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A comparative study of extracellular matrix remodeling in two murine models of emphysema

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Summary. A single instillation of porcine pancreatic elastase (PPE) results in significant airspace enlargement on the 28th day after instillation, whereas cigarette smoke (CS) exposure requires 6 months to produce mild emphysema in rodents. Considering that there are differences in the pathogenesis of parenchymal destruction in these different experimental models, it is likely that there may be different patterns of extracellular matrix (ECM) remodeling. To evaluate ECM remodeling, C57BL/6 mice were submitted to either a nasal drop of PPE (PPE 28 Days) or exposed for 6 months to cigarette smoke (CS 6 months). Control groups received either an intranasal instillation of saline solution (Saline 28 Days) or remained without any smoke inhalation for six months (Control 6 months). We measured the mean linear intercept and the volume proportion of collagen type I, collagen type III, elastin and fibrillin. We used emission-scanning confocal microscopy to verify the fiber distribution. Both models induced increased mean linear intercept in relation to the respective controls, being larger in the elastase model in relation to the CS model. In the CS model, emphysema was associated with an increase in the volume proportion of fibrillin, whereas in the PPE model there was an increase in the parenchymal elastin content. In both models, there was an increase in collagen type III, which was higher in the CS-exposed mice. We concluded that ECM remodeling is different in the two most used experimental models of emphysema.

Key words: Emphysema, Extracellular matrix remodeling, Collagen, Elastin. Fibrillin-1

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Introduction

Chronic Obstructive Pulmonary Disease (COPD) remains a major public health problem, and it is the fourth leading cause of death throughout the world. Further increases are predicted in the disease prevalence and its associated mortality in the coming decades (GOLD, 2010). COPD is characterized by a chronic airflow limitation caused by small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema). The airflow limitation is usually progressive and associated with an abnormal lung inflammatory response to noxious particles or gases (GOLD, 2010).

The progressive chronic inflammatory response in lung tissues of COPD patients is associated with a dynamic tissue repair and remodeling process, which involves a structural reorganization of extracellular matrix (ECM) components (Abraham and Hogg, 2000). Changes in major lung ECM components, such as collagen types I and III and elastin, could interfere in the mechanical properties of the lung (Shifren et al., 2007). Indeed, biochemical and histological studies suggest that both the organization and the amount of ECM fibers are involved in the loss of elasticity in emphysema (Kuhn et al., 1976; Kononov et al., 2001; Suki and Bates, 2008; Koenders et al., 2009).

Animal models of emphysema have been extensively used to study the physiopathology of this disease, including the remodeling process. Emphysema can be modeled in many ways, such as by exogenous administration of proteinases, chemicals, and particulates and by exposure to cigarette smoke, resulting in features similar to human COPD (Mahadeva and Shapiro, 2002).

Cigarette smoke (CS) exposure models appear to best represent human COPD, considering that they

present emphysema, airway and vascular (depending on the species) remodeling, and physiological alterations similar to humans (Wright et al., 2008). However, the most important limitation of CS models is that no matter how long these animals are exposed, the resulting emphysema is mild, probably equivalent to human GOLD stage 1 or 2, without being disabling as observed in humans with severe emphysema, corresponding to GOLD stages 3 or 4 (Chen et al., 1998; Fabbri et al., 2003; Wright et al., 2008).

The exogenous administration of proteases, such as porcine pancreatic elastase (PPE) and neutrophil elastase, is another experimental approach to induce emphysema in animals (Wright et al., 2008). The major advantage of these protease-induced models is that emphysema can be rapidly induced by a single dose of protease, which allows for better evaluations of different treatment effects (Chen et al., 1998; Ito et al., 2005). Studies have shown that the synthesis of elastic and collagen fibers increases over time after emphysema induction with elastases (Kononov et al., 2001; Suki et al., 2003), enabling studies on alveolar repair and regeneration.

Although it is known that both PPE instillation and CS exposure produce emphysema through the release of proteases that destroy the lung matrix, the mechanisms of these processes are likely to be very different (Wright et al., 2008).To our knowledge, no previous study has compared the compositions of lung parenchyma in different animal models of emphysema.

