

Alterations at the Synthesis and Degradation of E-cadherin in the Human Lungs with Emphysema

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ABSTRACT

Pulmonary emphysema leads to a cascade of events starting with enlarged alveoli, loss of alveoli and, subsequently to the damage and disruption of pulmonary epithelium. The integrity of the pulmonary epithelium, which is constituted by pneumocytes linked to each other through E-cadherin proteins, is important for respiration. The aim of the present study was to detect the content and destruction of E-cadherin protein and to investigate the contribution of E-cadherin to pulmonary emphysema pathogenesis.

The structural changes, reparative capacity of the pulmonary epithelium, amount of E-cadherin protein and, the immunoreactivity of neural precursor cell expressed developmentally down-regulated protein 9 (NEDD9) were evaluated in emphysematous (n=7) and non-emphysematous (n=6) areas of lung samples taken from patients with chronic obstructive pulmonary disease. Emphysematous areas are characterized by enlarged alveoli, disrupted alveolar walls and epithelium, increased type 2 pneumocytes and NEDD9 immunoreactivity, and reduced E-cadherin proteins.

Our data shows that E-cadherin levels are decreased in emphysematous areas due to its degradation by NEDD9. Decreased E-cadherin levels also lead to the disintegration of the pulmonary epithelium by causing the presence of weakness intercellular connections or the absence of intercellular connections. The repair of the pulmonary epithelium could not complete due to the reduced E-cadherin, because type 2 pneumocytes could not differentiate into type 1 pneumocytes. In conclusion, the reduced E-cadherin levels lead to emphysematous alterations in human lungs and contributes to pulmonary emphysema pathogenesis.

Keywords: Pulmonary emphysema, COPD, E-cadherin, pulmonary epithelium

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by persistent alveolar loss, abnormal inflammatory response, airflow limitation and tissue remodeling, as well as symptoms such as chronic bronchitis and emphysema (1). Pulmonary emphysema is seen in 20% of COPD patients. It is characterized by inflated alveoli with excessive air, irreversible loss of alveoli, damage of the pulmonary epithelium, and reduced respiratory surface area depending on the extent of injury (2). The destruction of the alveolar structure can occur due to the protease-antiprotease imbalance, degradation of elastic fibers and loss of the tissue elasticity (3,4). Pulmonary epithelial cell loss, failure to achieve pulmonary epithelium integrity, chronic inflammation, and deterioration of repair

mechanisms following pulmonary epithelial injury can result in emphysema (3,5,6).

The experimental and clinical studies performed for the pathogenesis of pulmonary emphysema indicate clearly that endogenous repair, following pulmonary epithelial injury, could not occur in the pulmonary emphysema (7). Regeneration of the damaged pulmonary epithelium and ensuring the pulmonary epithelial integrity still remains a challenge in treating the pulmonary emphysema. Therapeutic approaches performed to repair alveolar epithelial and to achieve the integrity of pulmonary epithelium are of a great importance for the renewal and integrity of pulmonary epithelium. Currently there is no therapeutic approach or clinical trial efficient in the repair and keeping the integrity of the pulmonary epithelium for the emphy-



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sema patients. E-cadherin molecule is a cell adhesion protein that is effective on binding of epithelial cells to each other and on the protection of epithelial integrity (8). Oldenburger et al. (9) found that the amount of E-cadherin protein decreased in the lungs of COPD patients compared to control individuals. We suggest that decreases in the levels of E-cadherin protein may result in the failure of binding of pulmonary epithelial cells to each other, and in the disruption of the integrity of pulmonary epithelium. The aims of the present study were to identify the alterations in the levels of E-cadherin protein in the pulmonary emphysematous regions of COPD patients, to explain the possible causes of such alterations, and to determine the relation between E-cadherin and the integrity of pulmonary epithelium.

MATERIALS AND METHODS

Clinical Specimens

The study was ethically reviewed and approved by the Clinical Research Ethics Committee of Istanbul University Cerrahpasa Medical Faculty, Turkey (Diary No a-15/01.10.2013). COPD patients diagnosed with lung cancer were included in the study. COPD diagnosis was made according to the 2016 GOLD criteria. Specimens were obtained from 35 smokers (smoking history: 30 packs/year) and male patients with medium age 48 ± 16 . Lung specimens around the tumor areas were removed by thoracoscopic surgery from COPD patients. They were examined microscopically and specimens of emphysematous and non-emphysematous tissue that were free of carcinoma cells were identified in these samples. Two study groups were formed from the samples: Non-emphysematous tissue specimens ($n=6$, control group) and "Emphysematous Tissue Specimens ($n=7$)".

Histology and Immunohistochemistry

The lung specimens were fixed in 10% buffered formalin for 24 h, dehydrated in ascending alcohols and embedded in paraffin. Sections 5 μm thick underwent to hematoxylin-eosin and Verhoeff's elastic stainings (the latter-staining-elasticin in blue-black) and were examined under a light microscope.

