Endothelial Cell Death and Decreased Expression of Vascular Endothelial Growth Factor and Vascular Endothelial Growth Factor Receptor 2 in Emphysema

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Emphysema due to cigarette smoking is characterized by a loss of alveolar structures. We hypothesize that the disappearance of alveoli involves apoptosis of septal endothelial cells and a decreased expression of lung vascular endothelial growth factor (VEGF) and its receptor 2 (VEGF R2). By terminal transferase dUTP nick end labeling (TUNEL) in combination with immunohistochemistry, we found that the number of TUNEL+ septal epithelial and endothelial cells/lung tissue nucleic acid (μg) was increased in the alveolar septa of emphysema lungs (14.2 ± 2.0/μg, n = 6) when compared with normal lungs (6.8 ± 1.3/μg, n = 7) ($p < 0.01$) and with primary pulmonary hypertensive lungs (2.3 ± 0.8/μg, n = 5) ($p < 0.001$). The cell death events were not significantly different between healthy nonsmoker (7.4 ± 1.9/μg) and smoker (5.7 ± 0.7/μg) control subjects. The TUNEL results were confirmed by single-stranded DNA and active caspase-3 immunohistochemistry, and by DNA ligation assay. Emphysema lungs (n = 12) had increased levels of oligonucleosomal-length DNA fragmentation when compared with normal lungs (n = 11). VEGF, VEGF R2 protein, and mRNA expression were significantly reduced in emphysema. We propose that epithelial and endothelial alveolar septal death due to a decrease of endothelial cell maintenance factors may be part of the pathogenesis of emphysema.

Emphysema is a major component of the morbidity and mortality in chronic obstructive pulmonary disease (COPD), which is estimated to affect at least 14 million people in the United States (1, 2). Although chronic cigarette smoking is the most important risk factor for this global health issue, the pathogenesis of pulmonary emphysema remains enigmatic. Emphysema is a heterogeneous disease in regards to the degree of alveolar loss, the pattern of localization in the lung, and the several predisposing genetic risk factors involved in its pathogenesis. Eriksson's (3) discovery of the association between panacinar emphysema and a hereditary deficiency of α_1-antitrypsin led to the development of the protease–antiprotease imbalance hypothesis of emphysema (4, 5). This hypothesis is supported by animal experimental data (6) and by studies that compare cells and bronchoalveolar lavage fluid in smokers and nonsmokers (7). Cigarette smoking may cause emphysema by stimulating neutrophils and macrophages located in the walls of the membranous bronchioles to increase the production of proteinases (8–10). However, one of the limitations of this concept is that other inflammatory lung diseases characterized by dense accumulation of lymphocytes and macrophages (such as hypersensitivity pneumonitis), or by neutrophil infiltration (such as bronchopneumonia or acute respiratory distress syndrome), are not associated with the development of emphysema. Another concept is the persistence of childhood respiratory infections (for example, infection with adenovirus) that might cause impairment of lung growth, and during adolescence, increased susceptibility to injury by cigarette smoking (11–13). Even in the genetically susceptible group of individuals with α_1-antitrypsin deficiency, smoking greatly increases the severity of the emphysema (14).

Preceding Eriksson's discovery, Liebow (15), based on histological examination of emphysema lungs, pointed out that the alveolar septa in centrilobular emphysema appear to be remarkably thin and almost avascular. He also considered that a reduction in the blood supply of the small precapillary blood vessels might induce the disappearance of alveolar septa. Because the current hypothesis of protease–antiprotease imbalance or inflammation does not completely explain the loss of lung tissue in cigarette smoking-induced emphysema, we revisit the early vascular concept and propose that the disappearance of lung tissue in emphysema may involve the progressive loss of capillary endothelial and epithelial cells associated with the loss of the extracellular matrix. The endothelial cell loss might occur through the process of programmed cell death, apoptosis. Apoptosis has now been recognized to play an important role in the loss of cardiomyocytes following myocardial ischemia (16) and in the resolution of inflammation (17–19).

Because vascular endothelial cell growth factor (VEGF) induces endothelial cell growth (20) and withdrawal of VEGF leads to endothelial cell apoptosis *in vitro* (21, 22) and *in vivo* (23), VEGF is a trophic factor required for the survival of endothelial cells. VEGF is highly abundant in the lung, but its physiological action in the normal lung is not understood. Cigarette smoking may act by decreasing the expression of VEGF and its receptor 2 (VEGF R2 or KDR/Flk-1), thus resulting in lung septal endothelial cell death. Given this concept, we hypothesize that there is an increased number of apoptotic cells in the smoker's lungs with centrilobular emphysema, involving both epithelial and endothelial lung cells. The loss of endothelial cells may be caused both by loss of VEGF or a "faulty" VEGF signaling.

In this study we quantify the apoptotic events in emphysema lungs by measuring terminal transferase dUTP nick end labeling (TUNEL) and oligonucleosomal-length DNA fragmentation. We also determine the presence of active caspase-3 positive and single-stranded DNA expressing alveolar septal cells, and investigate the expression of VEGF and its receptor 2 in pulmonary emphysema.

METHODS

Patients

Archival tissue studies (immunohistochemistry, in situ hybridization). We obtained lungs from six patients with severe emphysema (one male,

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five females; 62.3 ± 1.7 [SEM] yr) who were undergoing lung transplantation, lung volume reduction surgery, or lobectomy (Table 1). All six were chronic cigarette smokers with obstructive changes documented by pulmonary function studies. Normal lung tissue was obtained from 11 patients undergoing open lung biopsy for diagnostic purposes (localized inflammation [n = 1] or primary or metastatic malignancies [n = 10]; six males, five females; 49.9 ± 3.5 [SEM] yr). Patients with histologically normal lung tissue had no clinical evidence of emphysema. We used two groups of control subjects. The first consisted of seven subjects who were nonsmokers or remote smokers (0–15 pack-years smoking). The second group consisted of four subjects with normal pulmonary function who had a history of more than 40 pack-years smoking. The primary pulmonary hypertension lung specimens were obtained from five patients (two males, three females; 31.8 ± 5.2 [SEM] yr) with severely elevated pulmonary artery pressure documented by right heart catheterization.

Fresh tissue studies (oligonucleosomal-length DNA laddering and Western blot). Fresh frozen lung tissue from patients with advanced smoking-induced emphysema was obtained at lung transplantation (n = 12, 56 ± 2.2 [SEM] yr) and normal lung tissue was obtained at the time of tumor resection (n = 11, 60 ± 5.5 [SEM] yr). The fresh lung tissue samples were obtained from different patients than those taken from our lung archive.