Considering that there are differences in the pathogenesis of parenchymal destruction in these two different experimental models, we hypothesized that the patterns of remodeling would also be different. Hence, the purpose of the present study was to compare the PPE and CS models of emphysema in mice analyzing fibrillar collagens and elastic fiber components quantitatively and qualitatively after lung injury induction.

Materials and methods

This study was approved by the review board for human and animal studies of the School of Medicine of University of São Paulo (São Paulo, Brazil). All animals in the study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985).

Animals and study design

Six to eight week old male C57BL/6 mice (g) were divided randomly into four groups: PPE 28 days, in which the animals received an instillation of porcine pancreatic elastase (n=7); CS 6 months, in which the animals were exposed for 6 months to cigarette smoke (n=18); Saline 28 Days, in which the animals received an instillation of saline solution (n=9); and Control 6 months, in which the animals remained exposed to normal air in the room during six months (n=12). Part of these animals have been used in another study (Toledo et al., 2012).

PPE instillation

All animals in the PPE 28 day group received a nasal instillation of 50 μl (0,667 IU) (6) of PPE (6.6 units/mg, E-1250, Type I) (Sigma, St. Louis, MO) (Ito et al., 2005). The control group, Saline 28 Days, received 50 μl of 0.9% NaCl (saline solution), the vehicle of PPE. After 28 days, the animals were anesthetized and sacrificed to remove the lungs for further analysis.

CS exposure protocol

All animals in the CS 6 months group were exposed, as described by Toledo et al. (2011), to 12 (±1) commercially filtered cigarettes (0.8 mg of nicotine, 10 mg of tar and 10 mg of carbon monoxide per cigarette) for a total particulate matter concentration of 354.8±50.3 $\mu g/m^3$ per day. These mice were kept in the exposure chamber for 30 minutes a day for 5 days a week for 24 weeks. The mice in the Control 6 months group were exposed to the ambient air in the room.

Immunohistochemistry and immunofluorescence

The lungs were removed and fixed with 10% buffered formalin infused through the trachea at 20 cm H_2O for 24 hours, and then they were embedded in paraffin. Next, sections were processed, and 5 μm sections were obtained and stained with H&E to measure the mean linear intercept (Lm), an indicator of the mean diameter of airspaces (Margraf et al., 1991).

Immunohistochemistry and immunofluorescence were performed with the following antibodies: anti-collagen I (rabbit polyclonal, 1:800), anti-fibrillin I (rabbit polyclonal, 1:200) (ABCAM, MD, USA), anti-collagen type III (rabbit polyclonal, 1:50) (Sigma Aldrich, St. Louis, MO, USA), anti-elastin (goat polyclonal, 1:150) (Santa Cruz Biotechnology Inc., CA, USA).

Immunohistochemistry was performed using the biotin-streptavidin peroxidase method. A VECTASTAIN ABC Kit (Vector Elite PK-6105 or PK-6101) was used for the addition of the secondary antibody, and 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA) was used as the chromogen. The sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany). For confocal analysis, the slides were incubated with the fluorescent secondary antibodies goat anti-rabbit Alexa Fluor 488 (against anti-collagen type I, anti-collagen type III and anti-fibrillin) and rabbit anti-goat Alexa Fluor 546 (against anti-elastin) for 2 hours at room temperature in the dark. The sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, USA). For the negative controls, the primary antibody was omitted from the procedure and replaced with BSA.

Qualitative and quantitative analysis

For conventional morphometry, an eye-piece with a coherent system of 50 lines, 100 points and a known area attached to the microscope ocular was used. The Lm measurements were performed in H&E stained slides. We quantified twenty non-overlapping fields of lung parenchyma per animal at x200 magnification, and the results were expressed in micrometers.

The volume proportion of collagen types I and III, elastin and fibrillin in alveolar parenchyma were determined using the same eye-piece. We counted the number of points hitting a specific fiber in the alveolar parenchyma and compared that with the number of points hitting the alveolar tissue in each field to generate proportions. We assessed 20 non-overlapping fields of lung parenchyma per animal at x400 magnification.

