Apoptosis in microvascular endothelial cells of perfused rabbit lungs with acute hydrostatic edema

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Gotoh, Naoki, Kenjiro Kambara, Xiao-Wen Jiang, Michiya Ohno, Shoichi Emura, Takako Fujiiwara, and Hisayoshi Fujiiwara. Apoptosis in microvascular endothelial cells of perfused rabbit lungs with acute hydrostatic edema. J. Appl. Physiol. 88: 518–526, 2000.—We test the hypothesis that microvascular endothelial cells may undergo apoptosis in response to acute pulmonary venous hypertension. The isolated rabbit lungs were perfused in situ for 4 h with left atrial pressure of 0, 10, or 20 mmHg at a constant blood flow. Edema formation was monitored by lung weight gain. To assay for apoptosis, we performed agarose gel electrophoresis of DNA, in situ nick end labeling of DNA strand breaks, and electron microscopy. We also examined the levels of expression of Bcl-2, a suppressor of apoptosis, in microvascular endothelial cells using an immunohistochemical technique. In a vascular pressure-dependent fashion, we found apoptosis in endothelial cells of alveolar septal capillaries, as well as expression of Bcl-2 in arteriolar and venular endothelial cells. We conclude that acute pulmonary venous hypertension induces apoptosis in capillary endothelial cells but not in arteriolar and venular endothelial cells, suggesting that microvascular endothelial cell apoptosis is dependent on the levels of Bcl-2 expression and influences the formation or resolution of acute hydrostatic lung edema.

Congestive heart failure; in situ nick end labeling; Bcl-2; electron microscopy; immunohistochemistry

There has been considerable interest in the fine structural changes in the lung in patients with congestive heart failure. Several investigators have examined the ultrastructure of the lung in patients with mitral stenosis (14, 16) and in animals with experimentally induced hydrostatic lung edema (2, 7, 19, 20). These studies demonstrated the swelling and vacuolization of capillary endothelial cells, with thickening of their basal laminae, and a proliferation of reticular and elastic fibers in thicker portions of the alveolar interstitium. Furthermore, over wide areas of the alveolar walls, type I pneumocytes have been lost and replaced by type II pneumocytes. Although interstitial pulmonary edema was usually confined to the thicker collagen-containing portions of the alveolar wall, endothelial cell basal lamina was occasionally split to enclose edema fluid (endothelial blebs) and disintegrating fragments of extravasated erythrocytes, leading to detachment of the endothelial cell from the basal lamina. These findings indicate that sustained pulmonary venous hypertension induces severe injury and death in alveolar epithelial and capillary endothelial cells. However, there have been no studies on the mechanisms of cell homeostasis and tissue remodeling in alveolar-capillary wall during the formation or resolution of hydrostatic lung edema.

Apoptosis, or programmed cell death, has a critical role in normal cell homeostasis and tissue remodeling, but it may contribute to the pathogenesis of many diseases when dysregulated (10, 23). Despite recurrent exposure to cellular toxins from the circulation and tissue, endothelial cells are remarkably resistant to cell death (13). However, failure to adhere to the extracellular matrix or inhibition of certain integrin-mediated signaling has been shown to activate a suicide process in anchorage-dependent cells (9, 18, 24), because the extracellular matrix is critical for endothelial cell survival (12).

From these observations, we hypothesized that 1) pulmonary microvascular endothelial cells might undergo cell death with the morphological and biochemical characteristics of apoptosis in response to acute elevation of pulmonary venous pressure and that 2) a suppressor of apoptosis, such as Bcl-2, would provide protection against microvascular endothelial cell apoptosis. Bcl-2 is an intracellular membrane-associated protein that functions to block apoptotic cell death in a variety of physiological and pathological contexts (11, 27).

Thus in this study we investigated whether apoptosis and Bcl-2 protein are induced in lung fluid-exchange vessels during the formation of acute hydrostatic edema in situ blood-perfused rabbit lungs.