Computed Tomographic Scan Analysis

The computed tomographic (CT) images of each lung used in the archival tissue studies were divided into three craniocaudal zones of equal length. The extent of emphysema in each zone of each lung was visually estimated to the nearest 25% (using the scoring system established by the National Emphysema Treatment Trial Advisory Committee on Radiology [*Chair*: P. Friedman]). From the six scores for the lungs, an overall percentage of emphysema was calculated.

Tissue Processing

Frozen tissue. Fresh lungs were maintained on ice for approximately 15 to 45 min, and then expanded and cryoprotected as previously described (24). Lung fragments were frozen in Tissue Tek embedding medium (Sakura, Torrance, CA) and maintained at −70° C until sectioning. Seven-micron sections were mounted onto Superfrost slides (Fisher Scientific, Pittsburgh, PA).

Paraffin-embedded tissue. Random sections of the resected specimens were obtained from formalin-fixed lung tissue. Five-micron sections were mounted onto Superfrost slides. One slide per case was selected for TUNEL studies based on the assessment of alveolar expansion, lack of an inflammatory or infectious process, and sampling of peripheral lung tissue. The diagnosis of primary pulmonary hypertension (PPH) was established on the basis of structural pulmonary vascular lesions in association with the clinical and hemodynamic findings (25).

Apoptosis Assays

TUNEL. TUNEL was performed with TACS 2 TdT DAB kit (Trevigen, Gaithersburg, MD), following the manufacturer's instructions. Briefly, after deparaffinization and dehydration, sections were digested with proteinase K at a concentration of 20 μg/ml for 20 min. Endogenous peroxidase activity was quenched with 2% (vol/vol) H_2O_2 for 5 min. The slides were immersed in terminal deoxynucleotidyltransferase (TdT) buffer. TdT, 1 mM Mn^{2+}, and biotinylated dNTP in TdT buffer were then added to cover the sections and incubated in a humid atmosphere at 37° C for 60 min. The slides were washed with phosphate-buffered saline (PBS) and incubated with streptavidin–horseradish peroxidase for 15 min. After rinsing with PBS, the slides were immersed in diaminobenzidine (DAB) solution. The slides were counterstained for 1 min with 0.5% methyl green.

The number of TUNEL positive cells in the alveolar septa was normalized by the amount of nucleic acid (DNA+RNA) extracted from the serial section (5 μm in distance) adjacent to the TUNEL section. The immediate serial section of the TUNEL processed slides was scraped off from the slides, collected into a microcentrifuge tube, and digested with proteinase K (400 μg/ml) in 1 mM EDTA, 50 mM Tris (pH 8.5), and 0.5% Tween 20 buffer overnight at 37° C. The digests were briefly centrifuged to remove cellular debris. Absorbance at 260 nm was measured in order to determine the concentration of nucleic acid. Proteins have a negligible absorbancy at 260 nm wavelength, with most of the absorbency at this wavelength attributable to nucleic acids. This novel approach to normalize the tissue expression data was used because the lungs were not expanded with formalin, thus precluding the use of lung volume or septal length as a means to normalize the morphometric data.

Double staining. To identify which cell types were apoptotic, double staining with immunohistochemistry followed by TUNEL was

TABLE 1. CHARACTERISTICS OF SUBJECTS

Patients	Sex	Age	Diagnosis	Smoking (*pack-years*)	FEV_1 (%)	$D_{L_{CO}}$ (%)	CT score (%)
1	F	65	Emphysema	25	34	105	
2	F	63	Emphysema, adeno ce	40	36		
3	F	63	Emphysema	60	55	66	75
4	F	54	Emphysema	60	43	106	63
5	F	66	Emphysema	80	21	59	67
6	M	63	Emphysema, adeno ce	110	44	32	21
7	F	64	Meta adeno ce	0	95	89	NE
8	M	39	Normal	0			
9	M	25	Carcinoid	5			
10	M	46	Meta adeno ca, colon primary	5	88		
11	M	58	Meta Leiomyosarcoma	7	92		NE
12	F	52	Meta Leiomyosarcoma	15	83	110	NE
13	M	62	Meta hepatocellular ca	15			NE
14	M	48	Meta renal cell ca	40	90		NE
15	F	45	Large cell lung ca	50	85	93	
16	F	49	Rheumatoid arthritis, adeno ca	60	89	76	NE
17	F	51	Bronchioloalveolar ca	88	78		
18	F	48	PPH				
19	M	38	PPH				
20	M	25	PPH				
21	F	18	PPH				
22	F	30	PPH				

Definition of abbreviations: CT score = overall extent of emphysema (%) by chest CT; Meta = Metastatic disease; NE = no evidence of emphysema by CT scan; PPH = primary pulmonary hypertension.

Patients 1–6 were patients with emphysema; Patients 7–13 were nonsmokers or patients with a short smoking history; Patients 11–17 had a long smoking history but did not have significant histological emphysema; and Patients 18–22 had a primary diagnosis of primary pulmonary hypertension.

performed. The immunohistochemical staining was performed using the modified avidin–biotin–alkaline phosphatase system (Vecstatin ABC-AP kit; Vector Laboratories, Burlingame, CA). Nonspecific antibody binding was blocked using 2% normal goat serum incubated for 20 min. The slides were incubated with either a monoclonal anticytokeratin (1:6 dilution, CAM 5.2; Becton Dickinson, San Jose, CA) or a polyclonal anti-Factor VIII related antigen (Factor VIII r.Ag) (1:400 dilution, A0082; Dako Corp., Carpinteria, CA) antibody in a humid chamber at room temperature for 30 min. The following steps were then performed: biotinylated horse anti-mouse antibody for monoclonals (BA-2000; Vector Laboratories) or biotinylated goat anti-rabbit antibody for polyclonals (BA-1000; Vector Laboratories) at room temperature for 10 min; and alkaline phosphatase avidin–biotin complex in PBS at room temperature for 5 min; then the reaction was visualized by Vector red in 120 mM Tris–HCl (pH 8.3). After staining for epithelial or endothelial cells, sections were washed in PBS, and TUNEL was carried out as described above.

