model, we analyzed lung lysates by Western blot (Figure 2). This analysis revealed an increase in phosphorylated-ERK and phosphorylated-JNK in the lungs of smoke-exposed guinea pigs after 4 and 12 weeks of exposure, compared with non-exposed controls (Figure 2A and B). In contrast, exposure to cigarette smoke did not affect the phosphorylation of p38 (Figure 2B) as is the case in the mouse model of smoke exposure [15].

Development of emphysema post cigarette smoke exposure in guinea pigs

The impact of cigarette smoke on the development of emphysema in guinea pigs was determined by measuring the mean linear intercept of the airspaces in lungs of smoke-exposed ($n = 4$) and control animals ($n = 4$) (Figure 3A). Our morphometric analysis showed that guinea pigs exposed to cigarette smoke for 12 weeks developed statistically significant airspace enlargement compared with the controls ($32.5\mu\text{m}$ vs. $25\mu\text{m}$ for the controls, $p < 0.001$) (Figure 3A). A destructive index analysis showed a similar pattern when compared to the morphometric assessment of the guinea pig lungs (67% vs. 51% for the controls, $p < 0.001$) (Figure 3A). In addition, a marked decrease in the alveolar surface area was observed in the lungs of smoke-exposed animals (Table 1). Animals exposed to cigarette smoke for 8 weeks did not exhibit emphysematous changes in their lungs (data not shown). No evidence of apoptosis was detected by TUNEL assay and there was no increase in caspase activity (data not shown).

Decrease in pulmonary collagen and elastin in smoke-exposed guinea pigs

To assess the influence of cigarette smoke on the lung extracellular matrix, histological staining for collagen and elastin was performed. We observed a significant decrease in signal intensity in the alveolar walls of smoke-exposed animals, where fibers of collagen and elastin were thinner (Figure 3B and 3C). Quantification of the stained collagen using video-microscopy demonstrated a very significant decrease of collagen content due to smoke exposure ($12.7 \pm 6\%$ of total tissue area for the smoke-exposed animals, compared to $37.6 \pm 18\%$ for the controls; Figure 3B). Proteolytic degradation of type III collagen has been seen in mouse models of emphysema [8]. In the guinea pig smoke exposure model, immunohistochemistry demonstrated a marked decrease in type III collagen on lung sections of smoke-exposed guinea pigs (Figure 3C). These data provide evidence that cigarette smoke affects the integrity of the lung extracellular matrix in the guinea pig, causing a decrease in elastin and interstitial collagen as is seen in mouse models.

Increased cathepsin K activity in the lungs of smoke-exposed guinea pigs

Proteases play a critical role in the pathogenesis of smoke-induced emphysema. To detect the activity of collagenolytic MMPs in the lungs of smoke-exposed guinea pigs we performed quantitative RT-PCR analysis. MMP expression (Mcol-A, MMP1, MMP8, MMP12, MMP13, and MMP14) at the mRNA level was unchanged (data not shown). As the complete sequences of guinea pig MMPs have not been determined we tested assays that have been developed based on the human and mouse sequences. To investigate the effect of cigarette smoke on the

activity of other collagenolytic enzymes in the guinea pig lung, we measured the activities of cathepsins K, S and L in lung lysates using a specific activity assay. The activity of cathepsin K was significantly higher in the lung extracts of smoke-exposed guinea pigs compared with control animals (Figure 4A). To determine the expression pattern of cathepsin K in the guinea pig lung, we performed immunostaining which exhibited an increase in signal intensity in the lungs of smoke-exposed guinea pigs. The stronger signal localized to the alveolar macrophages when compared to the epithelial cells (4B). Furthermore, an increase in cathepsin S activity was detected in the lung lysates of smoke-exposed guinea pigs, though the difference between the smoke-exposed and control group was not significant (Figure 4A). The activity of cathepsin L was unchanged for both groups (Figure 4A).

Increase in Cathepsin K expression in the lungs of the patients with emphysema

Our data suggests that cathepsin K is involved in the destruction and remodeling processes of the lung in the guinea pig model of smoke-induced emphysema. To determine whether cathepsin K levels were altered in the lungs of patients with emphysema, we analyzed the lysates the lungs of normal and emphysema patients by Western blotting. A marked increase in cathepsin K expression was seen in the lungs of patients with emphysema compared to normal subjects (Figure 5).

Discussion

In our in vivo guinea pig model of smoke-induced emphysema, exposure to cigarette smoke caused lung inflammation, with a significant increase in the recruitment of alveolar macrophages. After 12 weeks of exposure, the lungs exhibited emphysematous changes, with dilation of the alveolar wall and loss of the extracellular matrix. We also observed increased activation of two major MAP kinases pathways, ERK and JNK, due to smoke exposure. While collagenolytic MMPs were not significantly modulated by smoke, elevated cathepsin K activity was present in the lungs of smoke-exposed animals. Cathepsin K is a potent elastase and collagenase, and therefore likely contributes to the observed loss of lung extracellular matrix in this model.