For the qualitative analyses of collagens and elastic fibers, tissue sections were analyzed under a Zeiss 510 Meta confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) attached to a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Oberkochen, Germany). Images were captured using a x20 or x63 magnification oil immersion lens at a pixel resolution of 512x512, and LSM 5 software was used to evaluate the expression of collagen types I and III, elastin and fibrillin (488 nm).

Statistical analysis

Statistical analyses were performed using SigmaStat software (SPSS Inc., Chicago, USA). The Lm values and the volume proportions of collagen types I and III, fibrillin-1 and elastin fibers were compared using one-way analysis of variance followed by all pairwise multiple comparison procedures (Holm-Sidak method). A p value of less than 0.05 was considered significant.

Results

Animals

The final weight of the animals that received intranasal instillation, PPE and Saline 28 day groups, were 26.244±1.183 g and 26.871±1.204 g, respectively. The animals from the Smoke 6 months and Control 6 months showed 33.025±2.142 g and 33.055±2.536 g as a final weight, respectively. There was a difference between the 6 month and the 28 day groups when we compared the weight of the animals.

Mean Linear Intercept (Lm)

PPE instillation and CS exposure both resulted in a substantial enlargement of distal air spaces, compatible with emphysema. In both models, the emphysematous lesions showed a heterogeneous distribution within the lung parenchyma. Fig. 1 shows the mean linear intercept values (Lm) measured in the four experimental groups. The PPE 28 day and CS 6 month groups presented a significant increase in the mean values of Lm ($p<0.05$) compared with the control groups. The mean Lm values were higher in the PPE 28 day group ($p<0.001$) than in the CS 6 month group.

Analysis of the LM values revealed no significant statitistical difference (p=0,111) when we compared the control groups (Saline-28 day and Control-6 month) at the end of their respective exposures.

Immunohistochemical and immunofluorescence analyses

The pattern of labeling for elastin, fibrillin and collagens was similar between immunohistochemistry and immunofluorescence stained slides. Confocal analysis of stained slides gave a better detail on fiber morphology. For this reason, we present the qualitative data based on the confocal findings and the quantitative data based on the immunohistochemical analyses.

Fibrillin-1 and elastin

In Figure 2A, fibrillin-1 appears as slender fibers that are labeled with green fluorescence. In the CS 6 month group, we observed higher amounts of these fibers with a heterogeneous distribution. There was an increase in the volume proportion of fibrillin-1 measured in the PPE 28 day and Saline 28 day groups when compared with the Control 6 month group ($p\leq0.001$). The highest values were observed for the CS 6 month group when compared with the other groups ($p\leq0.001$) (Fig. 2B).

Elastin is labeled with red fluorescence (Fig. 2C). In the PPE 28 day group, these fibers were thicker, whereas in the CS 6 month group we observed reduced and

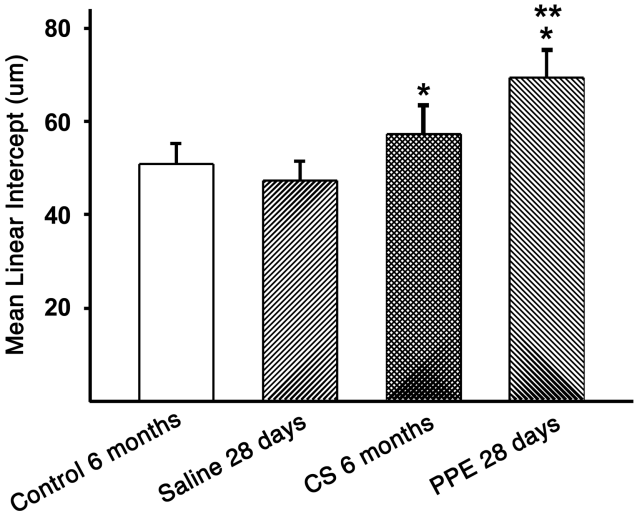

Fig. 1. Mean linear intercept values measured in the four experimental groups. Values are given as means ± SD; *p<0.05 compared with the control groups; **p<0.001 compared with the Smoke 6 months group.

fragmented red fibers. A significant increase in the volume proportion of elastin was observed in the PPE 28 day group when compared with the other experimental groups ($p \leq 0.001$) (Fig. 2D).