Sections 4 μm thick were stained using immunoperoxidase-based procedures. After antigen retrieval (10), the sections were incubated in 3% hydrogen peroxide in 1:1 methanol/phosphate-buffered saline mixture, incubated with the indicated antibodies overnight at 4 °C, and then treated with Histostain Plus-peroxidase kit according to the manufacturer's instructions. The indicated primary antibodies are rabbit anti-ki67 (a proliferation marker, diluted to 1:50, Millipore), -prosurfactant C (proSPC, type 2 pneumocyte marker, diluted to 1:500, Millipore) and -neural precursor cell expressed developmentally down-regulated 9 (NEDD9, a digestive protein for E-cadherin, diluted to 1:500, Abcam). The peroxidase activity was revealed by a 3-amino-9-ethylcarbazole substrate kit. Slides were counterstained with Mayer's hematoxylin. For negative controls, phosphate-buffered saline solution was used instead of the primary antibodies.

Five microscopic fields were randomly selected from alveolar areas without bronchioles from sections of each sample. Digi-

tal images of these fields were captured at a magnification of 400 and overlaid with transparent grids (1 mm^2). The number of anti-ki67 or anti-proSPC immunoreactive cells was calculated and reported as percentage of the total number of cells.

Western Blotting

Lung samples were snap frozen in liquid nitrogen and stored at -86 °C. Lung samples weighing 200 mg were homogenized in lysis buffer. The lysates were then centrifuged at 13,000g for 10 min at 4 °C, and the supernatants were collected and stored at -20 °C. The total protein concentrations were determined using Bradford method (11). Then 80 μg samples were loaded in 10% SDS-PAGE electrophoresis. Next, the protein was transferred onto nitrocellulose membranes and blocked with 5 % non-fat dried milk for one hour. Later on, the protein was incubated overnight at 4 °C with the primary antibodies against E-cadherin and advanced glycosylation end-product specific receptor (AGER, type 1 pneumocyte marker) diluted 1:500 and 1:100 respectively. The membrane was washed, and incubated with the secondary antibody (a goat anti rabbit Ig-G-HRP, diluted 1:500). Finally, the blots were developed with luminol reagent (Santa Cruz, CA, USA). The intensities of the protein bands were quantified using molecular imaging software (Kodak GL 1500, CT, USA) being normalized to β -actin protein bands.

Statistical Analysis

The results were analyzed by *Student T*-test and *Mann Whitney U-2 tail* test to compare differences among groups by using GraphPad Prism software, version 5.00 (San Diego, CA). P values of <0.05 were considered significant.

RESULTS

Histology of Human Lung Specimens

The alveolar structure in the non-emphysematous areas was generally preserved when compared to the alveolar structure of emphysematous areas. Enlarged alveoli were rarely seen in the non-emphysematous areas. In these areas, the integrity of pulmonary epithelium was preserved, and also there were no thinning or breaking of the pulmonary epithelium. The accumulation of macrophages, neutrophils and erythrocytes in the lumens of alveoli were commonly observed in the human lung without emphysema (Figure 1a). It was noteworthy the disrupted lung structure throughout the tissue in the emphysematous areas of human lung. In the emphysematous areas, larger alveoli were observed instead of the small normal-sized alveoli observed in the non-emphysematous areas. There were thinning and breaking in the walls of the enlarged alveoli (Figure 1b). Therefore, the pulmonary epithelial integrity could not be preserved in the human lungs with emphysema. The numbers of accumulated macrophages, neutrophils and erythrocytes in the alveolar lumens were high in the human lung with emphysema.

Elastic fibers were predominantly localized in the alveolar wall, pulmonary interstitial tissue, and the walls of pulmonary arteries and arterioles in the human lung. They were seen as a very curved and long fibers in the lung without emphysema (Figure 2a). There