Methods

In Situ Perfused Rabbit Lung Preparation

Male Japanese White rabbits weighing 1.8–2.2 kg were given 3,000 units of heparin sodium via an ear vein and were anesthetized with pentobarbital sodium (25 mg/kg). The chest was opened, and the animal was rapidly exsanguinated from the left ventricle. After right and left parasternal incisions were made, the whole lungs were exposed, and
Experimental Protocols

The animals were randomly assigned to three groups (n = 10 each). After a 10-min stabilization period, the rabbit lungs were perfused for 4 h with left atrial pressures (Pla) of 0 (group 1), 10 (group 2), or 20 mmHg (group 3) by adjusting the height of the outflow reservoir. Pulmonary arterial pressure (Ppa), airway pressures, and Pla were continuously monitored by pressure transducers (UK215, Baxter) referenced to the top of the lung and were recorded on a multichannel polygraph (RMC-1100, Nihon Kohden). After 4 h, pulmonary capillary pressure was estimated by the double occlusion pressure (Pdo). During simultaneous occlusion of both inflow and outflow cannulas, Ppa and Pla rapidly equilibrated to Pdo, which has been shown to be an excellent estimate of the existing capillary pressure (26). Double occlusion was performed rapidly and maintained for 3–4 s. The respirator was turned off at end expiration before each occlusion. Total pulmonary vascular resistance was partitioned into arterial (Rpa) and venous resistances (Rpv) by the Pdo.

Effluent perfusate was collected at baseline and at the end of perfusion to measure leukocytes, arterial partial pressure of oxygen (PaO₂), and lactate dehydrogenase (LDH) as a measure of cell injury. The LDH activity was determined by using a radioimmunoassay.

After the completion of perfusion, both lungs were excised from mediastinal structures. The left lungs were used for measuring the wet-to-dry weight ratio as an index of lung edema. The right lungs were used for histological examinations.

Histological Studies

Electron microscopic study. Tissues for ultrastructural evaluation were randomly collected from the different regions of the surface of the right lower lobe. The tissue samples were immediately immersed in 2.5% glutaraldehyde solution in 0.1 M Millonig’s buffer at pH 7.4 and cut into 1-mm cubes. The cubes were placed in fresh fixative for an additional hour. The tissue was washed twice in Millonig’s buffer, postfixed with 1% osmium tetroxide in the same buffer for 1 h, dehydrated through ascending concentrations of acetone, and embedded in Epon 812. Thin sections were cut with a Porter-Blum MT-1 ultramicrotome, mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi-H700H electron microscope.

The electron microscopic morphological criteria of apoptosis and necrosis in the present study are as follows. Early apoptosis is characterized by compaction and segregation of chromatin in sharply circumscribed masses that abut the inner surface of the nuclear envelope, convolution of nuclear outline, condensation of the cytoplasm with preservation of the integrity of organelles, and the beginning of convolution of the cell surface. In the next phase, the nuclear fragments and further condensation of the cytoplasm are associated with extensive cell surface protrusion, followed by separation of the surface protuberances to produce membrane-bounded apoptotic bodies of varying size and composition. These bodies are phagocytosed by nearby cells and are degraded by lysosomal enzymes, being rapidly reduced to nondescript residues within telolysosomes. The onset of necrosis manifests as irregular dumping of chromatin without radical change in its distribution, gross swelling of mitochondria with the appearance of flocculent densities in matrices, dissolution of ribosomes, and focal rupture of membranes. At a more advanced stage of this process, all cellular components disintegrate (15).

In situ detection of apoptosis. For light microscopic studies, the middle portion of the lower lobe was removed from the right lung. The sample was fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemical detection of apoptosis and Bcl-2.

After they were deparaffinized, 5-µm sections were digested by proteinase K for 15 min at room temperature, and intrinsic peroxidase activity was quenched by the addition of 0.3% hydrogen peroxide in phosphate-buffered saline for 5 min. Staining procedures followed those of an ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). General procedures were also used for color development by 3,3′-diaminobenzidine tetrahydrochloride in immunohistochemistry. Finally, the sections were counterstained with hematoxylin. This technique has been applied to the study of several disorders, including lungs with acute injury (3).

Immunohistochemical detection of Bcl-2. To gain further evidence of the occurrence of apoptosis, we analyzed the level of expression of the cytochrome c inhibitor Bcl-2 in the lung tissue using an immunohistochemical technique. Bcl-2 is a cytosolic protein with a lipid anchoring domain that can target it to the nuclear envelope, parts of the endoplasmic reticulum, and the outer mitochondrial membrane and plays a role in the inhibition of apoptosis (23).