Oligonucleosomal-length DNA laddering. The presence of oligonucleosomal-length DNA cleavage was investigated with the Apoalert LM-PCR ladder assay kit (CLONTECH Laboratories Inc., Palo Alto, CA). In brief, DNA was isolated from lung tissue samples using proteinase K-phenol extraction. Dephosphorylated adaptors were ligated to 5′ phosphorylated blunt ends with T4 DNA ligase to 500 ng of lung sample DNA (during 16 h at 16° C). They then served as primers in LM-PCR under the following conditions: hot start (72° C for 8 min), 30 cycles (94° C for 1 min, and 72° C for 3 min), and extension (72° C for 15 min). Every reaction set included thymus DNA (25 ng) as positive control and for normalization of the amount of reaction product. Amplified DNA was subjected to gel electrophoresis on a 1.2% agarose gel containing ethidium bromide. Images were scanned and the DNA fragmentation levels, based on the density of the second band bottom-up (approximately 400-bp product) relative to the thymus control DNA, were quantitated using SigmaGel (Jandel Corp.).

Immunohistochemistry for single-stranded DNA. Tissue sections were deparaffinized and rehydrated. The slides were then treated with saponin (0.1 mg/ml in PBS), 20 min at room temperature (RT), followed by proteinase K digest (20 μg/ml in PBS) for 20 min at RT, and 50% formamide (vol/vol in distilled water) preheated to 56–60° C in a warm bath for 20 min. The slides were then transferred to ice cold PBS for 5 min, treated with 3% H_2O_2 for 5 min at RT, blocked with 3% nonfat dry milk for 15 min at RT, and incubated with anti-single-stranded DNA antibody (Mab F7-26; Alexis Corp.) at 1/100 dilution for 15 min. Antibody binding was detected with peroxidase-anti-mouse IgM (Zymed) at 1/100 dilution for 15 min at RT and diaminobenzidine, and counterstained with methyl green. Negative control consisted of isotype-matched antibody.

Immunohistochemistry for CM1. CM1, anti-caspase-3 antibody (provided by Dr. Anu Srinivasan, Idun, La Jolla, CA), immunolocalization was performed in paraffin-embedded, formalin-fixed lungs. Methods described previously were used for immunohistochemistry studies (26). Briefly, after paraffin removal in xylene, the sections were rehydrated and submitted to microwave treatment (800 W/15 min) in 10 mM citric acid monohydrate. After quenching of endogenous peroxidase with 3% H_2O_2 for 20 min, the sections were exposed to the anti-CM1 polyclonal antibody (1:500 dilution) for 60 min. After incubation with the primary antibody, immunodetection was performed using biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin (Vector Laboratories), with diaminobenzidine chromogen as the substrate. Negative controls for nonspecific binding included normal rabbit serum without primary antibody or with secondary antibody alone.

In situ ligation of labeled DNA fragments on lung tissue. A 200-bp double-stranded DNA fragment was prepared by polymerase chain reaction with *Taq* polmerase using primers 5′-CACTAGAGACAG-TTTGCCATG-3′ and 5′-GTCCCACACCCTTTAGAGAAG-3′ complementary to human adenocarcinoma DNA. The deparaffinized 5-μm sections were subjected to *Taq* polymerase-based DNA *in situ* ligation assay using the DNA fragment labeled with digoxigenin according to the method described by Didenko and Hornsby (27). Briefly, sections were incubated for 90 min at 65° C in 10 mmol/L sodium citrate, pH 6.0, and then washed with water. Sections were incubated with 50 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) in PBS for 15 min, and then rinsed thoroughly with water. A mix of 50 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM adenosine triphosphate (ATP), 25 μg/ml bovine serum albumin (BSA), 15% polyethylene glycol with the digoxigenin-labeled DNA fragment at 2 μg/ml and DNA T4 ligase (BioLabs, Beverly, MA) at 10,000 U/ml was added. Sections were placed in a humidified box for 16 h. The sections were washed with several changes of water over 2 h. Sections labeled with digoxigenin were detected using the DIG nucleic acid detection kit (Boehringer Mannheim).

Western Blot Analysis of Lung Tissue for VEGF R2

Frozen fresh lung tissue was available from 10 patients with emphysema and from 6 normal subjects. Lung tissue was homogenized with 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.15 mM spermine, 0.5 mM spermidine, 0.25 M sucrose, 100 mM NaCl, 0.2 mM ethylendiaminetetraacetic acid (EDTA), 200 μM phenylmethlysulfonyl fluoride (PMSF), 0.5 mM DTT, 1 μg/μl leupeptin, and 1 μg/μl aprotinin, using a Tissuemizer (Tekmar, Cincinnati, OH) at 4° C. The homogenate was initially centrifuged at 10,000 × *g* for 10 min at 4° C to remove tissue fragments and the supernatant was collected. Direct protein quantitation was by the D_C protein assay kit (Bio-Rad, Hercules, CA). To ensure equal protein loading of all gels, each sample was quantified, and 50 μg of protein was loaded in each lane of a 4–15% sodium dodecyl sulfate (SDS)–polyacrylamide gel. Transfer of proteins to PVDF membrane (Bio-Rad, Hercules, CA) was performed by electrophoresis at 100 V for 1 h in a Bio-Rad Western blot apparatus. Membranes were then blocked for 1 h in a PBS–Tween 20 (0.1%) with 5% nonfat dried milk. The anti-KDR/Flk-1 polyclonal antibody (sc-504, Santa Cruz, CA) was used as the primary antibody at 1:500 dilution. After the primary antibody incubation, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG for 1 h and visualized by enhanced chemiluminescence (ECL plus kit; Amersham, Arlington Heights, IL). Immunoreactive proteins were measured by scanning with an UMAX model Astra 1200S scanner and analyzed by using NIH image 1.54 software. The protein samples were assayed for β-actin expression and the VEGF R2 expression was normalized for β-actin. To determine whether emphysema lungs have a selective decrease of protein concentration or have protein degradation, we compared the expression of β-actin and α-tubulin, two housekeeping gene products, in emphysema and normal lungs by Western blot. The Western blot was performed with the monoclonal anti-α-tubulin (Clone B-5-1-2, Sigma, MO, 1/2000 dilution) and anti-β-actin (clone AC15, Sigma, MO, 1/5000 dilution) added concomitantly in the first step reaction. The resulting bands were scanned and the densitometric ratios of β-actin/α-tubulin were determined.

ELISA of Lung Tissue for VEGF

The experimental samples were prepared using the procedure for Western blot analysis as described above. Frozen fresh lung tissue was available from 12 patients with emphysema and from 5 normal subjects. The enzyme-linked immunosorbent assay (ELISA) procedure for VEGF was as described previously (28). The reagents were matched antibody pairs from R&D Systems (Minneapolis, MN) and the samples were added to the wells at a protein concentration of 1.65 mg/ml diluted in immunoassay buffer. The sensitivity of the assay ranged from 20 to 50 pg/ml.

