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Three-dimensional architecture of elastin and collagen fiber networks in the human and rat lung

Masahiro Toshima, Yuko Ohtani, and Osamu Ohtani

Department of Anatomy, Toyama Medical and Pharmaceutical University, Toyama, Japan

Summary. Collagen and elastin fibers are the major components of the lung connective tissue, but their spatial organization has not been well documented. We have demonstrated the three-dimensional architecture of collagen and elastin fiber networks in the human and rat lung using scanning electron microscopy. These networks in their original forms were extracted by an alkali-water maceration technique and a formic acid treatment, respectively. The collagen fibers formed a continuum extending throughout the lung and pleura. They were condensed in the alveolar mouth and subdivided into smaller fibers in the alveolar septa, thus forming basket-like networks. Sizes of the alveolar pores in the collagen fiber network of the alveolar septa became larger with age. In the collapsed lung, collagen fibers in the alveolar mouths and septa took on wavelike configurations, while in the inflated lung they became straight. The elastin fibers also formed a continuum, rich in the alveolar mouths and poor in the alveolar septa, were quite straight without any wavelike configuration. Transmission electron microscopy showed that collagen and elastin fibers were intermingled, suggesting that both fiber systems may act as parallel mechanical elements to stress or strain applied. Our results suggest that at low levels of strain the wavy collagen fibers are easily extended to allow alveolar mouths and alveoli to expand, with most of the stress being borne by adjacent elastin fibers, while at higher levels collagen fibers become straight and limit any further distension of alveolar ducts and alveoli. The elastin fiber continuum appears to permit the lung to effectively recoil or retract. The present study has also shown that alveolar pores enlarge with age, suggesting that collagen remodeling may be related to the pathogenesis of emphysema.

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Address for correspondence: Professor Osamu Ohtani, Department of Anatomy, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama 930-0194, Japan
Tel: +81-76-434-7205; Fax: +81-76-434-5010
E-mail: osmotani@ms.toyama-mpu.ac.jp

Introduction

Elastin and collagen are the major components of the lung connective tissue network, and together provide the lung with its elasticity and tensile strength. A large number of light microscopy studies have demonstrated the organization of elastin and collagen in the human lung (Pierce and Ebert, 1965; Niewoehner and Kleinerman, 1977; Matsuda *et al.*, 1987; Mercer and Carpo, 1990; Mulkusch *et al.*, 1995). Light microscopy, however, cannot show the exact organization of collagen and elastin networks in three-dimenisions. Recently, Finlay *et al.* (1996) visualized the lamellar elastin network in the human lung by scanning electron microscopy of formic acid-treated freeze-dried samples. It is known that freeze-drying immediately after formic acid treatment changes elastin fibers into a lamellar structure (Ushiki, 1992). Mercer and Crapo (1990) have suggested that collagen fibers in the lung have a wavelike arrangement, as do those in the rat tail tendon which undergoes considerable extension (Diamant *et al.*, 1972). However, it has not been clearly demonstrated whether lung collagen fibers show configurational changes which are dependant on respiration. In the present study, we demonstrate the three-dimensional structure of elastin and collagen fiber networks in the human and rat lung. Elastin fibers were extracted by formic acid, freeze-dried after fixation with tannic acid, and observed under a scanning electron microscope. The collagen fiber networks of both collapsed and inflated lungs were studied using an alkali-water maceration/scanning electron microscope technique (Ohtani, 1987; Ohtani *et al.*, 1988).

Materials and Methods

The materials used were cancerous lungs surgically removed from 9 individuals, 31 to 81 years of age. Preoperative examinations of ventilatory function showed that 7 were normal, and 2 suffered from obstructive impairment. The lung tissue without any cancer was obtained from the peripheries of the resected lungs and used in the experiments. In order to assess the effects of respiration on collagen fiber configuration, 4 male Wistar rats were used; the lungs from two of them were fixed in a collapsed condition, while the remaining two were inflated with an infusion of approximately 1 m*l* of air before fixation.

Collagen fiber preparation

Collagen fiber specimens were prepared according to our alkali-water maceration technique (Ohtani, 1987; Ohtani *et al.*, 1988). The lung tissues were immersion-fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4), and cut into pieces of approximately $5 \times 5 \times 5$ mm^3. The tissue pieces were immersed in 2N-NaOH at room temperature for 5 to 7 days, followed by immersion in distilled water at room temperature for 3 to 4 days. Collagen fiber samples were fixed with 0.5% tannic acid in distilled water for 3 hours. After rinsing several times in distilled water, the pieces were further fixed by immersion in a 1% OsO_4 aqueous solution for 2 h and then dehydrated through graded concentrations of ethanol. They next, were freeze-cracked in liquid nitrogen and freeze-dried using t-butanol (Inoué and Osatake, 1988).

Elastin fiber preparation

Elastin fiber preparations were made according to a modification (Ushiki, 1992) of an original method described by Hass (1942). The lung tissues were cut into cubes of approximately $5 \times 5 \times 5$ mm^3, and fixed with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) overnight. The tissue pieces were treated in a 90% formic acid aqueous solution at 45℃ for 3 to 4 days. Next, they were rinsed in McIlvaine buffer diluted with the same volume of distilled water at room temperature for several hours and fixed with a 0.5% tannic acid in the same buffer for 2 h. After rinsing with distilled water, they were further fixed in a 1% OsO_4 aqueous solution for 2 h. They were then dehydrated through graded concentrations of ethanol, freeze-cracked in liquid nitrogen, and freeze-dried using t-butanol.