Briefly, after sections were deparaffinized, intrinsic peroxidase activity was inhibited by 0.3% hydrogen peroxide in methanol for 30 min, and nonspecific binding was blocked with normal goat serum. A 1:40 dilution of the primary antibody of monoclonal mouse anti-human Bcl-2 oncoprotein (M887, Dako) was incubated with the sections for 24 h at 4°C. The peroxidase conjugated F(ab)² fragment of the second antibody [goat anti-mouse IgG (H + L), Jackson Immunoresearch Laboratory] was incubated with the sections at a dilution of 1:500 for 40 min at room temperature. Sections were then stained with 0.4 mg/ml of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical, St Louis, MO) and 0.006% hydrogen peroxide in 50 mM Tris-HCl (pH 7.4) buffer for 5 min at room temperature. Between steps, the sections were washed with distilled water or 10 mM sodium phosphate-buffered saline. Finally, the sections were counterstained with hematoxylin. As a negative control, serial sections were stained without the primary antibody against Bcl-2.

To confirm antibody specificity, we compared the specificity of anti-human Bcl-2 oncoprotein between human and rabbit plastic cannulas were placed in the main pulmonary artery and the left atrium. A tracheostomy was made, and the lungs were mechanically ventilated with room air at 20 breaths/min (AR-300, Acoma, Tokyo, J apan). The tidal volume was set to achieve a peak airway pressure of 10 mmHg, and the end-expiratory pressure was set at 0 mmHg.

The lungs were perfused in situ at a constant flow of 40 ml/min and were covered with Saran wrap to prevent evaporation. The closed perfusion system included a roller pump (Mera HAD-100, Senko Ika Kogyo, Tokyo, J apan), a heat exchanger (Mera MHE-3-P), a bubble trap and filter (CX-BT15, Terumo, Tokyo, J apan), an electromagnetic blood flow-meter (MFV-3100, Nihon Kohden, Tokyo, J apan), and a reservoir. Changes in lung weight were continuously recorded as the converse of the change in the weight of the perfusate reservoir. The total volume of the perfusion system was 600 ml. The perfusion medium, which consisted of 100 ml of autologous blood, 500 ml of Krebs-Henseleit buffer containing 3% bovine albumin, and 20 µM of indomethacin, was continuously mixed by a magnetic stirrer in the reservoir.
lymph node, in each of which the presence of Bcl-2 had been confirmed, by a Western immunoblotting technique. Briefly, human and rabbit lymph node were homogenized at 4°C in radioimmunoprecipitation assay buffer. Primary antibodies were used for monoclonal mouse anti-human Bcl-2 oncprotein (M887, Dako), which were the same as those used in immunohistochemistry. Secondary antibodies were used for gold-conjugated goat anti-mouse IgG (H+L) (BioRad).

To further identify the microvascular endothelial cells undergoing apoptosis or expression of Bcl-2, the cells were stained with a monoclonal mouse antibody of human endothelial cell CD31 (monoclonal mouse anti-human endothelial cell, Dako) on formalin-fixed, paraffin-embedded tissue sections. The CD31/platelet endothelial cell adhesion molecule 1 is an adhesion molecule expressed by endothelial cells, platelets, monocytes, neutrophils, and native T lymphocytes (21). Although CD31 is not specific for microvascular endothelium, CD31 in endothelial cells is localized to sites of endothelial cell-to-endothelial cell and endothelial cell-to-basement membrane contact (21). Thus microvascular endothelial cells can be identified.

Identification of apoptotic cells and Bcl-2 expression. The sections were first examined by light microscopy at low magnification (×100), allowing estimation of the occurrence of apoptosis and Bcl-2 expression. Then 20–30 random fields per section were examined at a high magnification (×400) to calculate the prevalence of DNA nicking and Bcl-2 expression. The percentage of DNA nicking cells was determined by dividing the number of positive-staining nuclei by the total number of nuclei of the alveolar wall and multiplying that value by 100. The percentage of Bcl-2 expression was also determined by dividing the number of microvascular vessels with positive-staining endothelial cells by the total number of microvessels and multiplying that value by 100. Approximately 20,000 nucleated cells and 100 microvessels were counted in each section.

Agarose Gel Electrophoresis of DNA

All samples were frozen in liquid nitrogen and stored at −80°C until analysis. Frozen sections were mechanically homogenized on ice and lysed with lysis buffer containing 10% SDS, 10 mM Tris, and 1 mM EDTA (pH 7.8) and were digested with proteinase K at 200 µg/ml at 37°C for 16 h. DNA was purified by extraction with phenol/chloroform and then dissolved in Tris-EDTA buffer. The concentration and purity of DNA were determined by measurement of the optical density at 260 nm and the optical density ratio of 260 to 280 nm, respectively. Four-microgram aliquots of DNA were run on 2.0% agarose gels in Tris-borate-EDTA buffer. DNA was visualized with ethidium bromide.