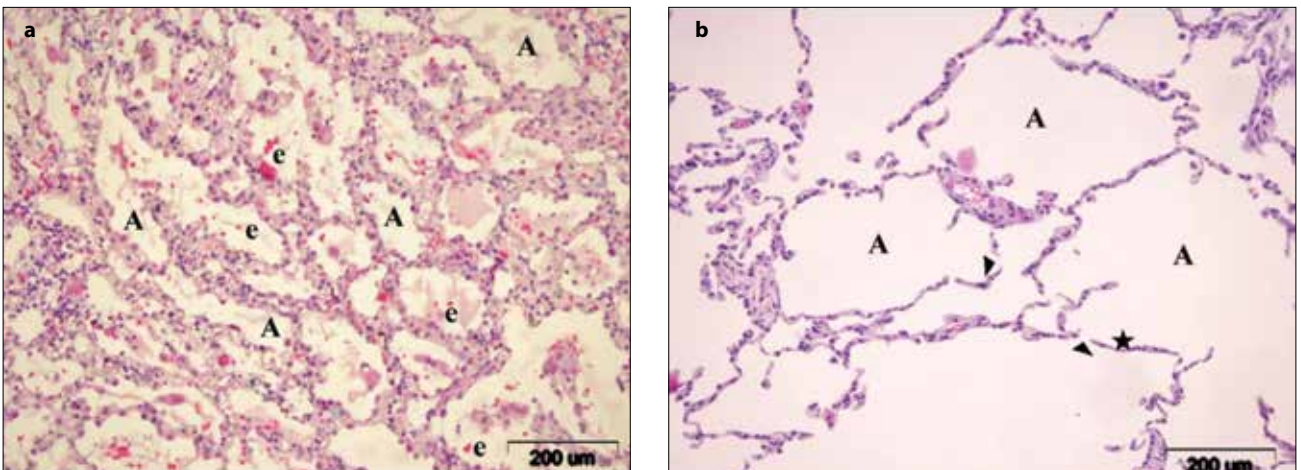


Figure 1. a, b. Histological section of human lung stained with hematoxylin-eosin. a) Lung sample without emphysema; (A) alveoli and (e) erythrocyte in the alveolar lumen; b) Lung sample with emphysema; (A) alveoli, (*) thinning of alveolar wall, and (▲) disruption of alveolar wall. Scale bars = 200 μm .

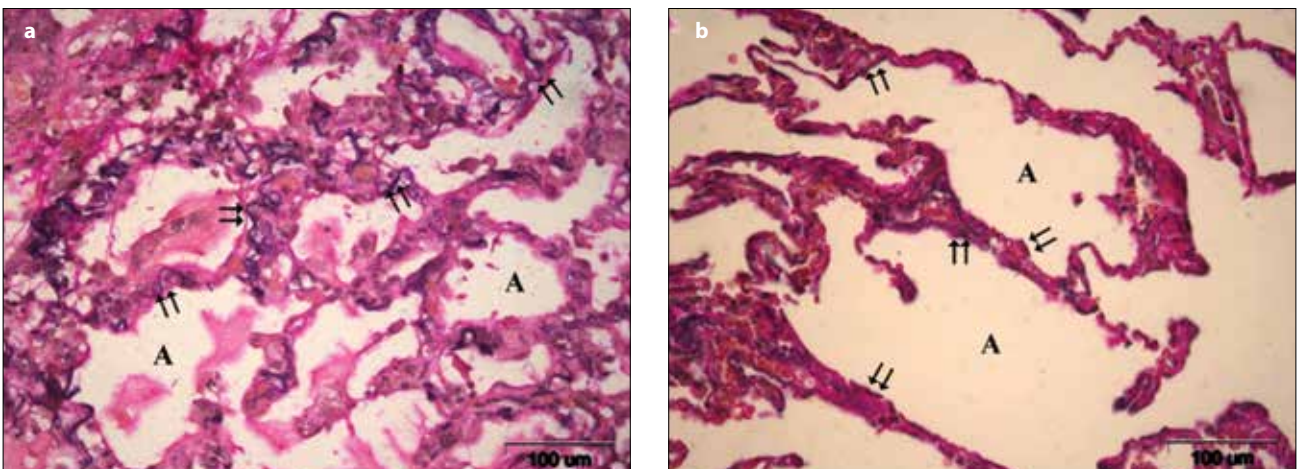


Figure 2. a, b. Elastic fibers of the human lung were seen as blue-black fibers stained with Verhoeff's Elastic Stain. They were more less and discontinuous than that of non-emphysematous areas. a) Lung sample without emphysema; b) Lung sample with emphysema. A: Alveoli, ⇔: Elastic fibers. Scale bars=100 μm .

was a significant decrease in the presence of elastic fibers in the alveolar wall of the emphysematous areas. The elastic fibers in these regions were much less pronounced, more flat and discontinuous compared to those of the non-emphysematous areas (Figure 2b).

Cell Proliferation and Repair of Alveolar Epithelium in Human Lungs with and without Emphysema

Ki67 immunoreactivity was observed in the nuclei of bronchial and pulmonary epithelial cells, and mesenchymal cells in the connective tissue. Ki67 immunoreactive cells were few in number in the non-emphysematous areas of human lung, and also their immunoreactivities were weak (Figure 3). However, there were more Ki67 immunoreactive cells in the emphysematous areas of the human lung. Especially, a lot of these cells in the pulmonary epithelium showed an intense Ki67 immunoreactivity (Figure 3).

ProSPC immunoreactivity was observed in the cytoplasm of some pulmonary epithelial cells in the emphysematous and

non-emphysematous areas (Figure 4). A lot of proSPC immunoreactive epithelial cells, strongly expressed proSPC in the emphysematous areas (Figure 4).

The levels of AGER were high in the lungs without emphysema versus the lungs with emphysema [($p < 0.01$) (Figure 5)].