In Situ Hybridization

Frozen sections of three lungs with emphysema and three normal lungs were subjected to *in situ* hybridization studies. The procedure for *in situ* hybridization for the detection of VEGF and VEGF R2 mRNA was as described previously (28). Antisense and sense cRNA probes for VEGF and VEGF R2 were labeled with digoxigenin-dUTP with the Genius IV kit (Boehringer Mannheim), and applied to serial sections mounted on the same slide. The hybridization reaction progressed overnight at 42° C and was developed under dark conditions for up to 12 h.

Statistics

Data were displayed as the mean ± SEM. The significance of differences observed between each group was calculated using the unpaired Student's *t* test.

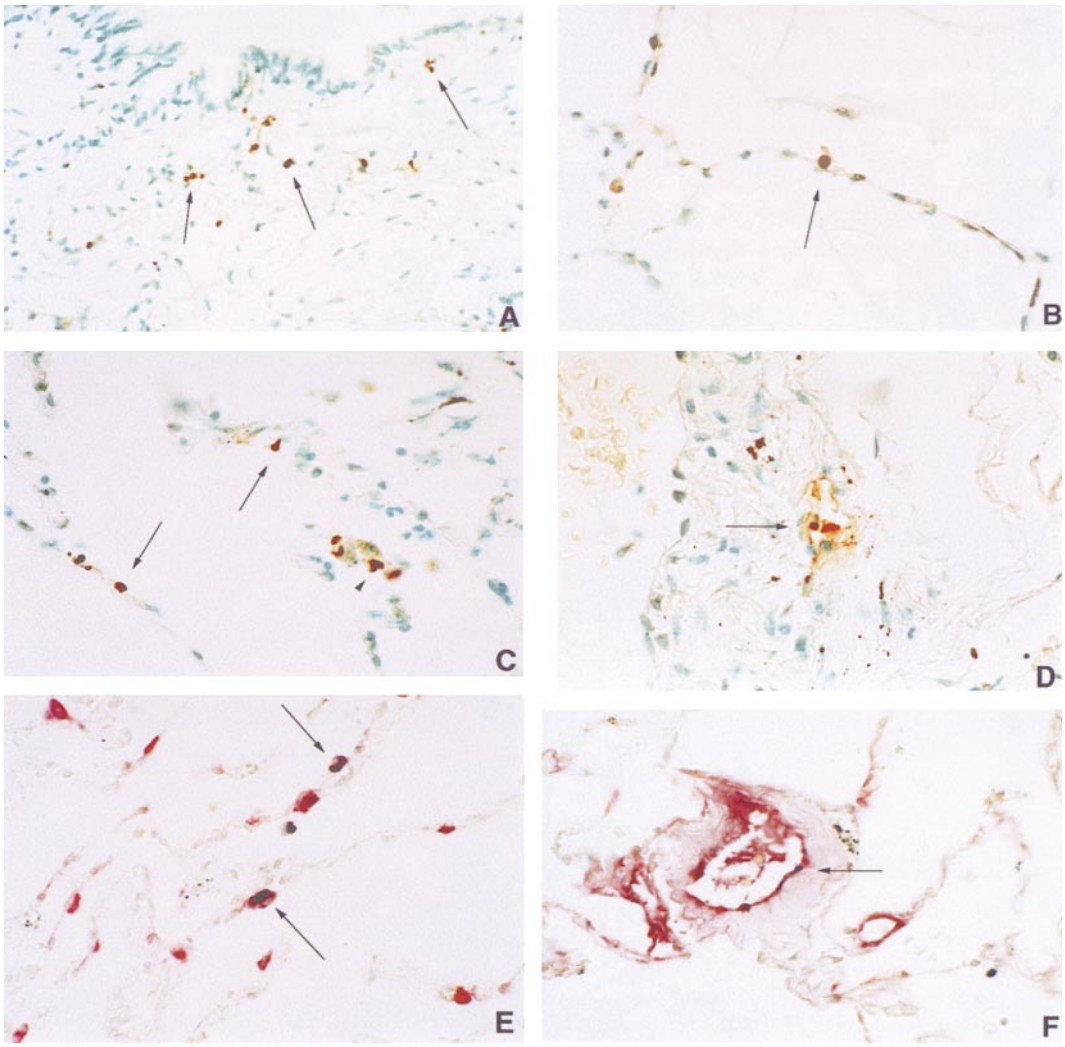

Figure 1. Lung sections stained by the TUNEL technique and immunohistochemistry. (*A*) TUNEL section of a nonsmoker (normal lung) counterstained with methyl green, showing specific staining for DNA strand breaks in the inflammatory cells (*arrows*) in the peribronchial lesion; original magnification: ×400. (*B*) TUNEL section of a nonsmoker (normal lung) demonstrates infrequent staining in the alveolar septa (*arrows*); original magnification: ×600. (*C*) TUNEL section of an emphysema lung shows abundant staining in the alveolar septa (*arrows*) and intraalveolar cells (*arrowhead*); original magnification: ×600. (*D*) TUNEL section of an emphysema lung labels endothelial cells of a pulmonary vessel (*arrow*); original magnification: ×600. (*E*) Double staining with TUNEL and cytokeratin, a marker of epithelial cells. Some epithelial cells show nuclear black staining (TUNEL+) and cytoplasmic red staining (cytokeratin +) in emphysema lung (*arrows*); original magnification: ×600. (*F*) Double staining with TUNEL and Factor VIII r.Ag, a marker of endothelial cells. Some endothelial cells show nuclear black staining (TUNEL+) and cytoplasmic red staining (Factor VIII r.Ag +) in emphysema lung (*arrow*); original magnification: ×600.

RESULTS

Characteristics of the Study Subjects

A clinical description of the patients and the results of the computer tomographic analysis are presented in Table 1. Using $FEV_{1.0\%}$ as the lung function criterion for chronic obstructive lung disease, there was a range from 21 to 55% of normal (predicted for sex, age, and body weight). Quantitative image analysis was performed in four patients with emphysema using CT. Their overall extent of emphysema was 75%, 63%, 67%, and 21%, respectively.