Scanning electron microscopy

Both collagen and elastin preparations were mounted on specimen holders with either silver paste or carbon-coated double sticky tape and coated with gold. Observations were made in a Hitachi S-3200S scanning electron microscope (Hitachi, Tokyo) at an acceleration voltage of 15 to 20 kV.

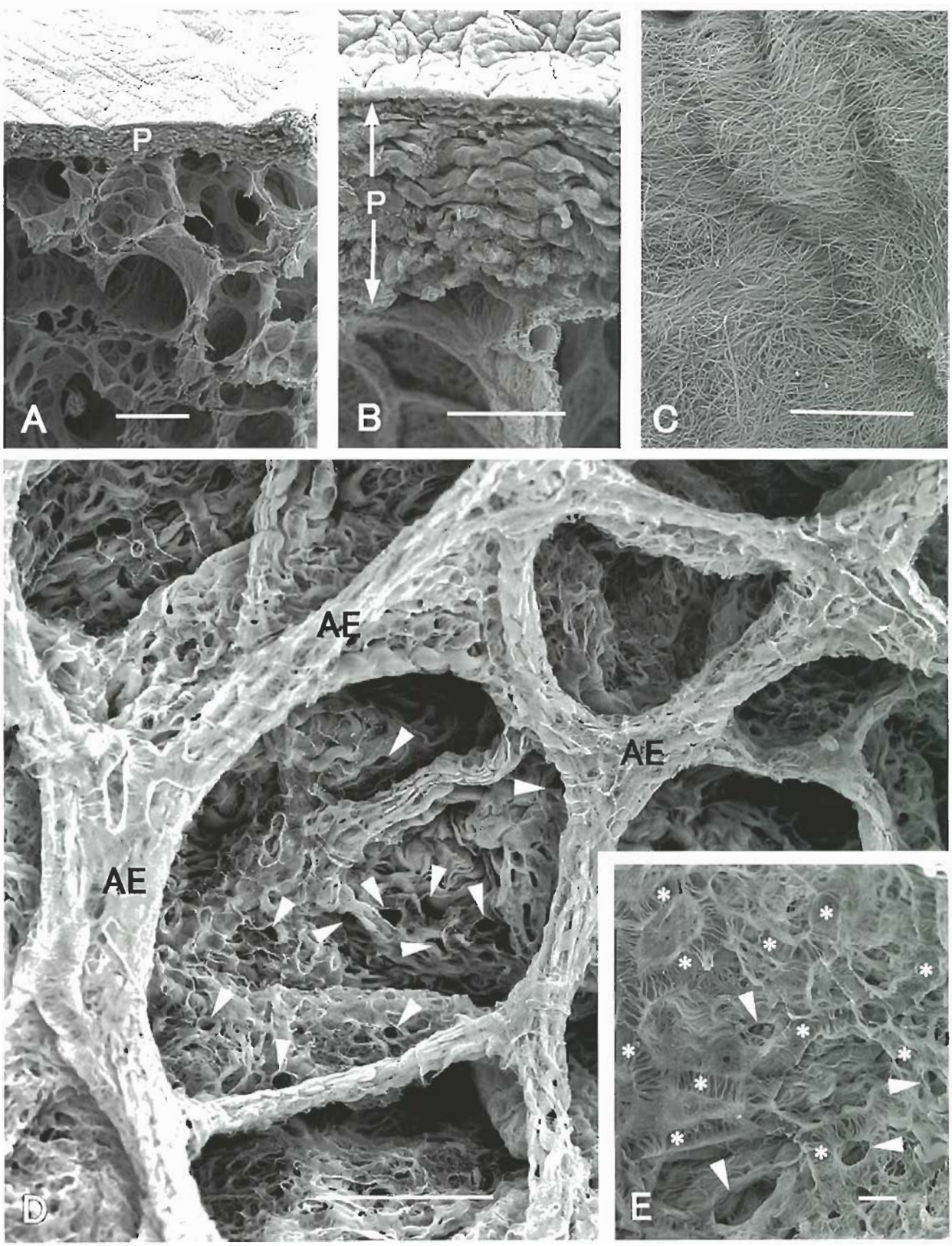
Transmission electron microscopy

Small pieces of the lung tissues were processed for transmission electron microscopy by being fixed in 2.5 % glutaraldehyde in a 0.1 M phosphate buffer and postfixed in 1% OsO_4. Pieces of elastin preparations fixed with 0.5% tannic acid were also postfixed in OsO_4 and then processed for transmission electron microscopy. After being dehydrated through graded concentrations of ethanol, all of the pieces were embedded in Epon 812. Ultrathin sections were stained with tannic acid-uranyl acetate and lead citrate (Kajikawa and Yamaguchi, 1975) and observed in a JEOL 200-CX transmission electron microscope (JEOL, Tokyo).

Statistics

Statistical analysis was done on the sizes of alveolar pores (of Kohn) as revealed by scanning electron microcopy of the collagen fiber preparation from individuals of 44, 65, and 81 years of age. The data were presented as the mean ± S.D. For statistical comparisons, the Kruskal-Wallis test was employed.