Statistics

Data are expressed as means ± SE. Differences among groups were analyzed by one-way ANOVA. If the F ratio indicated a statistical difference among groups, the Newman-Keuls test was used to compare between-group means. Within-group comparisons were analyzed by ANOVA for repeated measurements followed by the Newman-Keuls test. A P value <0.05 was accepted as indicating statistical significance.

RESULTS

Formation of Acute Hydrostatic Lung Edema

Ppa was almost stable throughout the perfusion period in all groups (Fig. 1). After 4 h, the Pdo was 5.1 ± 0.4 mmHg in group 1, 11.8 ± 0.2 mmHg in group 2, and 22.3 ± 0.3 mmHg in group 3. Compared with group 1, Ppa and Pdo increased in groups 2 and 3 by 6.7 and 17.2 mmHg, respectively. Total resistance decreased significantly in group 2 by the reduction of both Rpa and Rpv and in group 3 by the reduction of Rpv (Table 1). The ratio of Rpa to Rpv increased in proportion to the magnitude of elevated Pla. In group 3, the Rpa may

Table 1. Segmental distribution of pulmonary vascular resistance after 4-h perfusion

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<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tr>
<td>Total pulmonary vascular resistance, mmHg·ml⁻¹·min</td>
<td>0.310±0.017</td>
<td>0.157±0.013*</td>
<td>0.198±0.023*</td>
</tr>
<tr>
<td>Arterial resistance, mmHg·ml⁻¹·min</td>
<td>0.167±0.013</td>
<td>0.097±0.008*</td>
<td>0.138±0.016†</td>
</tr>
<tr>
<td>Venous resistance, mmHg·ml⁻¹·min</td>
<td>0.146±0.018</td>
<td>0.060±0.006*</td>
<td>0.061±0.008*</td>
</tr>
<tr>
<td>Ratio of arterial to venous resistance</td>
<td>1.227±0.149</td>
<td>1.708±0.138</td>
<td>2.389±0.216†</td>
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Values are means ± SE. *P < 0.05 vs. group 1; † P < 0.05 vs. group 2.
have been raised by accumulation of edema liquid or hypoxic pulmonary vasoconstriction.

The cumulative lung weight gains in groups 2 and 3 compared with group 1 (Fig. 1). Similarly, total lung weight, left dry lung weight, and wet-to-dry weight ratio increased in groups 2 and 3 compared with group 1 (Table 2), indicating that lung edema was produced in proportion to the magnitude of PLa elevation.

Table 3 summarizes the perfusate leukocyte counts, PaO₂, and LDH at baseline and after 4 h. There were no differences among the three groups at baseline. After 4 h, the leukocyte counts increased significantly in all groups compared with baseline values, but there were no differences in leukocyte counts among the three groups. Perfusate PaO₂ decreased during perfusion in all groups, but there were no differences in perfusate LDH among the three groups.

Apoptosis and Expression of Bcl-2 in Microvascular Endothelial Cells

Bcl-2 was barely detectable in the lung sections of group 1 (Figs. 2B and 3). As to the percentage of DNA nicking nuclei detected by in situ nick end-labeling assay, group 3 had significantly greater expression of apoptosis in mesenchymal nucleated cells at the level of alveolar septum than did groups 1 and 2 (Table 4).

The apoptotic cell type was not easily identifiable at the light microscopic level, even when the cell-cell adhesion molecule was stained with a monoclonal antibody to CD31. Therefore, electron microscopic analysis was performed. Electron microscopy showed the presence of capillary endothelial cells with typical apoptotic ultrastructures, such as the shrinkage of cytoplasm and specific chromatin condensation of nuclei in group 3 (Fig. 4A). Figure 4B shows the phagocytosis of the apoptotic cell with condensed chromatin and thin cytoplasm by alveolar macrophage. However, we found no morphological evidence of apoptosis in alveolar epithelial cells, fibroblasts, or endothelial cells of arterioles and venules in any of the groups. We also found by electron microscopy no evidence of necrosis in any group.