Multiple studies indicate that lung inflammation is a hallmark of smoke-induced emphysema [16,17,18]. Our data demonstrate that in response to cigarette smoke exposure, guinea pigs develop a marked increase in lung inflammation characterized by a significant elevation in the number of alveolar macrophages. In the guinea pig model elevated number of macrophages correlated with increased MMP-9 in bronchoalveolar lavage fluid. MMP-9 is a marker of lung inflammation and is expressed mainly in macrophages [14]. After 12 weeks of smoke exposure, guinea pigs developed emphysematous changes, with the characteristic destruction of the lung extracellular matrix and abnormal enlargement of the airspaces. Smoke-exposed animals exhibited a 30% increase in the mean linear intercept. In contrast, mice have been shown to be much more resistant to the development of smoke-induced emphysema. Consistent with these findings, we have demonstrated that mice exposed to cigarette smoke in the identical fashion as the guinea pigs in this study manifested a 16% increase in the mean linear intercept but after one year of exposure [19]. Our data therefore indicates that guinea pigs are more susceptible to emphysema formation in response to chronic smoke exposure. Although presence of apoptotic cells have been reported in mice post-smoke exposure, our laboratory did not observe any evidence of apoptosis in the lungs of mice [19] and in the present guinea pig model after exposure to smoke.

The investigation of the downstream MAPK signaling pathways in the lungs of smoke-exposed guinea pigs provides insight into the molecular mechanisms of smoke-induced emphysema. Our laboratory has demonstrated that ERK is activated in the lungs of patients with emphysema and induces the expression of MMP-1 in small airway epithelial cells [20], a protease that

causes emphysema in transgenic mice [6]. It has therefore been hypothesized that smoke-induced phosphorylation of mitogen activated protein kinases play an important role in the deregulation of the balance between proteases and antiproteases in the lung extracellular matrix. In the guinea pig model, we revealed an increase in ERK and JNK phosphorylation due to smoke exposure. The activation of ERK and JNK has already been demonstrated in rodent models of emphysema [20,21]. As demonstrated in the rodent model, smoke activation of both ERK and JNK in the lung of the guinea pig likely affects the inflammatory process leading to the development of emphysema.

In the guinea pig model, the development of emphysema was accompanied by increased destruction of the components of lung extracellular matrix. The marked destruction of pulmonary collagen and elastin is a hallmark of smoke-induced emphysema. In transgenic mice overexpressing MMP-1, the development of emphysema was attributed to the loss of type III collagen [8], suggesting that, in humans, the disruption of this fibrillar collagen is a crucial event leading to emphysema. A striking reduction of the type III collagen in the lungs of smoke-exposed guinea pigs was also observed in our study consistent with what is seen in the mouse model [8]. The loss of elastin and type III collagen can contribute to the change in compliance seen in the guinea pig smoke exposure model.

While MMP-1 is overexpressed in human emphysema [4] and augmented expression of MMP-13 has been seen in response to cigarette smoke in murine lungs [22], we did not detect any significant upregulation of collagenolytic MMPs (MMP-1, -8, -13, -14, and McolA) in the lungs of smoke-exposed guinea pigs. Interestingly, Selman et al. detected MMP-1 mRNA in alveolar macrophages and epithelial cells in the damaged lungs of the guinea pigs exposed to cigarette smoke [23]. These authors used human MMP-1 cDNA as a probe in their experiments since the cDNA sequence of guinea pig MMP-1 was not yet determined at that time. However, in the present study, we performed reverse-transcriptase-PCR using primers specific to guinea pig MMP-1 [24], and we did not detect MMP-1 mRNA expression in the lungs of control and smoke-exposed animals. Therefore, since collagenolytic MMPs were not significantly modulated in the lungs of guinea pigs exposed to smoke, the observed decrease in collagen and elastin content likely results from the upregulation of other classes of proteolytic enzymes.

It has been demonstrated that cathepsins degrade major proteins of extracellular matrix and limit the release of newly synthesized collagen and elastin [25,26]. In addition, Zheng et al. showed that IL-13-induced activity of cathepsins such as B, S, L, H and K leads to the development of emphysema in the adult murine lung [9]. In our study, we demonstrated increased activity of cathepsin K in the lungs of smoke-exposed guinea pigs, which likely contributes to the development and progression of cigarette smoke-induced emphysema. Specific enzymatic assays of lung extracts revealed a significant increase in the activity of cathepsin K in the smoke exposed guinea pigs. Cathepsin K is a cysteine protease, which possesses both collagenase and elastase activities, capable of degrading fibrillar collagen at several sites, contrary to MMPs that cleave collagen at one specific site [3]. Therefore, we believe that increased activity of such potent proteolytic enzyme is likely responsible for the development of emphysema in the guinea pig lung. The expression of cathepsin K was detected mainly in the alveolar macrophages of the smoke-exposed animals, whereas collagenolytic MMP-1 expression in humans was identified in the parenchymal epithelial cells. Additionally, Mercer et al. demonstrated that cigarette smoke induces MMP-1 expression in the small airway epithelial cells [20]. Moreover, the elevated expression of cathepsin K was detected in the human lung extracts of patients with emphysema confirming the clinical relevance of cathepsin K in human disease. Therefore, this finding is noteworthy for its contribution to the understanding of the pathogenesis of emphysema suggesting that dysregulated activity of cathepsins as well as MMPs might lead to the irreversible changes in the lung structure.