Fig. 1. Scanning electron micrographs of the collagen fiber network in the human lung. **A:** In the pleura (P), there is a collagen fiber layer of about 160 μm in thickness. Scale bar = 400 μm. **B:** The pleural layer consists of crisscrossing collagen fibers of about 15 μm in thickness. The outermost part is a sheet-like layer (6 – 7 μm thick) made up of collagen fibrils. Scale bar = 100 μm. **C:** The outermost layer of the pleural collagenous network consists of interwoven collagen fibrils approximately 80 nm in thickness. Scale bar = 100 μm. **D:** There are condensations of collagen fibers at the alveolar entrances (AE). They take wavelike courses and extend from one alveolar entrance to another. They also branch off from the numerous collagen fibers into the alveolar septa to form sac-like alveolar collagen networks. There are also alveolar pores (arrowheads) devoid of collagen fibers. Scale bar = 100 μm. **E:** High magnification of a collagen fiber network in the alveolar septum. There are ladder-like collagen fibrillar networks (*) surrounding capillaries. Scale bar = 10 μm



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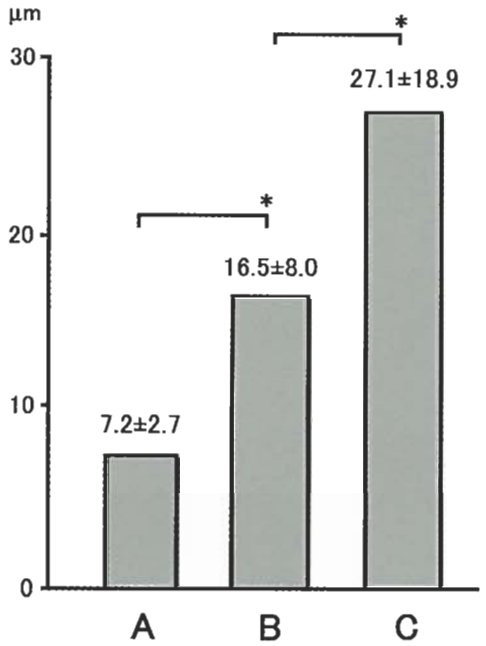


Fig. 2. Sizes of alveolar pores (of Kohn) (mean ± S.D.) from individuals of 44 (**A**), 65 (**B**), and 81 (**C**) years of age. Numbers of the alveolar pores measured are 63 (**A**), 58 (**B**), and 58 (**C**). * $p < 0.0001$ (Kruskal-Wallis test)

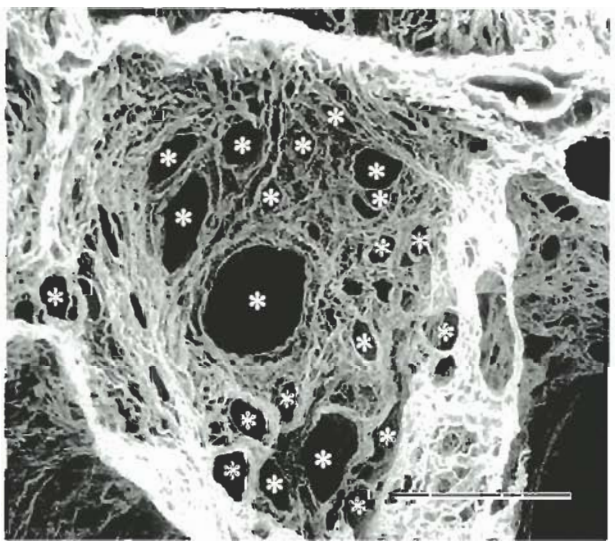


Fig. 3. Scanning electron micrograph of a collagen fiber network in the alveolar septa of the human emphysematous lung. There are many pores (*) devoid of collagen fibers. Scale bar = 100 μm

Results

Scanning electron microscopy of collagen fibers

The alkali-water maceration completely removed the tissue elements except for collagen fibers. Scanning electron microscopy of the specimens prepared by this method showed a continuum of the collagen fibrillar plexuses extending through the pleura and the lung parenchyma (Fig. 1).

In the human visceral pleura there was a dense collagen fiber layer approximately 160 μm in thickness (Fig. 1A). Collagen fibers of this layer measured about 15 μm in thickness, took wavelike courses, and crisscrossed each other (Fig. 1B). The collagen fibers of the pleura were continuous with those of lung parenchyma (Fig. 1A, B). On the outer surface of this layer was a sheet-like layer of 6 to 7 μm in thickness (Fig. 1B) made up of interwoven collagen fibrils of approximately 80 nm in diameter (Fig. 1C).

The collagen fibers in the human alveolar duct wall ran in various directions and condensed around the entrances of alveoli. The collagen fibers around the mouths of alveoli took wavy or helical courses and repeatedly divided and fused *en route*. They gave off small fibers that entered alveolar septa where the fibers again repeatedly divided and fused to form dense networks (Fig. 1D). The collagen fibers in the alveolar septa ran in various directions except for the portions of alveolar capillaries. On the epithelial side, capillaries were surrounded by collagen fibrils oriented transversely to the axis of the capillaries (Fig. 1E). The collagen fibrils, however, did not cover the entire surface of the capillaries, so that considerable areas of the capillary surface were devoid of these fibrils (Fig. 1E). The collagen fiber network in the alveolar septa formed round or oval pores corresponding to the alveolar pores of Kohn (Fig. 1D, E). The diameter of the alveolar pores became significantly larger with age (Fig. 2). The alveolar pores in an emphysematous lung of an individual over 80 years of age measured up to 60 μm in diameter and were more numerous than those in the normal lung (Fig. 3).