Levels of E-Cadherin Protein and Anti-NEDD9 Immunoreactivity in Human Lungs with and Without Emphysema

The levels of E-cadherin were higher in the lungs without emphysema than in the lungs with emphysema [($p < 0.05$) (Figure 6)].

NEDD9 immunoreactivity was also observed in the cytoplasm and nuclei of the bronchial and pulmonary epithelial cells, and in the mesenchymal cells of the pulmonary interstitium. NEDD9 immunoreactive cells were rarely found in the non-emphysematous areas of human lung (Figure 7). However, a large number of NEDD9 immunoreactive cells were identified in the pulmo-

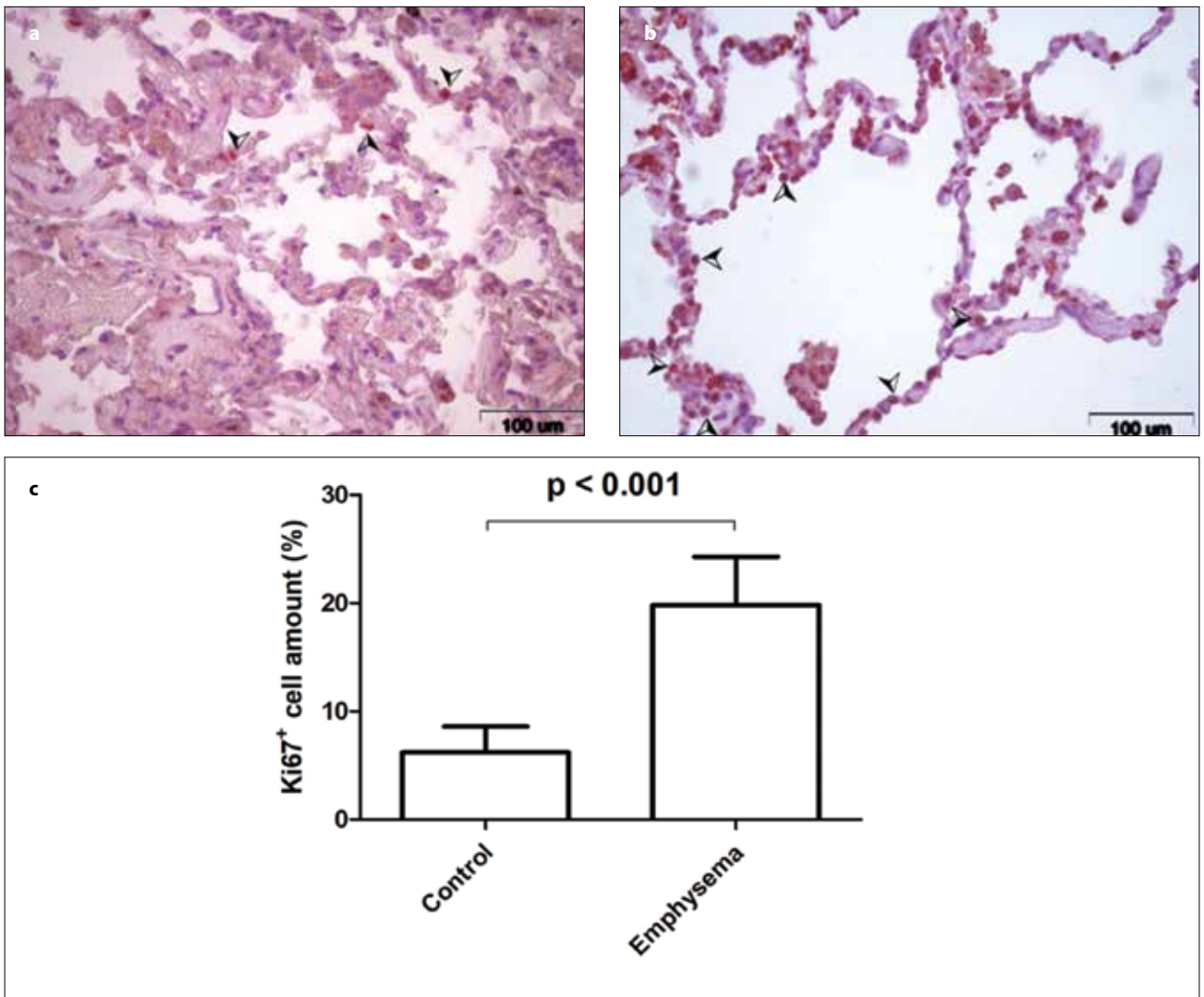


Figure 3. a-c. Ki67 immunoreactivity was marked by arrows in the alveolar epithelium of the non-emphysematous (a) and the emphysematous areas (b). Scale bars=50 µm. Mayer's hematoxylin counterstain. The number of proliferative cells (%) in the alveolar areas the non-emphysematous and the emphysematous regions was shown in "c".

nary epithelium of the emphysematous areas. The number of these cells was much higher than that of the non-emphysematous areas, and these cells were localized into groups at the corners of the alveoli in the pulmonary epithelium (Figure 7).