Collagens Type I and Type III

In Figure 3A, collagen type I fibers labeled with a green fluorescence (Alexa Fluor 488) are shown. These fibers were distributed with the same color intensity and a regular deposition in each of the four experimental groups. In Figure 3C, collagen type III fibers are labeled with green fluorescence. There were differences in the intensities of the green fibers among the groups. The PPE 28 day and CS 6 month groups showed larger areas with green-labeled fibers; however, the green-labeled

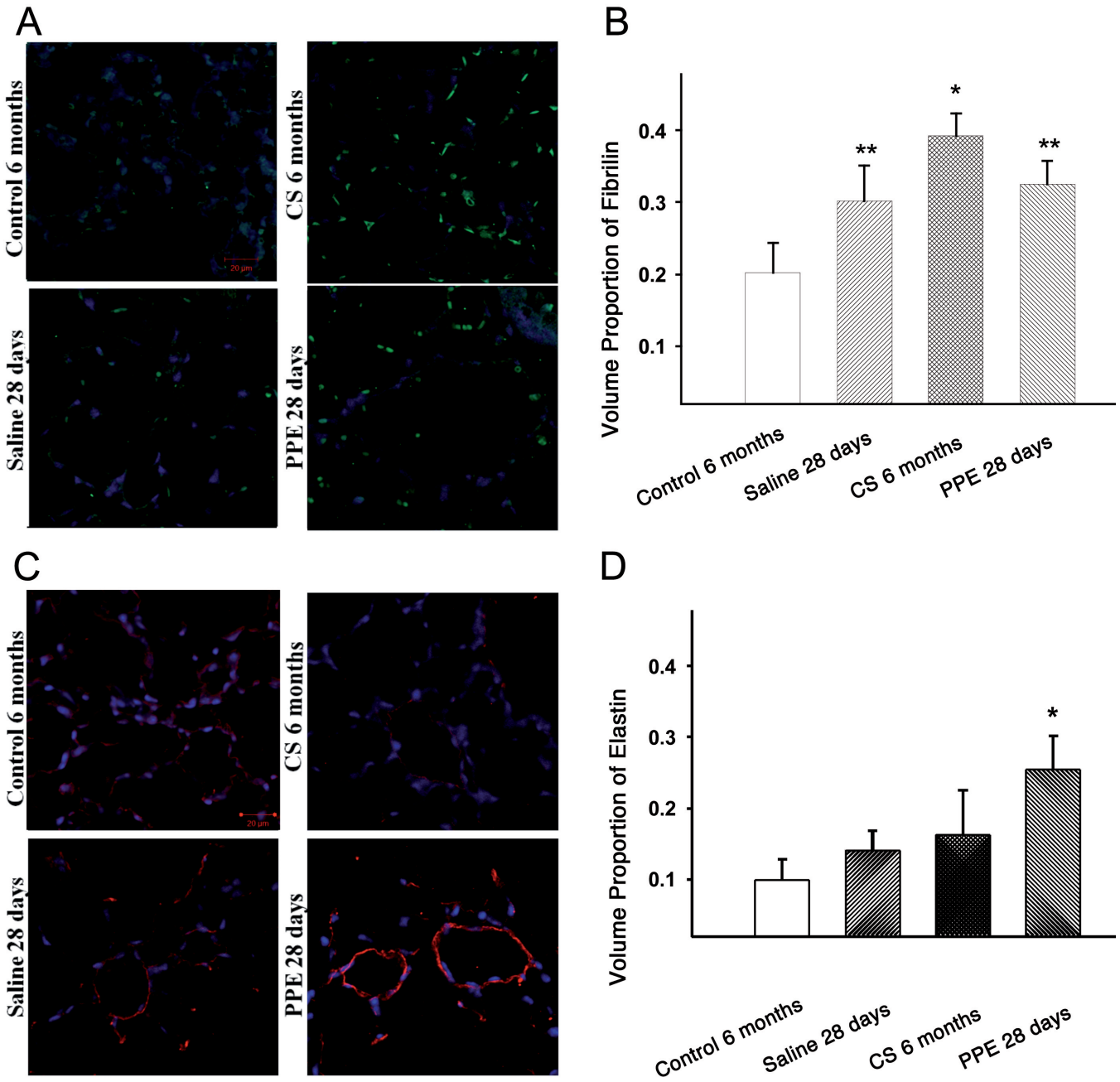

Fig. 2. A. Confocal photomicrographs of fibrillin-1 in parenchyma (20x magnification, 3x zoom, Alexa Fluor 488 was used as fluorochromo, counterstained with DAPI). **B.** Proportional volumes of fibrillin-1 in the alveolar tissue in the four experimental groups. Values are given as means ± SD; *p<0.001 compared with the other groups; **p<0.001 compared with the Control 6 months group. **C.** Confocal photo microscopy of elastin (20x magnification, 3x zoom). **D.** Proportional volumes of elastin in the alveolar tissue in the experimental groups. Values are given as means ± SD; *p<0.001 compared with the other groups.

fibers were thicker in the CS 6 month group. There was no difference among the four experimental groups in the volume proportion of collagen type I fibers quantified in immunohistochemistry stained slides in the alveolar parenchyma (Fig. 3B). However, there were increases in the volume proportion of collagen type III fibers in both the CS 6 month and PPE 28 day groups ($p \leq 0.05$) compared with their respective controls. Additionally, a