As shown in Fig. 5, separation of DNA fragments in agarose gels demonstrated the typical formation of 180-bp ladders in group 3 (lanes 2 and 3, Fig. 5). In contrast, we did not consistently observe a DNA ladder from tissues in group 1 (lane 1, Fig. 5).

Lung sections from all lungs revealed high levels of Bd-2 expression in vascular smooth muscle and respiratory smooth muscle cells, and there were no apparent differences among the three groups (data not shown). However, lung sections from groups 2 and 3 showed the high levels of immunoreactive Bd-2 in arteriolar and venular endothelial cells, and groups 2 and 3 had significantly more expression of Bd-2 than did group 1 (Table 4, Fig. 3).

Staining of cell-cell adhesion molecules with a monoclonal antibody to CD31 on serial sections confirmed that Bd-2 was confined to the microvascular endothelial cells in groups 2 and 3. Figure 6 illustrates the longitudinal distribution of Bd-2 expression in microvascular endothelial cells with an internal diameter of 100–500 µm or >50–100 µm, indicating that Bd-2 protein was expressed preferentially in endothelial cells with thickened smooth muscle cells, especially arteriolar and venular endothelial cells with an internal diameter of 50–100 µm.

Alternatively, we examined the effects of the cumulative lung weight gain and pulmonary capillary pressure on the occurrence of DNA nicking or the levels of Bd-2 expression. As shown in Fig. 7, the cumulative lung weight gain or pulmonary capillary pressure increased the incidence of positive nucleated cells by in situ nick end-labeling assay and the levels of Bd-2 expression in microvascular endothelial cells. These findings indicate that, in the perfused rabbit lung, acute hydrostatic lung edema is associated with endothelial cell apoptosis in alveolar septal capillaries and overexpression of Bd-2 protein in arteriolar and venular endothelial cells.

Table 2. Gravimetric analysis of lung weight

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<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tr>
<td>Total lung weight, g</td>
<td>11.97 ± 0.75</td>
<td>22.91 ± 2.69*</td>
<td>50.91 ± 3.55†</td>
</tr>
<tr>
<td>Left dry lung weight, g</td>
<td>0.66 ± 0.03</td>
<td>0.89 ± 0.05*</td>
<td>1.52 ± 0.10†</td>
</tr>
<tr>
<td>Wet-to-dry weight ratio, g/g</td>
<td>7.08 ± 0.30</td>
<td>10.13 ± 0.65*</td>
<td>13.37 ± 0.46†</td>
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Values are means ± SE. *P < 0.05 vs. group 1; †P < 0.05 vs. group 2.

Table 3. Perfusate leukocyte counts, partial pressure of oxygen, and lactate dehydrogenase at baseline and after 4 h

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<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tr>
<td>Leukocytes/μl</td>
<td>1,100 ± 130</td>
<td>1,130 ± 205</td>
<td>1,110 ± 109</td>
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<tr>
<td>Baseline</td>
<td></td>
<td></td>
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<tr>
<td>After 4 h</td>
<td>770 ± 104*</td>
<td>660 ± 113*</td>
<td>690 ± 67*</td>
</tr>
<tr>
<td>Arterial partial pressure of oxygen, Torr</td>
<td>106.1 ± 5.6</td>
<td>99.9 ± 5.7</td>
<td>119.8 ± 3.7</td>
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<tr>
<td>Baseline</td>
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<tr>
<td>After 4 h</td>
<td>83.1 ± 5.7</td>
<td>73.3 ± 4.7*</td>
<td>56.6 ± 8.4†</td>
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<tr>
<td>Lactate dehydrogenase, IU/l</td>
<td>98.6 ± 6.9</td>
<td>112.7 ± 14.7</td>
<td>125.8 ± 7.8</td>
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<tr>
<td>Baseline</td>
<td></td>
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<tr>
<td>After 4 h</td>
<td>177.5 ± 18.5*</td>
<td>195.2 ± 23.7*</td>
<td>164.5 ± 8.5*</td>
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Values are means ± SE. *P < 0.05 vs. baseline; †P < 0.05 vs. group 1.
DISCUSSION

Capillary Endothelial Cell Apoptosis in Acute Hydrostatic Lung Edema

Several lines of evidence indicate that pulmonary vascular endothelial cells can be induced to undergo apoptosis by exogenous toxic agents or insults, such as tumor necrosis factor-α (22, 28), lipopolysaccharide (12), hemorrhagic snake venom (1), and hyperoxia (6).