DISCUSSION

Age, gender, various respiratory diseases, smoking, various environmental factors and occupational exposures are found among the causes of pulmonary emphysema (12). In COPD patients, who are aged 45 to 60 and smokers, emphysematous areas are defined as the expansion of the airways and alveoli in areas ranging from distal airways to bronchioles and alveoli (13). In human and animal lungs exposed to cigarettes, emphysematous alterations, such as the enlarged alveoli, the thinned and occasionally disrupted alveolar epithelium, the inflammation in the alveolar spaces and pulmonary parenchyma, and

the destruction of the connective tissue have been determined (14,15). In the present study, most of the individuals were male COPD patients, who were with medium age 48 ± 16 , smokers and diagnosed with lung adenocarcinoma. Emphysematous areas in lung biopsies taken from these patients were distinguished by the characteristic properties of emphysema mentioned above, such as dilated alveoli, the thinning and occlusion of the alveolar epithelium and infiltration of inflammatory cells, the decreases in amount of elastic fibers, and disorganization of their order.

Responsible mechanisms for the formation of enlarged alveoli in the pulmonary emphysema include the disintegration and disruption of the alveolar epithelium following the injury of alveolar epithelium. The inflammation resulting from cigarette smoke exposure, subsequently, the released agents from the inflammatory cells, and the protease-antiprotease imbalance in