Apoptosis Assay

Labeling of the DNA strand breaks *in situ* by TUNEL demonstrated positive cells that were localized to the peribronchiolar, intraalveolar, and septal sites in both normal and emphysema lungs (Figures 1A–1D). The quantification of the number of apoptotic cells in the alveolar septa normalized by the amount of nucleic acid extracted from the serial section revealed an increase of TUNEL+ cells in emphysema lungs when compared with normal lungs (Figure 2). TUNEL+ cells/nucleic acid (μg) in the emphysema group (14.2 ± 2.0/μg) was significantly ($p < 0.01$) higher than that of the normal group (6.8 ± 1.3/μg). There were significantly more TUNEL+ cells in the emphysema group than in both healthy nonsmokers (7.4 ± 1.9/μg) ($p < 0.02$) and smokers (5.7 ± 0.7/μg) ($p < 0.01$). In addition, the PPH lungs that we used for comparison showed

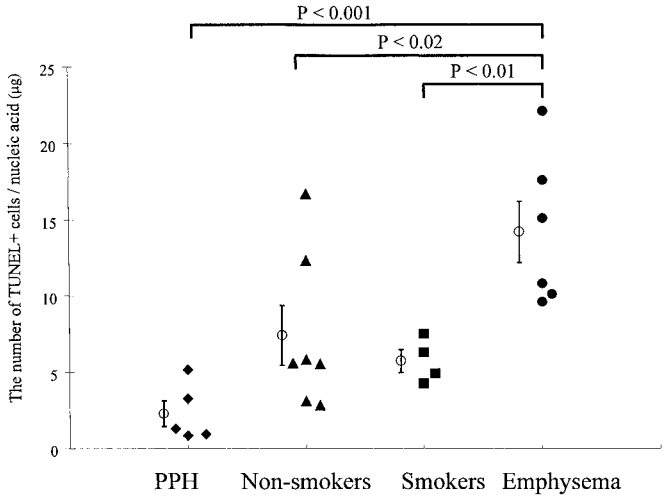

Figure 2. Comparison of TUNEL+ cells in alveolar septa normalized for the amount of nucleic acid extracted from the serial section that had been used for TUNEL. There are significantly more TUNEL+ cells in the emphysema lungs when compared with the lungs from nonsmokers, healthy smokers, and patients with PPH. Data are expressed as mean ± SEM.

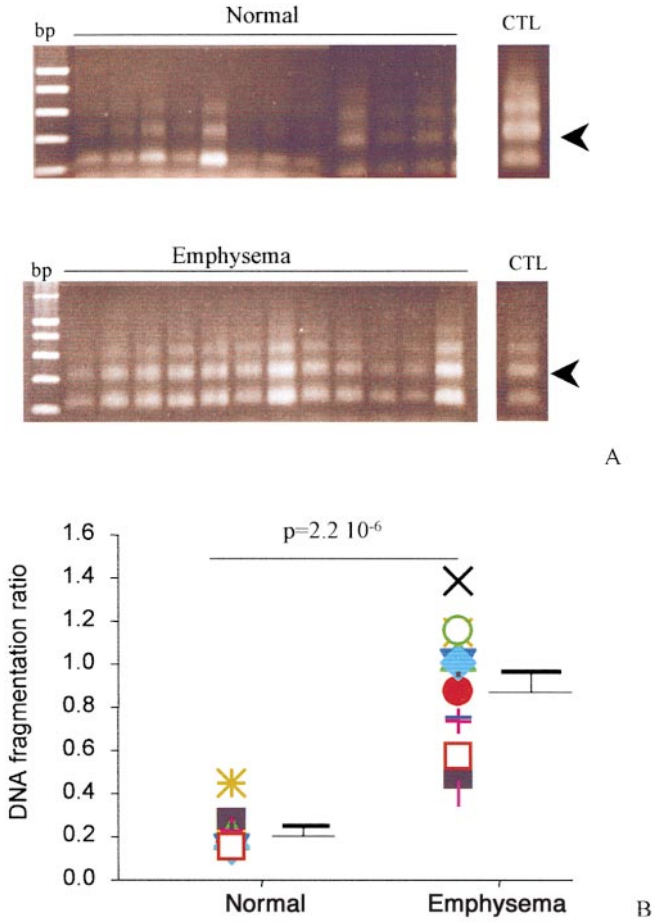

Figure 3. (*A*) Oligonucleosomal-length DNA laddering in normal (n = 11) and emphysema (n = 12) lungs; CTL (positive control thymus DNA); bp = 100 base pair ladder. (*B*) Quantification of DNA laddering based on scanning densitometry of the approximately 400-bp band (*arrowhead*) followed by normalization with the density obtained with the equivalent band of the thymus DNA positive control (lung sample/control = densitometric ratio), which was included in every oligonucleosomal DNA laddering assay. Each symbol represents a single lung sample; *bar* = mean ± SE.

significantly ($p < 0.001$) less ($2.3 \pm 0.8/\mu g$) TUNEL+ cells than emphysema lungs. There was no significant difference between healthy nonsmokers and smokers without emphysema in regard to the number of TUNEL+ cells/nucleic acid (μg).

To identify the proportion of septal epithelial and endothelial cell that were also TUNEL+, we performed double labeling with epithelial or endothelial cell markers. Both epithelial and endothelial cells were undergoing apoptosis in emphysema (Figures 1E and 1F). Among the entire lung fields examined, only a small fraction of the lung epithelial and endothelial cell populations was TUNEL+ in emphysema (approximately 0.8%) and in normal lungs (approximately 0.5%). In normal lungs, 18.2% of TUNEL+ cells were epithelial cells and 3.7% of TUNEL+ cells were endothelial cells. In emphysema lungs, 25.7% of TUNEL+ cells were epithelial cells and 5.5% of TUNEL+ cells were endothelial cells.

Emphysema lungs showed increased levels of oligonucleosomal-length DNA fragments when compared with normal lungs (Figure 3).

Due to early chromatin changes in apoptosis, DNA from apoptotic cells is more susceptible to thermal denaturation than DNA from viable cells, thus resulting in reactivity with a monoclonal antibody to single-stranded DNA. Immunohistochemistry localizing single-stranded DNA confirmed the presence of increased numbers of apoptotic septal cells in emphysema lungs when compared with normal lungs (Figures 4A and 4B).