In the rat, collagen fiber configuration in the collapsed lung differed considerably from that in the inflated lung (Fig. 4A, B). In the collapsed lung, collagen fibers generally took zigzag or helical courses. Thicker collagen fibers, located along the entrances of alveoli in particular, took predominantly wavy courses (Fig. 4A). On the other hand, collagen fibers in the inflated lung were straighter than those in the collapsed lung, and did not assume a wavelike configuration (Fig. 4B). Collagen fibers in the alveolar septa appeared denser in the collapsed lung than in the inflated lung (Fig. 4).

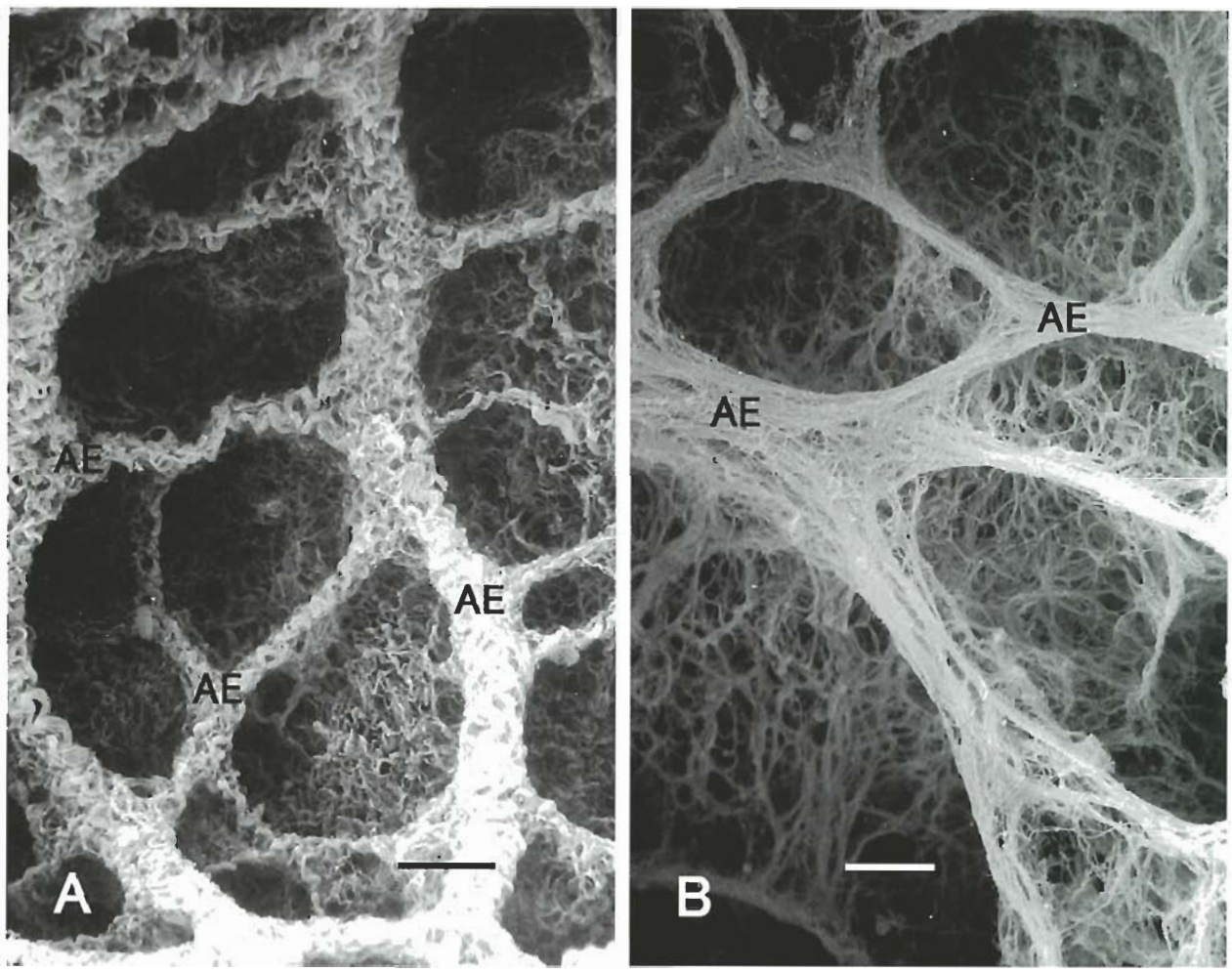


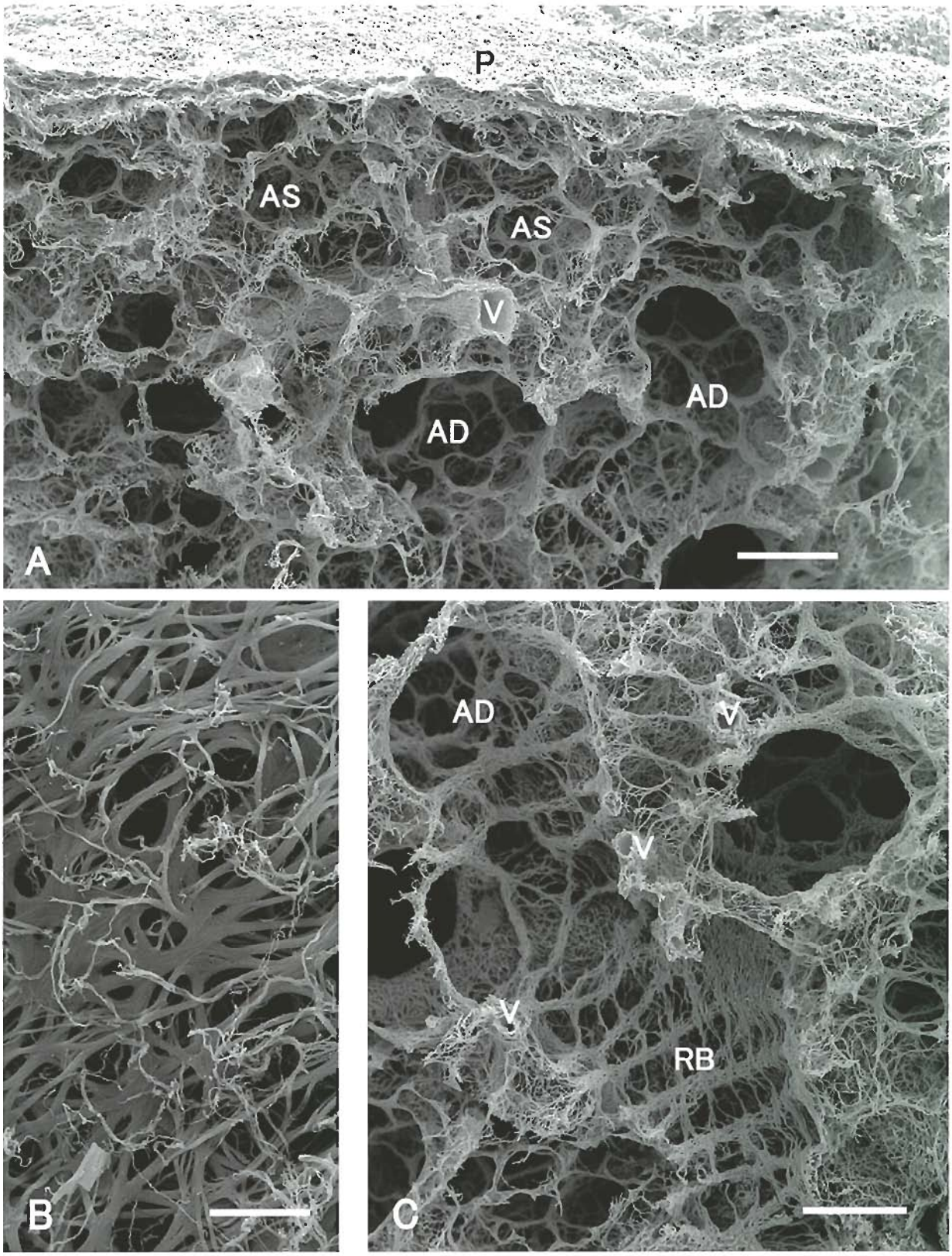
Fig. 4. Scanning electron micrographs of collagen fiber networks in rat lung. Collagen fibers at the alveolar entrances (AE) in the collapsed lung (**A**) take zigzag or helical courses, while those in the inflated lung (**B**) are straight. Scale bar = 100 μm

Scanning electron microscopy of elastin fibers

Formic acid-treatment of the human lung resolved all of the tissue components except for the elastin fibers, which under a scanning electron microscope showed a continuum of elastin fiber plexus extending through the pleura and the lung parenchyma (Fig. 5, 6). The lung elastin consisted of elastin fibers of varying thickness instead of elastin lamellae.

The pleura had two to three layers of interwoven elastin fibers (Fig. 5A) with many connections between these layers. Free endings of elastin fibers were also observed on the outermost elastin layer of the pleura (Fig. 5B).

Respiratory bronchioles appeared polygonal, forming a quadrilateral meshwork that primarily consisted of circularly oriented thicker fibers and longitudinal thinner fibers (Fig. 5C). Both kinds of elastin fibers were intermingled at their crisscrossing points (Fig. 5C). At the alveolar ducts and sacs, elastin fibers were condensed around the entrances of alveoli, thus forming round or oval networks (Fig. 5, 6). Elastin fibers repeatedly divided and fused to form band-like structures along the alveolar entrances (Fig. 6A). The band-like elastin fibers gave off thinner fibers which again repeatedly subdivided and fused to form coarse sac like networks in the alveolar septa (Fig. 6A, B). Thicker blood vessels possessed several lamellae consisting of elastin fibers, while thinner vessels had a single layer of an elastin fiber network (Fig. 6). Elastin fibers in the outermost layer of blood vessels were intermingled with those in