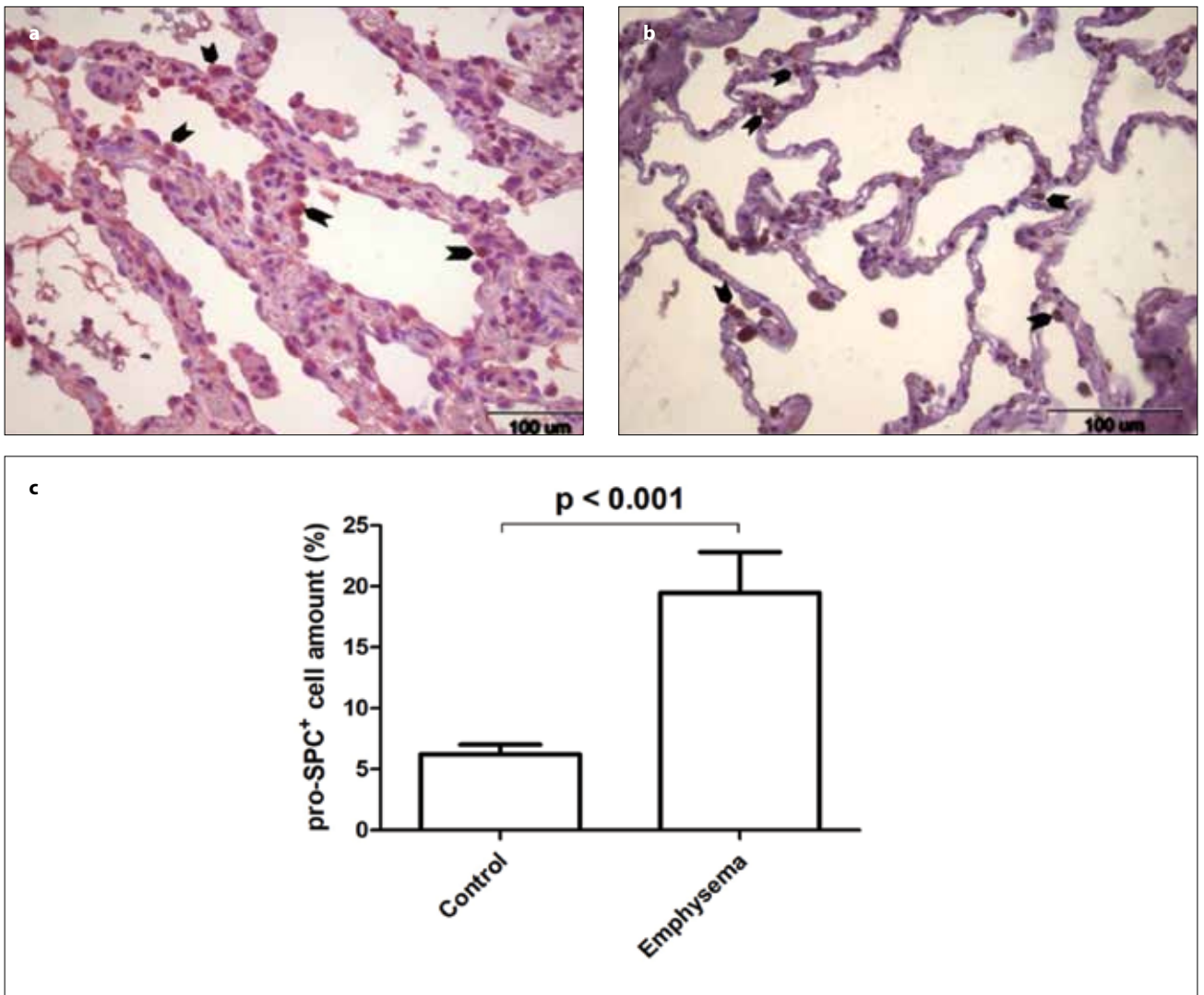


Figure 4. a-c. ProSPC immunoreactivity was marked by arrows in the alveolar epithelium of the non-emphysematous (a) and emphysematous areas (b). Scale bars = 50 μm. Mayer's hematoxylin counterstain. The number of proSPC immunoreactive cells (%) in the alveolar areas the non-emphysematous and the emphysematous regions was shown in "c".

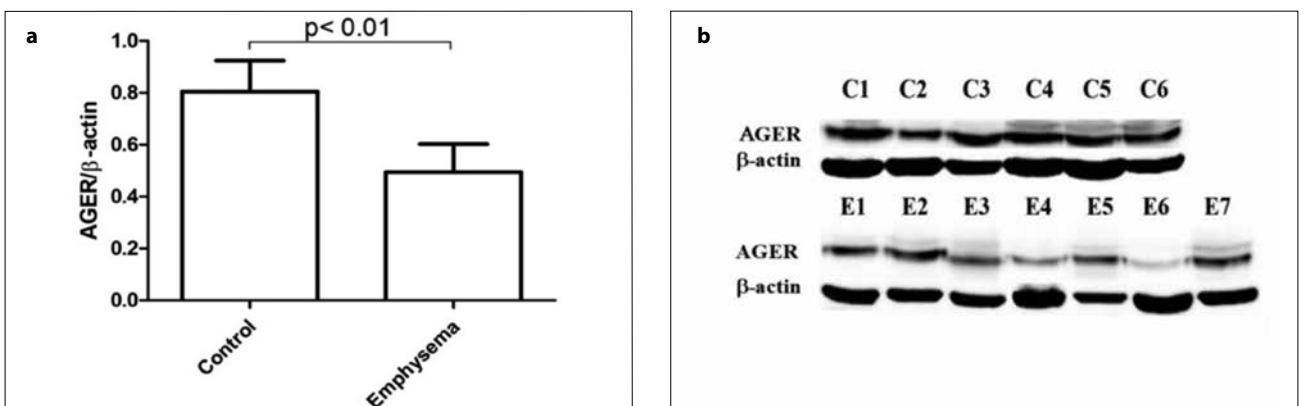


Figure 5. a, b. Alterations of AGER protein levels in non-emphysematous and emphysematous regions areas of lung of COPD patients. a) intensity analysis of the protein bands and b) AGER protein levels in the emphysematous lung samples (E) compared to the non-emphysematous areas (control group, C).

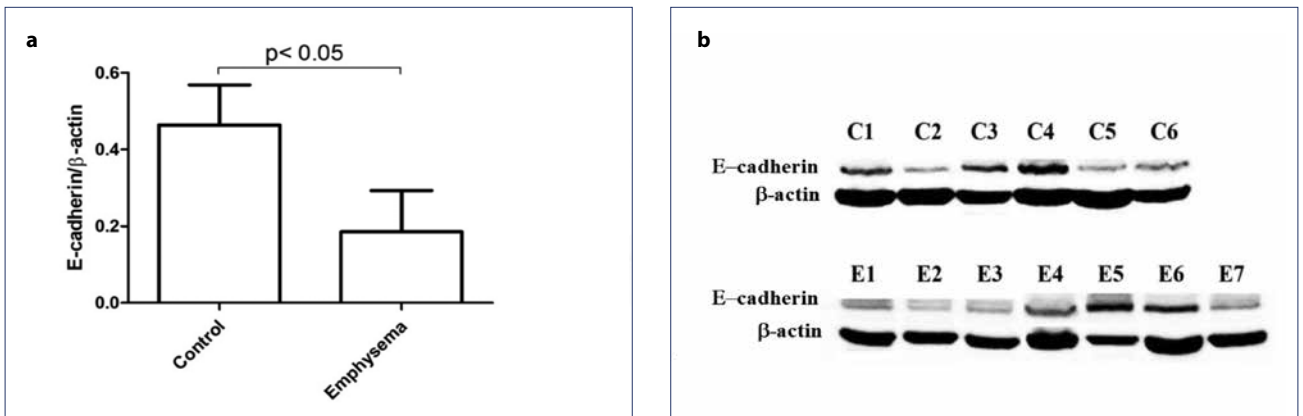


Figure 6. a, b. Alterations of E-cadherin protein levels in non-emphysematous and emphysematous regions areas of lung of COPD patients. a) intensity analysis of the protein bands and b) E-cadherin protein levels in the emphysematous areas (E) compared to the non-emphysematous areas (control group, C).