In addition to cathepsin K, cathepsin S activity was also detected in the lungs of smoke-exposed guinea pigs, but its modulation by smoke was not statistically significant. A recent study [27] showed an enhanced expression of cathepsin S in mice after cigarette smoke exposure. Lack of neutrophils, the absence of MMP-13 and cathepsin S upregulation, together with an increased susceptibility to emphysema in guinea pigs suggest that the mechanisms involved in the alveolar wall disruption differ between small rodents and guinea pigs.

The present study suggests that cathepsin K may contribute to the remodeling of the lung extracellular matrix after exposure to smoke. Cathepsins have been shown to be regulated by MAP kinases in several cell lines in culture [28]. Therefore, the modulation of ERK and JNK pathways due to smoke may be responsible for the upregulation of cathepsin K in the lungs. Further studies are needed to understand the precise role and regulation of cathepsin K during emphysema formation in this animal model and to identify if similar regulation occurs in the human disease.

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Figure 1. Increased macrophages and elevated MMP-9 activity in the lungs of smoke-exposed animals

A. A statistically significant increase in the number of macrophages was observed in the lungs of guinea pigs exposed to cigarette smoke for 12 weeks (18.64 ± 3.31 vs. 28.63 ± 4.47 macrophages per mm², $p < 0.001$). The number of neutrophils was similar in both groups of animals (2.39 ± 3.03 vs. 1.81 ± 1.43 neutrophils per mm²). **B.** MMP-9 activity was detected in the bronchioalveolar lavage (BAL) fluid of smoke-exposed (SM) guinea pigs at 2 and 4 weeks when compared to the control (CON).

Figure 2. ERK phosphorylation and JNK pathways in smoke-exposed guinea pig lungs
A. Increased phospho-ERK (pERK) was demonstrated after 2 weeks of smoke exposure and continues through until 4 weeks of exposure. **B.** Increase in phospho-JNK detected after 12 weeks of smoke exposure. Densitometric analysis of the signal was performed to determine JNK/actin ratio.

Figure 3. Emphysematous changes and decreased extracellular matrix content in the lungs of guinea pigs exposed to cigarette smoke for 12 weeks

A. The mean linear intercept (MLI) and destructive index (DI) were measured in H&E stained lung tissue sections from control and smoke-exposed guinea pigs. A statistically significant increase in MLI was observed in the lungs from guinea pigs exposed to cigarette smoke for 12 weeks (32.5µm vs. 25µm for the controls, $p < 0.001$). A destructive index (DI) analysis exhibited a similar pattern (67% vs. 51% for the controls, $p < 0.001$). **B.** Lung sections of control and smoke-exposed animals were stained for collagen (Masson's trichrome) and staining was quantified by videomicroscopy. A decrease in collagen content in the lungs of smoke-exposed animals was observed. **C.** Lung sections of the guinea pigs were stained for elastin (Elastica

Van Gieson) and type III collagen. Reduction of elastin and type III collagen was demonstrated in the lungs of guinea pigs exposed to cigarette smoke for 12 weeks.

Figure 4. Increased cathepsin K activity in guinea pig lung extracts after smoke exposure and
A. Cathepsin K, S and L activities in lung tissue lysates were assayed with specific fluorescent substrates. A significant increase ($p < 0.05$) in the activity of cathepsin K was demonstrated in the lung lysates of smoke-exposed animals. **B.** Cathepsin K was detected by immunohistochemistry in the alveolar macrophages (arrows) of the guinea pigs exposed to cigarette smoke. Non-immune rabbit serum was used as a negative control.

Active cathepsin K/actin ratio

Figure 5. Upregulation of cathepsin K in the lungs of patients with emphysema

Lung lysates were analyzed by Western blotting using a monoclonal antibody. The amount of cathepsin K was elevated in emphysematous lung samples compared with normal lungs (n=3 for each condition). Both the pro- (40 k) and active (25k) forms of cathepsin K were detected. Active cathepsin K/actin ratio was determined by densitometric analysis (n=3 for normal lungs and n=6 for emphysematous lungs) using Image Pro 4.5 software.

Table 1
Morphometry measurements of non-exposed and smoke-exposed guinea pigs (12 weeks of smoke exposure)

A statistically significant decrease of the alveolar surface area was detected in the smoke-exposed animals.

Guinea pigs	Surface Area/Unit Volume	Fractional Volume %
Non-exposed	79.5±5	42.1±8.4
Smoke-exposed	61.2±1.6*	42±8.6

*
p<0.003