In addition, bronchoalveolar lavage fluid from patients recovering from adult respiratory distress syndrome induced apoptosis in cultured bovine pulmonary artery endothelial cells (5, 22). These findings suggest that the pulmonary vascular endothelial cell is a target for soluble inducers of apoptosis in acute edematous lung injury. However, the biochemical mechanisms and the functional significance of endothelial cell apoptosis in acute lung injury are not well understood.

Fig. 2. Light micrographs demonstrating in situ nick end labeling and Bcl-2 expression. A: Western blot analysis of Bcl-2 protein in human (H) and rabbit lymph node (R). Lane M, marker. Bcl-2 in both human and rabbit lymph node was of predicted molecular size (26 kDa). B: In situ nick end labeling of portions of lung parenchyma in rabbits perfused at Pla of 0 and 20 mmHg. Ba: in situ nick end labeling showed exceedingly few cells undergoing apoptotic death in group 1. Cells with normal nuclei stained blue (immunoperoxidase staining with hematoxylin counterstaining). Bb: in situ nick end labeling showed nucleated cells undergoing apoptotic death in group 3. Cells with fragmented DNA stained dark brown (magnification ×400).

Fig. 3. Bcl-2 expression in portions of lung parenchyma in rabbits perfused at Pla of 0 and 20 mmHg. A and B: Bcl-2 positivity was visible in arteriolar endothelial cells at lower power views in group 3 (magnification ×40 (A), ×100 (B)). C: Bcl-2 positivity was exceedingly rare in group 1 (magnification ×400). D: Bcl-2 positivity was present in arteriolar endothelial cells in group 3 (magnification ×400). E: Endothelial cells stained with monoclonal antibody of CD31 on serial sections (magnification ×400).
The ultrastructure of the alveolar-capillary membrane has been studied in patients with functional evidence of mitral stenosis (14, 16) and in animal models with experimentally induced acute (2, 7, 19) or chronic (20) lung edema. Despite tremendous microvascular endothelial cellular damages, however, there has been no quantitative histological analysis of cell death in alveolar-capillary wall associated with acute or chronic hydrostatic lung edema. The present study is the first to demonstrate that acute hydrostatic lung edema due to pulmonary venous hypertension promotes apoptosis in capillary endothelial cells. Apoptosis was identified by DNA degradation on agarose gel electrophoresis, in situ nick end labeling of DNA strand breaks, and electron microscopy, which are commonly

Table 4. Incidence of in situ nick end labeling and Bcl-2

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<tr>
<td>In situ nick end labeling, %</td>
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<tr>
<td>Capillary</td>
<td>0.21 ± 0.04</td>
<td>0.54 ± 0.12*</td>
<td>1.59 ± 0.25†</td>
</tr>
<tr>
<td>Arteriole and venule</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Bcl-2, %</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Capillary</td>
<td>2.13 ± 0.80</td>
<td>26.08 ± 2.48*</td>
<td>50.63 ± 2.15†</td>
</tr>
<tr>
<td>Arteriole and venule</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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Values are means ± SE. ND, not detected. *P < 0.05 vs. group 1; †P < 0.05 vs. group 2.

The ultrastructure of the alveolar-capillary membrane has been studied in patients with functional evidence of mitral stenosis (14, 16) and in animal models with experimentally induced acute (2, 7, 19) or chronic (20) lung edema. Despite tremendous microvascular endothelial cellular damages, however, there has been no quantitative histological analysis of cell death in alveolar-capillary wall associated with acute or chronic hydrostatic lung edema. The present study is the first to demonstrate that acute hydrostatic lung edema due to pulmonary venous hypertension promotes apoptosis in capillary endothelial cells. Apoptosis was identified by DNA degradation on agarose gel electrophoresis, in situ nick end labeling of DNA strand breaks, and electron microscopy, which are commonly
accepted methods for the detection of the apoptotic process.

In our study, however, the triggering factors for the capillary endothelial apoptosis remain to be elucidated. Possible mechanisms for induction of apoptosis in capillary endothelial cells may include mechanical forces such as shear stress, circumferential wall tension by transmural distending pressure, and longitudinal tension in the alveolar wall associated with lung inflation, as well as exogenous factors such as hypoxia, oxygen radicals, enzymes, and cytokines derived from formation of acute hydrostatic lung edema. The latter factors have been shown to contribute to the induction of apoptosis of mammalian cells in many different situations (10).