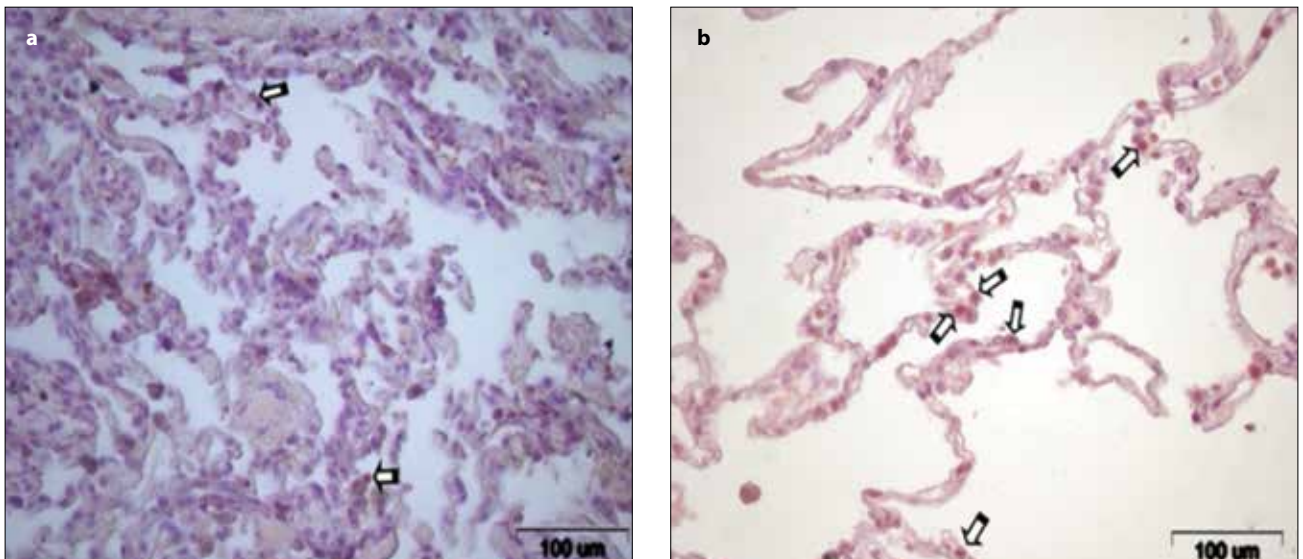


Figure 7. a, b. NEDD9 immunoreactivity was marked by arrows in the alveolar epithelium of the non-emphysematous (a) and emphysematous areas (b). Scale bars = 50 μm. Mayer's hematoxylin counterstain.

the lungs lead to the damage of the alveolar epithelium (2,6). The cell death in the alveolar epithelium cells has been shown in the experimental studies where pulmonary emphysema was induced by several substances such as cigarette smoke, elastase, and papain (7,14,16). It has been suggested that alveolar epithelial damage occurs irreversibly and that the repair of alveolar epithelium cannot be done in the pulmonary emphysema (7). In the present study, we found the disruption of alveolar epithelial integrity and the break of alveolar walls in the pulmonary emphysematous areas of COPD patients. The epithelial cells, which are firmly attached to each other by the E-cadherin molecules, provide the integrity and continuity of the epithelial layer. Type 1 and 2 pneumocytes cover the alveolar surface along the alveolar epithelium in the healthy human lung. Pneumocytes are attached to each other by a variety of tight and adherent cell connections. In a healthy human lung, these cell-cell connections are effective on the preservation and integration of the

alveolar epithelium, and in the construction of epithelial barrier function between the alveolar lumen and lung parenchyma (3). It has been reported that cigarettes reduce the expression of genes that encode cell-cell junction proteins, including zonula occludens-1 and E-cadherin and induces structural changes that disrupt the epithelial barriers in the mouse lungs (17). Oldenburger and coworkers (9) found that the amount of E-cadherin protein decreased in the lungs of COPD patients compared to control individuals. The study has also shown that reduction in the amount of E-cadherin protein resulted in the decrease of epithelial barrier functions in human bronchial epithelial cells exposed to cigarette extracts. The increased airway and alveolar epithelial permeability, and the stimulated inflammatory response are accepted as an important risk factors for COPD. The increases in the epithelial permeability and neutrophil infiltrations following cigarette smoke exposure were detected in A549 cells (18). Neutrophil numbers and elastase levels were also increased