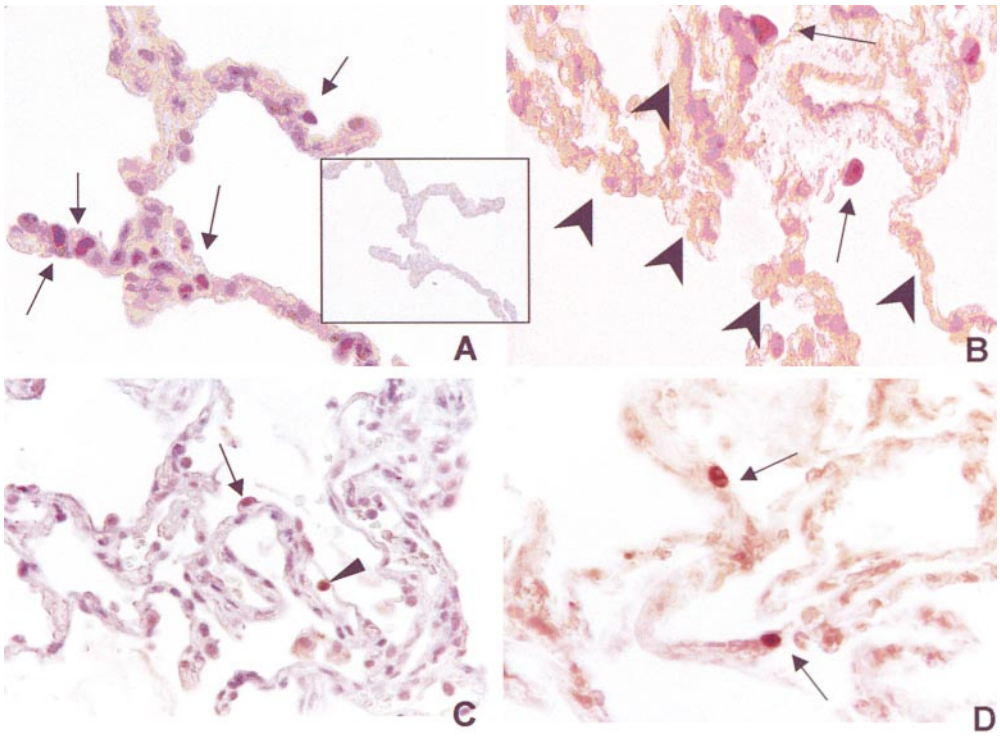

Figure 4. (*A*) Single-stranded DNA immunohistochemistry of emphysema lung, showing a large number of positive septal cells (*arrows*). Inset shows negative control of the same lung region; original magnification: ×400. (*B*) Single-stranded DNA immunohistochemistry of a normal lung, showing two positive alveolar cells (possibly macrophages, *arrows*), whereas cells in the alveolar septa are negative (*arrowheads*); original magnification: ×400. (*C*) Immunohistochemistry of the emphysematous lung counterstained with hematoxylin, showing specific staining for CM1 in the alveolar septa (*arrow*) and intraalveolar cell (*arrowhead*); original magnification: ×400. (*D*) *In situ* ligation of labeled DNA fragments assay of emphysematous lung. Positive reactions (*arrows*) were seen in alveolar septa; original magnification: ×600.

Since CM1 preferentially recognizes processed caspase-3, CM1 immunoreactive cells are those that undergo apoptosis. CM1 immunostaining was detected in bronchial epithelial cells and intraalveolar and septal cells in both emphysema and normal lungs, yet more positive cells were identified in emphysema lungs (Figure 4C). Overall the number of CM1 immunoreactive cells in the alveolar septa was smaller than the number of cells identified with the TUNEL technique.

The *in situ* ligation of labeled DNA fragments assay specifically detects double-strand DNA breaks with single-base 3′ overhangs in cells undergoing apoptosis. This assay is less sensitive, but more specific for apoptosis than TUNEL. Positive reactions were observed in alveolar cells in both emphysema and normal lungs (Figure 4D).

Western Blot Analysis of Lung tissue for VEGF R2

Because we noticed the presence of endothelial cell death in emphysematous lungs, we investigated the expression of the VEGF R2 in normal and emphysema lungs. We detected a weak VEGF R2 protein signal in the lungs of patients with emphysema (2321 ± 228 square pixels) in comparison with a stronger signal in the normal lung samples (3649 ± 398 square pixels) ($p < 0.01$) (Figure 5). Both α-tubulin and β-actin were expressed in a ratio of approximately 1 in both normal and emphysema lung samples, making the possibility of overall protein down-regulation or protein degradation in emphysema lungs unlikely.

ELISA of Lung Tissue for VEGF

VEGF protein measured in whole lung homogenates was significantly ($p < 0.01$) decreased in emphysema lungs (118.5 ± 16.8 pg/mg) when compared with lungs of normal subjects (209.2 ± 15.9 pg/mg) (Figure 6).

In Situ Hybridization

There was a decrease in the hybridization signal for VEGF and VEGF R2 mRNA in alveolar septal cells in emphysema lungs when compared with normal lungs and PPH lungs. In emphysema lungs, VEGF transcripts were preserved in the lymphoid cells and alveolar macrophages, and VEGF R2 mRNA was expressed in subpleural small vessels (Figure 7).

DISCUSSION

The novel aspect of this investigation is the finding of increased apoptotic events in the lung tissue samples obtained from patients with emphysema compared with the lung samples from either nonsmokers or smokers without emphysema. Of further importance are the decreased protein and mRNA expression of both VEGF and VEGF R2 in the lungs from patients with emphysema. TUNEL, oligonucleosomal-length DNA laddering, single-stranded DNA and anti-caspase-3 immunohistochemistry, and the *in situ* ligation of labeled DNA fragments identified apoptotic events in alveolar macrophages and inflammatory cells of all lungs examined, yet the number of apoptotic events in the alveolar septal cells—in both pneumocytes and endothelial cells—was greater in the lungs from patients with emphysema when compared with the lungs from actively smoking patients without emphysema, patients with primary pulmonary hypertension, and patients with normal lungs. Although inflammation may be one of the causes of apoptosis in the emphysema lung tissue (17, 18), the septal structures in the emphysematous areas examined for the presence of apoptotic cells did not show clear signs of acute or chronic inflammation. In marked contrast, the samples from PPH lungs that histologically show accumulation of inflammatory cells

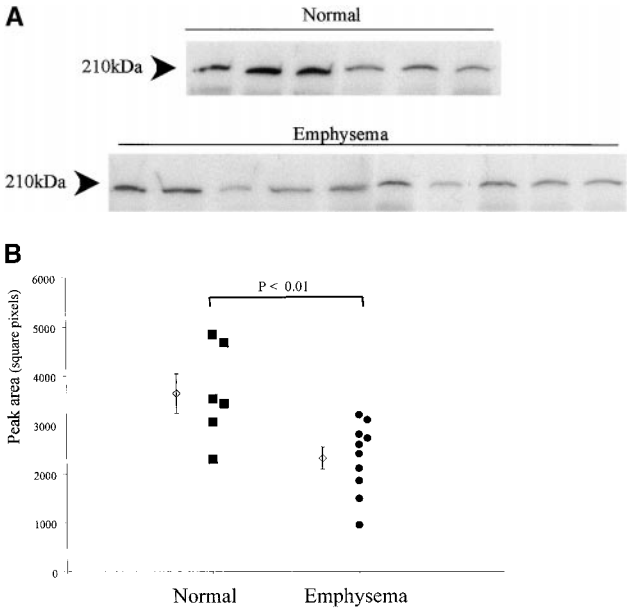

Figure 5. (*A*) Western blot analysis for VEGF R2 in the whole lung extract in normal lungs (n = 6) and in emphysema lungs (n = 10). Band at 210 kD is consistent with the size of KDR. (*B*) Quantification of the Western blot analysis for VEGF R2. VEGF R2 protein expression is significantly ($p < 0.01$) reduced in emphysema. Data are expressed as mean ± SEM.