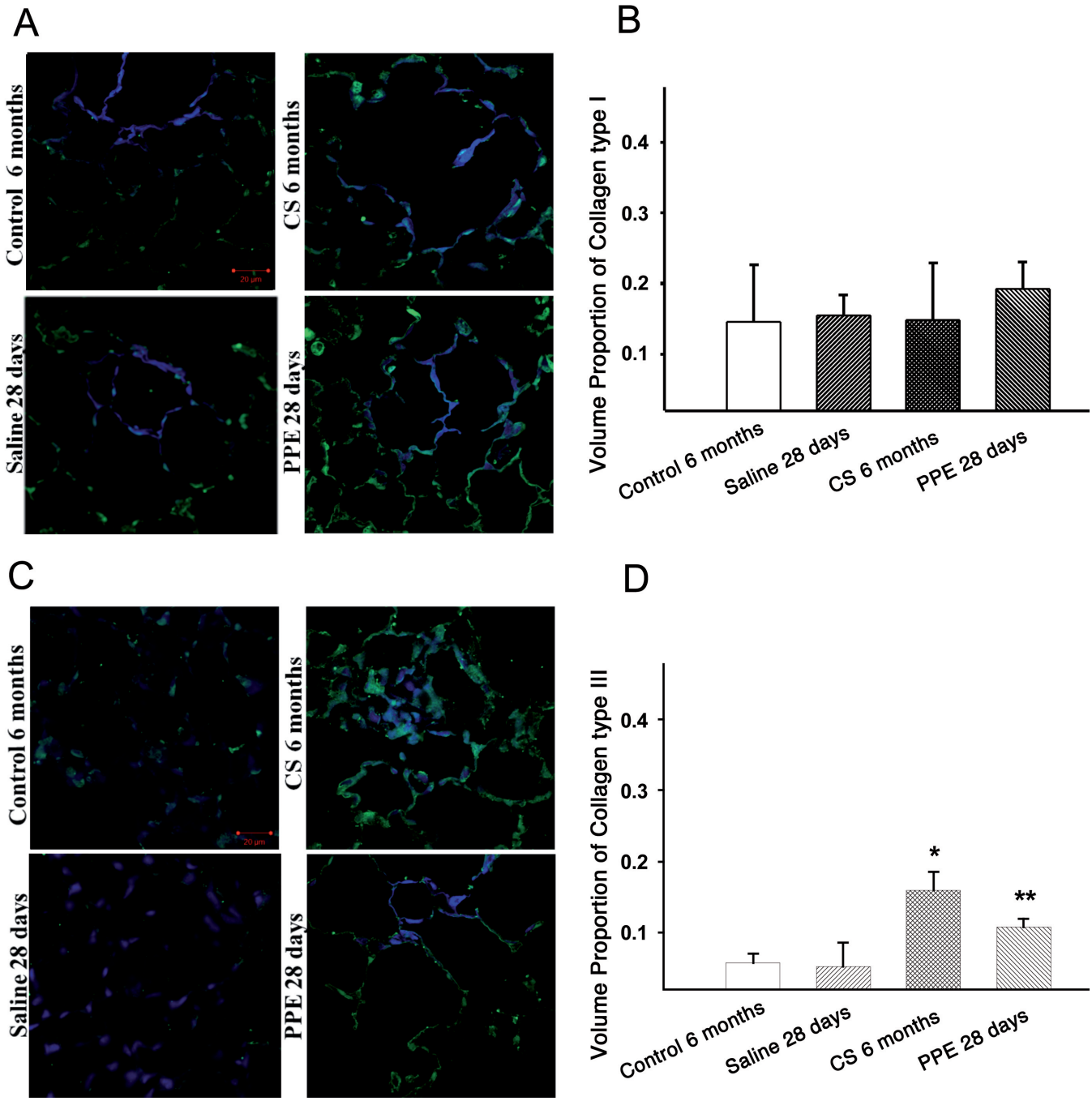

Fig. 3. A. Confocal photomicrographs of collagen type I fibers in parenchyma (20x magnification, 3x zoom. Alexa Fluor 488 was used as fluorochromo, counterstained with DAPI). **B.** Volume proportion of collagen type I fibers in alveolar tissue in the four experimental groups. Values are given as means ± SD. **C.** Confocal photomicrographs of collagen type III fibers in parenchyma (20x magnification, 3x zoom). **D.** Volume proportion of collagen type III fibers in alveolar tissue in the four experimental groups. Values are given as means ± SD; *$p \leq 0.05$ compared with the Control 6 months, Saline 28 days and PPE 28 days groups; **$p \leq 0.05$ compared with the Control 6 months, Saline 28 days and Smoke 6 months groups.

higher volume fraction of collagen type III was observed in animals that were submitted to CS exposure compared with the group that received PPE instillation ($p \leq 0.05$) (Fig. 3D).

Discussion

In this study, we demonstrated that the remodeling patterns in PPE- and CS-induced emphysema models are different in relation to the major lung ECM components: the elastic fibers and the fibrillar collagens. In animals exposed for 6 months to CS, the resulting emphysema was associated with an increase in the volume proportion of fibrillin, whereas in the PPE model there was an increase in the parenchymal elastin content. In both models, there was an increase in collagen type III in the alveolar parenchyma, which was higher in the CS-exposed mice. To our knowledge, this is the first study comparing remodeling patterns after lung disease was already established.

We compared the pattern of ECM remodeling in the two most used experimental models of emphysema in the literature, having as background the advantages and disadvantages of each model. In the PPE-induced model, severe emphysema is achieved within 28 days with a single and inexpensive treatment (Ito et al., 2005; Wright et al., 2008), but it may not mimic adequately human emphysema in its pathogenesis. On the other hand, the CS exposure model may mimic better human disease pathogenesis, but it requires lengthy experiments that lead in most cases to mild disease (Wright et al., 2008). Since these models use a different time point of analysis, we decided to compare them as such. There was alveolar enlargement in both models with a higher mean linear intercept in the PPE model. There was no significant statitistical difference when we compared the control groups (Saline-28 day and Control-6 month) at the end of their respective exposures despite the age differences. Elastic fibers are considered to be the major components responsible for the elastic recoil properties of the lungs (Shifren and Mecham, 2006; Robbesom et al., 2008). These fibers are composed of at least two morphologically distinguishable components: elastin and microfibrils (fibrillins, microfibril-associated glycoproteins, TGFbeta binding proteins), among which fibrillins are the major components (Shifren and Mecham, 2006).