in bronchoalveolar lavage fluids collected from COPD patients and residues of digested E-cadherin proteins were found in these fluids (19). Existing reports in the literature suggest that neutrophil-derived elastase may cause the destruction of E-cadherin in human lungs. In the present study, we also found that the levels of E-cadherin protein were reduced and that a large number of neutrophil cells were present in emphysematous areas. Thus, elastases released from these cells may have resulted in the destruction of the E-cadherin. On the other hand, one of the responsible molecules for the intracellular destruction of the E-cadherin protein is NEDD9 protein. Weak expression of NEDD9 in non-neoplastic lung specimens and increased expression of NEDD9 in lung adenocarcinoma cases have been previously reported (20). NEDD9 promotes metastasis in cancer cells by the inhibition of cell adhesion (21). Changes in the protein expressions of NEDD9 family reduced the expression of α -catenin, β -catenin, p120 catenin, and the localization and stabilization of E-cadherin in the cell membrane (22). NEDD9 is an effective protein in transporting E-cadherin to lysosomes and it regulates the lysosomal digestion of the E-cadherin (22). In the present study, NEDD9 immunoreactivity was observed rarely in the alveolar epithelium in non-emphysematous areas, whereas a significant increase in the number of NEDD9 immunoreactive cells was observed in the alveolar epithelium of the emphysematous areas. Therefore, intracellular digestion of E-cadherin induced by NEDD9 may be effective in reducing the amount of E-cadherin proteins in the emphysematous areas.

Our data suggest that the E-cadherin protein contributes to the pathogenesis of pulmonary emphysema via several ways. The first and second ways are the reduced E-cadherin-mediated inflammatory response, which appears due to the increase in the epithelial permeability and the disintegration of the epithelium, which appears due to inability to establish cell-cell connections, respectively. The third way is the successfully uncompleted epithelial repair due to a decreased E-cadherin protein levels. In a damaged tissue, if there are reserve cells or if the cells are capable of proliferation, they first proliferate, and then contribute to the complementation of the tissue repair by differentiating into many specialized types of cells. The cumulative data show a relationship between E-cadherin and regulation of cell proliferation. The cytoplasmic portion of the E-cadherin molecule binds to the cytoplasmic β -catenin via mediating proteins. These connection allows the stabilization and localization of E-cadherin molecule in the cell membrane. The disappearing of E-cadherin- β -catenin connection causes the release and transfer of β -catenin from cytoplasm to nucleus, with the subsequent transcription of genes involved in cell proliferation (23). Our findings demonstrate that the alveolar epithelium is damaged, and the alveolar wall is disrupted somewhere. The microscopic data of the present study show that a repair mechanism engages in fact primarily in the alveolar epithelium of the emphysematous areas. We detected the increased number of type 2 pneumocytes and proliferative cells (Ki67 immunoreactive cells) in response to alveolar epithelial damage in the emphysematous areas characterized by reduced E-cadherin levels. The proliferative type 2 pneumocytes differentiate into type 1

and type 2 pneumocytes in the correct repair of the alveolar epithelium (24). Thus, alveolar epithelial repair is completed. However, in the present study, the amounts of AGER and E-cadherin proteins were decreased in the emphysematous areas while the number of type 2 cells was increased. The data suggests that type 2 pneumocytes could not differentiate into type 1 pneumocytes, although the reduced E-cadherin-induced cell proliferation leads to an increased number of type 2 pneumocytes. That might suggest that the epithelial repair has not been completed. Nagaoka and coworkers (25) identified the decrease of E-cadherin levels by Western Blotting in an epithelial cell line transfected with anti-miR-200a, a gene involved in the epithelial cell differentiation and polarization. They noted that the E-cadherin molecule is effective molecule for the epithelial cell differentiation and polarization. Our data suggest that the reduction at the E-cadherin protein levels in the emphysematous areas of the lungs of COPD patients may contribute to the pathogenesis of pulmonary emphysema by causing delays in the differentiation of alveolar epithelial cells in these regions. This situation explains why pulmonary emphysema is characterized by an irreversible loss of alveolar structure.

Pulmonary emphysema is seen in 20% of COPD patients. The loss of transport of respiratory gases due to damage and loss of pulmonary epithelium is shown among the causes of death in COPD patients. The cumulative data have been suggested that pulmonary epithelial damage occurs irreversibly and that pulmonary epithelial repair cannot be performed in the pulmonary emphysema (7). The resolve of the underlying mechanisms related to both pulmonary epithelial injury and the deterioration of its repair in pulmonary emphysema is required for the elucidation of the pathogenesis of pulmonary emphysema. The achievement of the pathogenesis of pulmonary emphysema. The achievement of proliferation and differentiation of pulmonary epithelial cells in addition to the ensure of the repair and integrity of pulmonary epithelium have been suggested to be an effective approach in improving the pathogenesis of emphysema (26). The data of the present study demonstrate that reductions in protein level of E-cadherin can contribute to the pathogenesis of pulmonary emphysema, leading to emphysematous changes in the alveolar areas of the lung. Approaches to alleviate the increase of E-cadherin protein synthesis or the decrease of its digestion might be utilized in order to reduce the epithelial permeability, preservation of the epithelial integrity, and completion of the epithelial repair in the alveolar areas for the prevention of pulmonary emphysema pathogenesis.

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