(25) had the lowest apoptosis rates in the alveolar septal structures. Because the number of apoptotic events in the alveolar septal cells was lower in the samples from nonemphysematous smokers when compared with emphysematous smokers, some differences in the regulation of apoptosis may exist between smokers with and without emphysema. It is well appreciated that emphysema affects different lung regions to a varying extent. Because only one tissue sample per patient was available for examination, the number of apoptotic septal cell events reflects regional disease activity and may not be representative of the entire lung. This is of course also true for the amount of VEGF and VEGF R2 expression in the samples.

Overall, alveolar septal cell apoptosis in the samples of the entire cohort (n = 22) seemed to increase with age (data not shown). The patients with emphysema were the oldest patients in the cohort. Although the ages of three patients in the nonsmoker group were 62, 64, and 58 yr, the lung samples

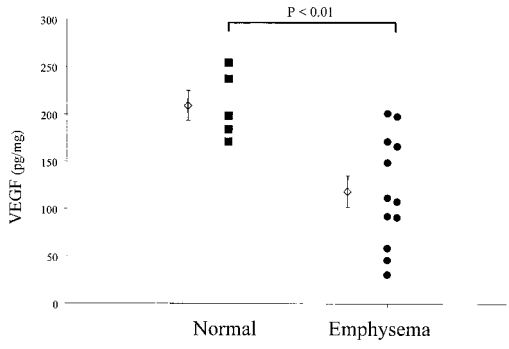

Figure 6. Measurement of lung tissue VEGF by ELISA. The result is displayed per 1 mg of total lung protein. VEGF protein expression is significantly ($p < 0.01$) reduced in emphysema. Data are expressed as mean ± SEM.

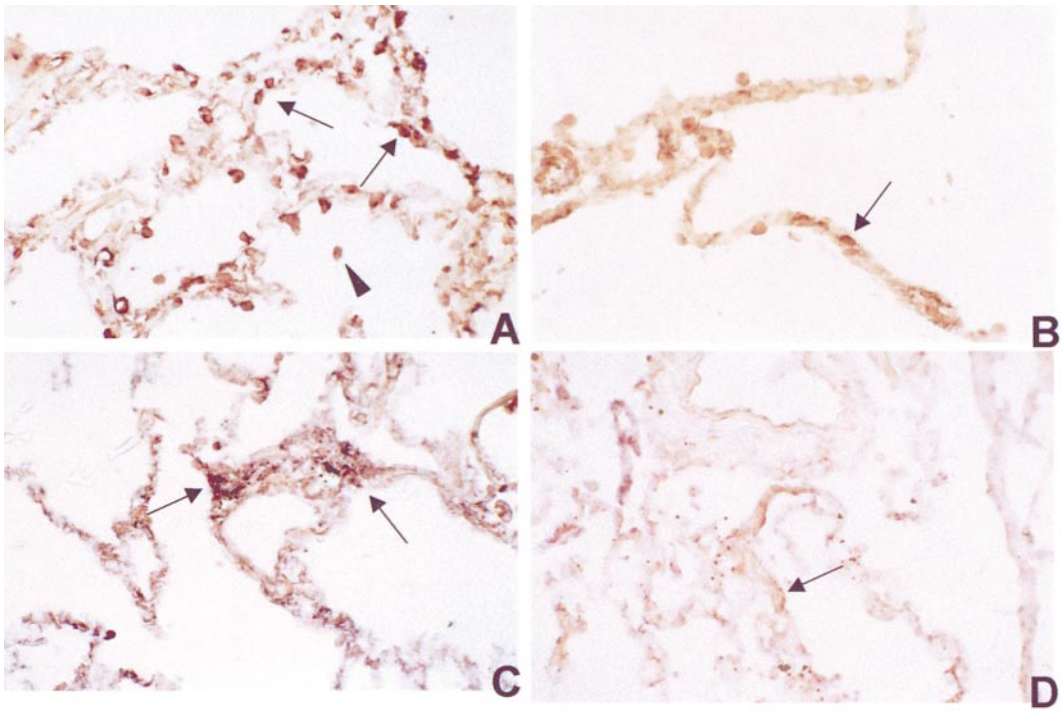

Figure 7. *In situ* hybridization for VEGF (*A–C*) and VEGF R2 (*D*) mRNA in lung tissue. (*A*) Normal lung shows specific hybridization signal for VEGF mRNA in the alveolar septa, possibly type I and II cells (*arrows*), and in intraalveolar cells, possibly macrophages (*arrowhead*) (antisense probe). (*B*) Emphysema lung shows a decrease in the hybridization signal for VEGF mRNA in alveolar cells (*arrow*) and intraalveolar cells when compared with normal lung (antisense probe). (*C*) Normal lung shows VEGF R2 mRNA signal in the alveolar septa, possibly capillary (*arrows*) (antisense probe). (*D*) Emphysema lung shows a decrease in VEGF R2 mRNA signal in the alveolar septa (*arrow*) when compared with normal lung (antisense probe). *A–D*: original magnification: ×400.

from these patients had relatively few (3–6/μg) alveolar septal cell apoptotic events.