Fibrillins assist in the formation of elastin polymers by providing a scaffold that directs elastin aggregation. In addition, fibrillin -1 decreases the activation of TGF-beta through its association with the large latent complex LTBP-1. Mice lacking elastin or elastic fiber proteins, such as fibrillin-1, showed an emphysema-like lung at birth (Siracusa et al., 1996; Neptune et al., 2003; Zacchigna et al., 2006). Aberrant expression of fibrillin-1 (fragmentation) has been described even in mild cases of cigarette induced emphysema in humans (Koenders et al., 2009).The assembly of elastic fibers is a complex task requiring a coordinated temporal sequence of all the molecules that compose the microfibrils and the cross-linking elastin. In situations of parenchymal lung injury, as in our models, the repair of elastic fibers is probably defective, resulting in non-functional fibers, especially in adults. We observed that elastic fiber remodeling differed between the CS- and PPE-exposed animals. In the CS-exposed animals, elastic fiber remodeling was associated with an increase in the fibrillar component (fibrillin) of the elastic fiber associated to a fragmented aspect, not accompanied by an increase in elastin. On the other hand in the PPE-exposed animals, there was an increase in elastin, suggesting that in this model the elastic fiber repair is more efficient in terms of fiber assembly. Such differences could be associated to the more pronounced chronic inflammation and oxidative stress associated with cigarette smoke (Rangasamy et al., 2009).

In line with our findings, hamsters that received elastase treatment and were subsequently exposed to CS to induce emphysema had a reduction in lysyl oxidase (enzymes that catalyze the cross-linking of collagen and elastin) levels in their lungs and an impairment in elastic fiber resynthesis; conversely, animals that received only elastase treatment showed an increase in lysyl oxidase activity (Osman et al., 1985). Chen et al. (2005) showed that cigarette-smoke-condensate-treated cells had a decrease in lysyl oxidase levels with a consecutive reduction in the catalytic activity of the protein compared with smoke-condensate-free controls.

Abnormal collagen remodeling could explain the lung functional changes and mechanical forces leading to emphysema development and progression. Several studies have reported an increase in collagen in both emphysema models and humans. Kononov et al. (2001) found that in the elastase-treated tissue there was significant remodeling, resulting in thickened elastin and collagen fibers and that during stretching the newly deposited fibers underwent substantially larger distortions than those in normal tissue. In another study, Ito et al. (2005) showed an increase in the whole lung hydroxyproline content in the mice that received an elastase instillation, and the tension at which isolated parenchymal fibers broke during stretching was significantly lower in these animals.

The lung parenchymal interstitium consists mostly of collagen types I and III, which provide the scaffold for the alveolar walls (Suki et al., 2003). We found an increase in collagen type III but not in collagen type I, which was more marked in the CS-induced model. Collagen fiber stiffness depends on the relative amounts of collagen types I and III. It has been previously shown that collagen type I is stiffer than collagen type III (Silver et al., 2002). Our findings might help explain why in animal models of emphysema, collagen fibers break at tensions that correspond with normal breathing (Ito et al., 2005).

Abraham and Hogg (2000) described the structural reorganization of the ECM in human lungs that were undergoing severe emphysematous destruction. The

alveolar walls showed an increase in thickness with a composite structure of collagen bands and relatively fine elastic fibers. Both collagen and elastin had altered configurations, especially collagen, which showed considerable variations in fiber size. In addition, Hogg et al. (2000) described that the progression of COPD severity was linked to a relative increase in the collagen type III/I ratio.

Our study has limitations. Due to the different times of exposure in the two models, the CS/control groups were older and heavier than the PPE/saline control groups at the end of the experiments, but controls did not differ in Lm values. Although in humans Robbesom et al. (2008) could not observe a correlation between aberrant (fragmented) fibrillin expression and age, we cannot exclude that the different ages of the study groups is an influencing factor for this finding. We did not compare lung function parameters in the two groups, so we do not know if there were different functional changes that could be attributed to different remodeling patterns. However, both models are known to induce lung function abnormalities that seem to appear earlier in the elastase model than in cigarette exposed animals (Wright et al. 2008).

In summary, we showed that both emphysema models were associated with the remodeling of elastin, fibrillin-1 and collagen types I and III; however, the remodeling was different between the models. The PPE model elicited more immediate remodeling, visible 28 days after a single elastase instillation, which is useful for assessing parameters of lung function, imaging and drug deposition. In the CS-induced model, remodeling is probably more defective than in the PPE model because of the chronic, persistent inflammatory injury caused by cigarettes. Therefore, our data contribute to a better understanding of the remodeling process in both experimental models of emphysema and may help researchers to choose the most appropriate model based on their expected outcomes.

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