Mechanical stresses alter the structural and functional properties of endothelial cells at the molecular and genetic level (4). Mechanical forces may stimulate several intracellular mediators, including ions, G proteins, phospholipids, and their products, kinases and phosphatases (4), and dysregulate the role of integrin signaling in controlling the apoptotic response in anchoragedependent cells (24). In this study, capillary endothelial cells undergoing apoptosis were found in a vascular pressure- and a fluid filtration rate-dependent fashion, which suggests a causal relationship between endothelial apoptosis and mechanical forces.

A recent study has provided the evidence that most types of normal cells require an attachment to the extracellular matrix to be able to proliferate and differentiate and that cells denied anchorage can undergo apoptosis (24). Furthermore, integrin-mediated signaling may be the control factor for the apoptotic response in anchorage-dependent cells, because integrins are primarily responsible for adhesion to extracellular matrices (9, 18, 24). For example, Hoyt et al. (12) demonstrated that collagen was a survival factor against lipopolysaccharide-induced apoptosis in cultured sheep pulmonary artery endothelial cells. Although we obtained no clear evidence of detached endothelium undergoing apoptosis in the present study, our results raise the possibility that pulmonary capillary endothelial cells may have lost contact with their underlying matrix in some locations by disengagement of cell adhesion molecules under the conditions of acute pulmonary venous hypertension and underwent apoptosis for the control of inappropriate cell positioning.

Expression of Bcl-2 Protein in Microvascular Endothelial Cells

The present study also demonstrates that acute hydrostatic lung edema induces high levels of immunoreactive Bcl-2 in arteriolar and venular endothelial cells in a vascular pressure- and a fluid filtration rate-dependent fashion. In an in vivo study of rats, it has been reported that immunoreactivity of Bcl-2 is expressed after 3 h of ischemia in myocytes (17). To our knowledge, the present study is the first report on Bcl-2 expression in a model of perfused lung. The most important findings were that a high degree of Bcl-2 expression was found in arteriolar and venular endothelial cells with thickened smooth muscle cells, although endothelial cell apoptosis was restricted to alveolar wall capillaries. However, it is uncertain whether Bcl-2 is expressed later in the capillary endothelium than in larger vessels. Bcl-2 is an intracellular membrane-associated protein that functions to inhibit most types of apoptotic cell death (27) by regulating mitochondrial transmembrane potential (29). For example, Strömblad et al. (25) showed that cell attachment mediated by αvβ3 integrin protected endothelial cells from apoptosis through upregulation of Bcl-2 during angiogenesis, suggesting that Bcl-2 plays a role in maintaining endothelial survival.

Interestingly, Dimmeler et al. (8) reported that shear stress inhibited apoptosis in cultured human umbilical venous endothelial cells induced by tumor necrosis factor-α or growth factor withdrawal. However, the signal transduction mechanism by which the shear stress protects endothelial cells from apoptosis has yet to be identified.
Study Limitations

In the present study, tissue samples were taken from the right lower lobe of the lung, because the macroscopic edema was most severe in the lower lobe, compared with the upper and middle lobes. Therefore, the relationship between acute congestion and apoptosis in the upper and middle lobes remains unknown.

This study demonstrated that apoptosis and expression of Bcl-2 in microvascular endothelial cells were features of perfused rabbit lungs with acute hydrostatic lung edema. The frequency of apoptotic endothelial cell death and the levels of Bcl-2 expression in microvascular endothelial cells were dependent on the magnitude of edema liquid accumulation and of hemodynamic force. However, neither the functions of apoptosis and Bcl-2 protein nor their potential roles in the pathogenesis of acute hydrostatic lung edema are defined.

Moreover, it is uncertain whether the disruption of the balance between endothelial cell survival and death plays a critical role in the formation or resolution of hydrostatic pulmonary edema. In this study, an isolated perfused rabbit lung was used to eliminate the effects of other organs and extrinsic innervation to the lung as contributors to the alteration of cellular architecture. In addition, bronchial circulation was not perfused in our models of acute hydrostatic lung edema. Thus there remains some doubt as to whether endothelial cell apoptosis and Bcl-2 expression are universally true of severe chronic congestive heart failure.

Another question is why endothelial cell apoptosis found in the present study has not been documented in previous morphological studies on acute hemodynamic lung edema, including perfused lung experiments (2, 19). Thus these apoptotic events may be dependent on the animal species or the experimental design (e.g., perfusion time, perfusate medium).

REFERENCES