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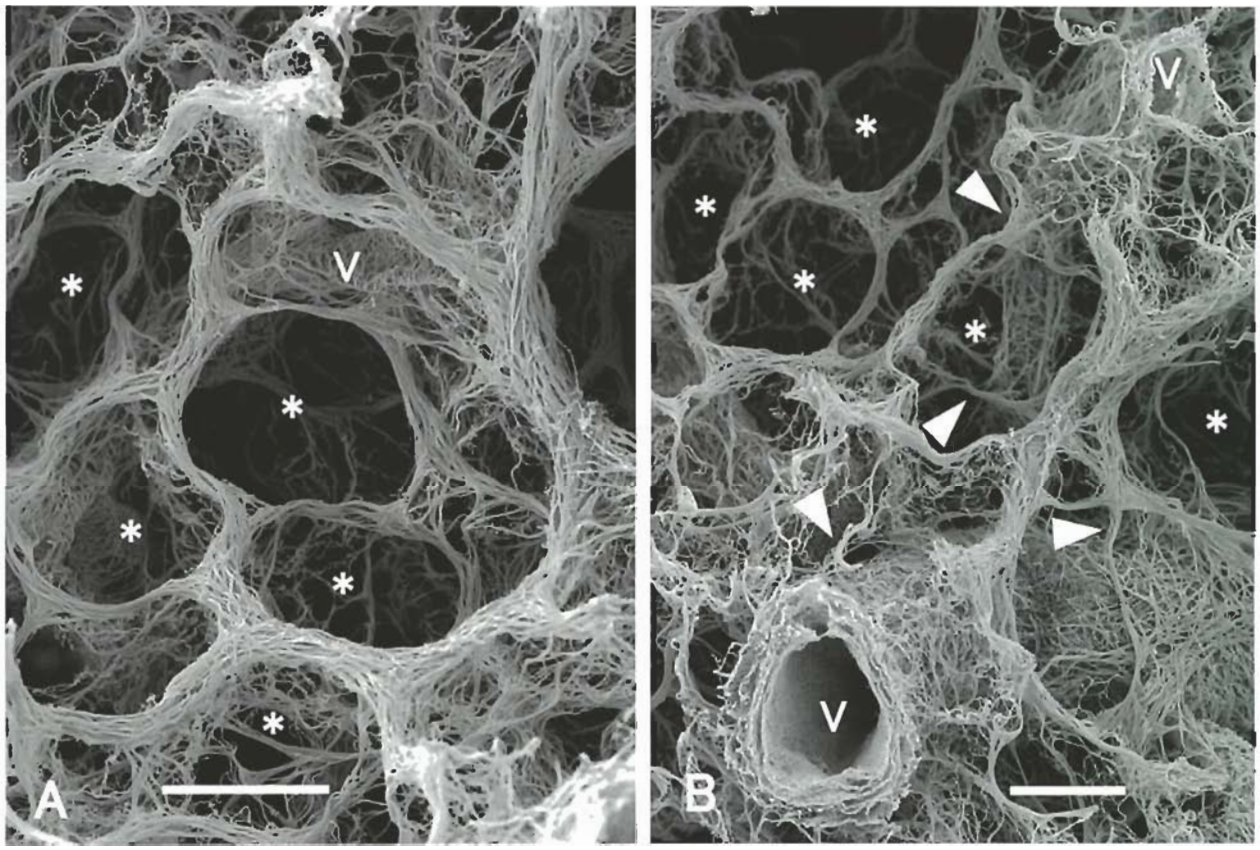

Fig. 6. Scanning electron micrographs of elastin fiber networks in the human lung. There are bands of elastin fibers at entrances of alveoli (*). The elastin fibers crisscross at the point where three alveolar entrances meet. The bands give off smaller elastic fibers to form a coarse network in the alveolar septum. Blood vessels (V) possess single or multiple layers of dense elastin fiber networks. The outermost elastin fibers of blood vessels are continuous with alveolar elastin fibers (arrowheads). Scale bar = 100 μm

alveoli, in particular with those in the alveolar entrances (Fig. 6B).

Transmission electron microscopy

Transmission electron microscopy of the human lung treated by the alkali-water maceration technique showed only collagen fibers (Fig. 7A), while that of the formic acid treated human lung showed only elastin fibers in their original locations (Fig. 7B). Microfibrils described by Low (1962) or fibrillin, a 350-kD glycoprotein (Sakai *et al.*, 1986), were not observed in our preparations. Staining of ultrathin sections of untreated lung tissues with tannic acid-uranyl acetate and lead citrate clearly showed elastin and collagen fibers by transmission electron microscopy (Fig. 8, 9). Elastin fibers of variable sizes were densely located at

Fig. 5. Scanning electron micrographs of elastin fiber networks in the human lung. **A:** Low magnification shows a continuum of the elastin fiber network. Scale bar = 200 μm. **B:** Elastin fiber network in the outer surface of the pleura. Scale bar = 10 μm. **C:** Elastin fiber network in the respiratory bronchiole (RB) and in the alveolar duct (AD). Scale bar = 200 μm. AS: alveolar sac, P: pleura, V: blood vessel