The comparison of the expressions of VEGF and VEGF R2 protein in the samples from patients with and without emphysema demonstrated that samples from emphysematous lungs contained a lower amount of the growth factor and its receptor. Because patients with emphysema tend to be hypoxemic and because hypoxia is a strong inducer of both the VEGF and VEGF R2 gene (28, 29), this finding is particularly interesting. We propose that the increase in alveolar septal cell apoptotic events and the relative lack of VEGF and VEGF R2 in emphysema may be related, yet the present human study cannot directly address this hypothesis. Gerber and coworkers (21, 22) recently reported that VEGF induces the expression of antiapoptotic proteins, and Alon and coworkers (23) found that VEGF acts as a survival factor for endothelial cells. If the amount of VEGF is sufficiently reduced and the signal transduction via VEGF R2 is impaired, either because of a reduction in the number of endothelial cell VEGF R2 receptors or due to impairment of the VEGF R2/tyrosine kinase activity, then alveolar endothelial cells could indeed die as a consequence of a failing VEGF-dependent endothelial cell maintenance program. We recently observed that rats treated with the VEGF R2 inhibitor SU5416 develop emphysema, which is preceded by alveolar septal cell death (32). Therefore, VEGF/VEGF R2 reduction in lungs of patients with emphysema could be one of several factors facilitating alveolar septal cell apoptosis.

Emphysema probably constitutes a disease of all cells in the alveolar septum, including the septal epithelial cells. Altered function of type I and II cells (which are main sites of VEGF production) induced by smoking may account for our finding of decreased expression of VEGF and increased epithelial cell apoptosis in emphysema. The loss of septal (endothelial and epithelial) cell antiproteases may further contribute to an imbalance between protease and antiprotease activity leading to matrix destruction, which, in turn, may promote the death of lung alveolar cells. The finding of endothelial cell apoptosis in emphysema allows us now to connect our concept with the ideas of earlier investigators (13, 30) that vascular factors have a role in the pathogenesis of emphysema. Indeed, in cell culture studies with lung cells, endothelial cells undergo apoptosis to a greater extent than lung epithelial cells when exposed to cigarette smoke extract (31).

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References

1. Feinleib M, Rosenberg HM, Collins JG, Delozier JE, Pokras R, Chevarley FM. Trends in COPD morbidity and mortality in the United States. *Am Rev Respir Dis* 1989;140(3, Pt 2):S9–S18.
2. Anonymous. American Thoracic Society: standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1995;152(5, Pt 2):S77–S121.
3. Eriksson S. Studies in alpha-1-antitrypsin. *Acta Med Scand* 1965;177(Suppl 432):1–85.
4. Snider GL. Chronic obstructive pulmonary disease: risk factors, pathophysiology and pathogenesis. *Annu Rev Med* 1989;40:411–429.
5. Hutchison DC. Pulmonary emphysema. *BMJ* 1994;309(6964):1244–1245.
6. Snider GL, Lucey EC, Stone PJ. Animal models of emphysema. *Am Rev Respir Dis* 1986;133:149–169.
7. Yoshioka A, Betsuyaku T, Nishimura M, Miyamoto K, Kondo T, Kawakami Y. Excessive neutrophil elastase in bronchoalveolar lavage fluid in subclinical emphysema. *Am J Respir Crit Care Med* 1995;152(6, Pt 1): 2127–2132.
8. Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997;277(5334):2002–2004.
9. MacNee W, Wiggs B, Belzberg AS, Hogg JC. The effect of cigarette smoking on neutrophil kinetics in human lungs. *N Engl J Med* 1989; 321:924–928.
10. Wright JL, Hobson JE, Wiggs B, Pare PD, Hogg JC. Airway inflammation and peribronchiolar attachments in the lungs of nonsmokers, current and ex-smokers. *Lung* 1988;166:277–286.
11. Barker DJP, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO. Relation of birth weight and childhood respiratory infection to adult

lung function and death from chronic obstructive lung disease. *BMJ* 1991;303:671–675.
12. Samet JM, Tager IB, Speizer FE. The relationship between respiratory illness in childhood and chronic air-flow obstruction in adulthood. *Am Rev Respir Dis* 1983;127:508–523.
13. Matsuse T, Hayashi S, Kuwano K, Keunecke H, Jefferies WA, Hogg JC. Latent adenoviral infection in the pathogenesis of chronic airways obstruction. *Am Rev Respir Dis* 1992;146:177–184.
14. Seersholm N, Kok-Jensen A. Clinical features and prognosis of life time non-smokers with severe alpha 1-antitrypsin deficiency. *Thorax* 1998; 53:265–268.
15. Liebow AA. Pulmonary emphysema with special reference to vascular changes. *Am Rev Respir Dis* 1959;80:67–93.
16. Anversa P, Kajstura J. Myocyte cell death in the diseased heart. *Circ Res* 1998;82:1231–1233.
17. Bardales RH, Xie SS, Schaefer RF, Hsu SM. Apoptosis is a major pathway responsible for the resolution of type II pneumocytes in acute lung injury. *Am J Pathol* 1996;149:845–852.
18. Kuwano K, Kunitake R, Kawasaki M, Nomoto Y, Hagimoto N, Nakanishi Y, Hara N. P21Waf1/Cip1/Sdi1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1996;154(2, Pt 1):477–483.
19. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995;267(5203):1456–1462.
20. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 1999;13:9–22.
21. Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* 1998;273:13313–13316.
22. Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway: requirement for Flk-1/KDR activation. *J Biol Chem* 1998;273:30336–30343.
23. Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nature Med* 1995; 1:1024–1028.
24. Halbower AC, Mason RJ, Abman SH, Tuder RM. Agarose infiltration improves morphology of cryostat sections of lung. *Lab Invest* 1994; 71:149–153.
25. Tuder RM, Groves B, Badesch DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol* 1994;144:275–285.
26. Srinivasan A, Roth KA, Sayers RO, Shindler KS, Wong AM, Fritz LC, Tomaselli KJ. In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. *Cell Death Differentiation* 1998;5:1004–1016.
27. Didenko VV, Hornsby PJ. Presence of double-strand breaks with single-base 3′ overhangs in cells undergoing apoptosis but not necrosis. *J Cell Biol* 1996;135:1369–1376.
28. Tuder RM, Flook BE, Voelkel NF. Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia: modulation of gene expression by nitric oxide. *J Clin Invest* 1995;95:1798–1807.
29. Minchenko A, Bauer T, Salceda S, Caro J. Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. *Lab Invest* 1994;71:374–379.
30. Issaksohn. Pathologisch-anatomische Veränderungen der Lungengefässe beim Emphysem. *Virchows Arch Pathol Anat* 1871;53:466.
31. Tuder RM, Wood K, Taraseviciene L, Flores S, Voelkel NF. Cigarette smoke extract decreases the expression of vascular endothelial growth factor by cultured cells and triggers apoptosis of pulmonary endothelial cells. Thomas L. Petty 42nd Annual Aspen Lung Conference: Mechanisms of COPD, Supplement to *Chest* Mar; 2000;117:241S–242S.
32. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 2000; 106:1311–1319.