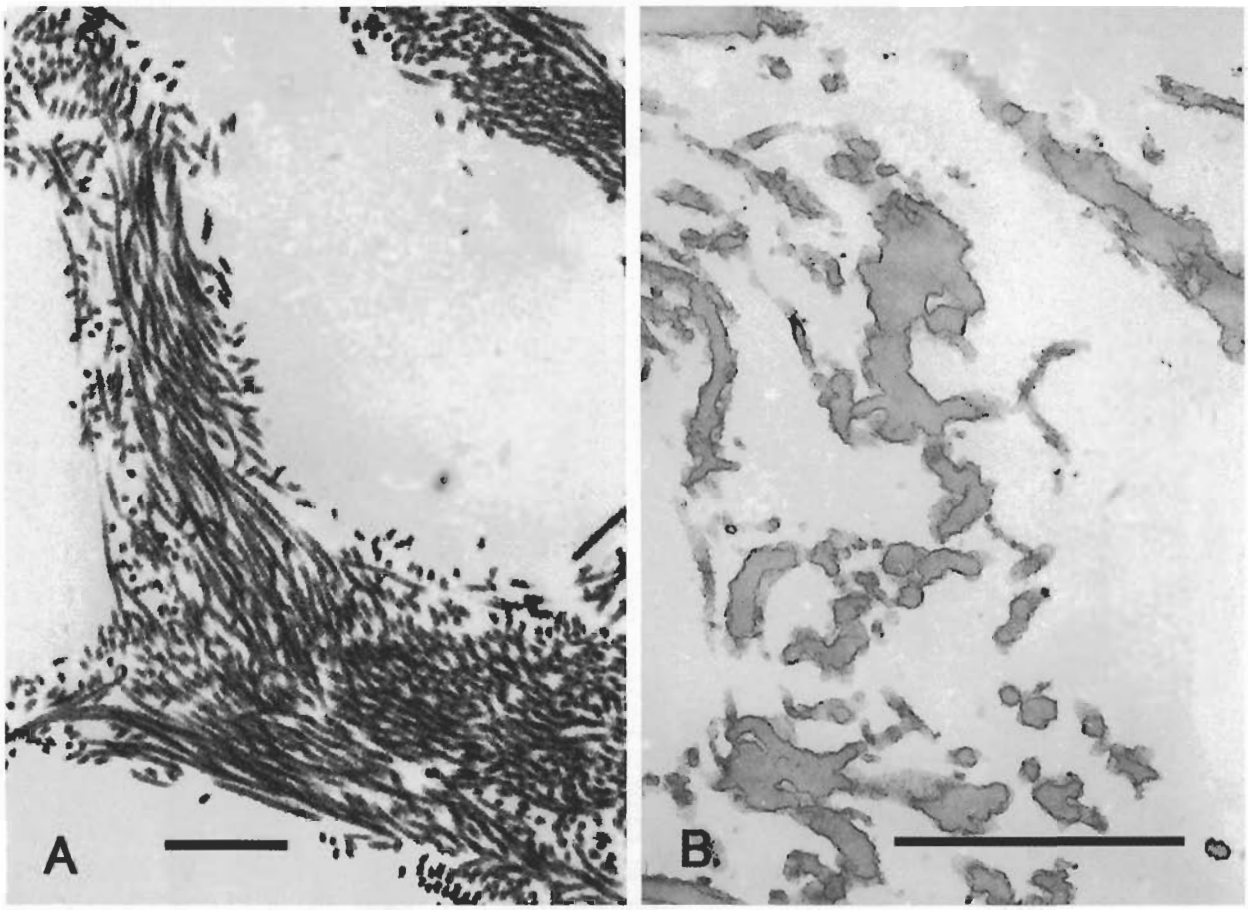

Fig. 7. Transmission electron micrographs of alkali-water macerated (**A**) and formic acid-treated (**B**) human lungs. Only collagen fibers are seen in the alkali-water macerated lung (**A**), while only elatin fibers are seen after formic acid treatment (**B**). Scale bar = 1 μm

the alveolar entrances, while they were only sparsely distributed in the alveolar septa (Fig. 9), and at the entrances they intermingled with collagen fibers (Fig. 8). Collagen fibers were much denser than elastin fibers in the alveolar septa (Fig. 8, 9).

Discussion

Our alkali-water maceration method (Ohtani, 1987; Ohtani *et al.*, 1988) enabled us to isolate collagen fibrillar networks of the human and rat lung. SEM of the lung treated by this method showed the three-dimensional architecture of the lung collagen fibrillar network. The formic acid treatment introduced by Hass (1942) eliminated cellular and collagenous elements and left only the elastin component. SEM of the elastin component revealed its three-dimensional organization. The elastin component fixed by tannic acid showed fibrous but not lamellar structures (Ushiki, 1992). The reported lamellar configurations do not represent the natural structure of lung elastin fibers (Finlay *et al.*, 1996).

Interestingly, collagen and elastin fibers in the alveolar ducts and septa are connected with those in blood vessels and in the pleura. Each fiber system forms a continuum; the continuum of the collagen fibers perhaps provides the lung with a mechanical framework to limit its excess distension, while that of elastin fibers seems to permit the lung to effectively recoil.

The present study has confirmed that collagen fibers in the alveolar mouths subdivide into finer collagen fibers that pass out to the alveolar septa, as noted by Mercer and

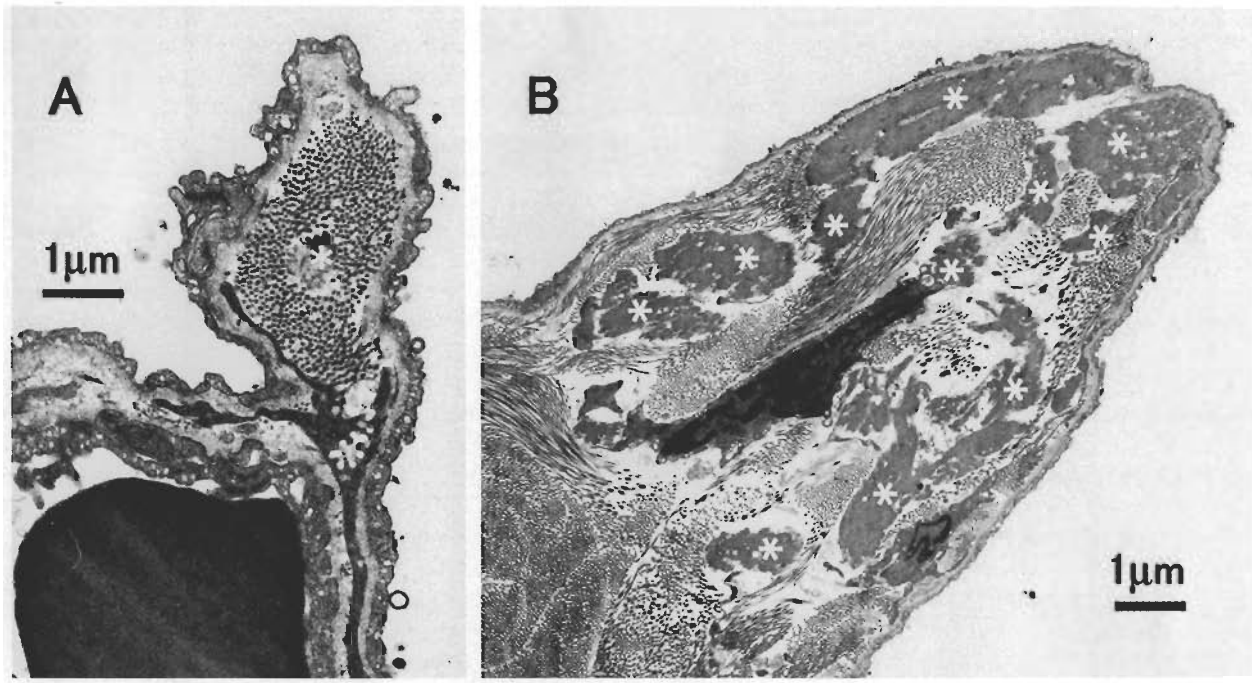

Fig. 8. Transmission electron micrographs of alveolar entrances of intact human lungs. At the small edge of the alveolar septum are elastin fibers surrounded by collagen fibers (**A**). Elastin (*) and collagen fibers are intermingled with each other at the alveolar entrance (**B**). Scale bar = 1 μm

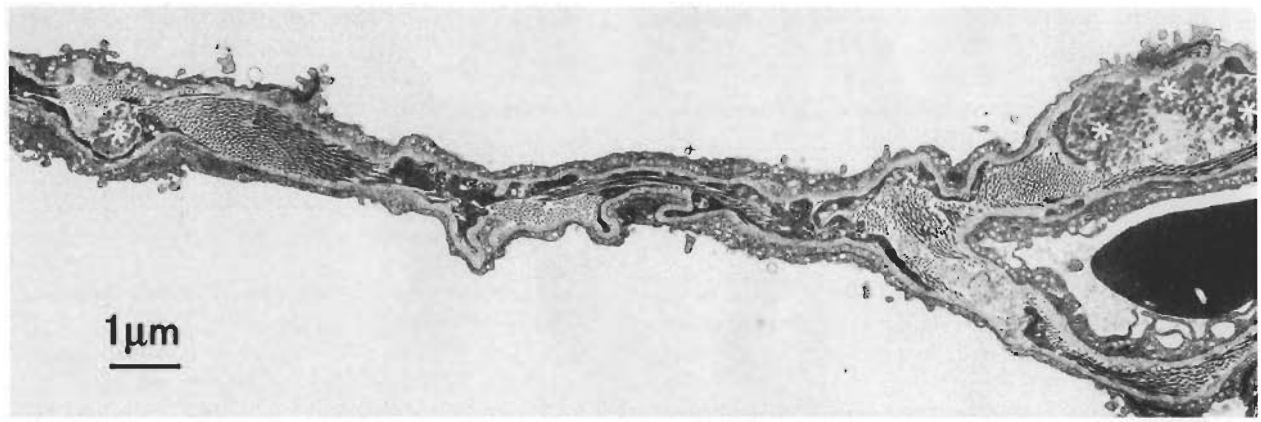

Fig. 9. Transmission electron micrograph of the alveolar septum of the intact human lung. Collagen fibers are distributed throughout the alveolar septum, while elastin fibers (*) are sparse. Scale bar = 1 μm

Carpo (1990). These same authors, in their transmission electron microscopic studies, also showed wavy configurations of collagen fibrils in alveolar mouths. Our SEM has clearly demonstrated that collagen fibers in alveolar mouths and septa took zigzag or wavelike courses in the collapsed lung of the human and rat as shown in the monkey (Ohtani and Nakatani, 1994), but became straight in the inflated rat lung. This finding suggests that the wavy structure of collagen fibers gives them a substantial degree of extensibility, which presumably permits the alveolar mouths and alveoli to expand during inspiration. Likewise, wavy collagen fibers in the pleura allow the lung to expand during inspiration

until the fibers straighten and thus limit further expansion.

Like collagen fibers, elastin fibers in the alveolar mouths subdivided into finer elastin fibers that entered the alveolar septa. However, the elastin fiber network in the alveolar septa was much coarser than the collagen fiber network. Transmission electron microscopy by us and others (Mercer and Carpo, 1990) has shown that the elastin fibers in the alveolar mouths and septa are interwoven with collagen fibers. The close proximity of elastin fibers with collagen fibers suggests that both fibers are mechanically interconnected.

Our findings have also shown that collagen and elastin fibers together probably act as parallel mechanical elements to applied stress or strain. As Mercer and Crapo (1990) suggested, the extension of connective tissue fibers in the alveolar ducts and alveoli may be in two stages. At low levels of strain, the wavy collagen fibers are easily extended, with most of the stress being borne by the adjacent elastin fibers. At higher levels, the collagen fibers become straight and act to limit any further distension of the alveolar ducts and alveoli.

Noteworthily, alveolar pores observed in the collagen fiber network significantly increased in size with age. The emphysematous lung particularly possessed larger and more alveolar pores than the normal lung. These findings are consistent with the condition of the emphysematous lung, which is characterized by the abnormal and permanent enlargement of the air spaces distal to the terminal bronchiole, accompanied by destruction of their walls without obvious fibrosis (Snider *et al.*, 1985). In this context, collagen remodeling may be related to the pathogenesis of emphysema (Finlay *et al.*, 1996).

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