Med Electron Microsc (2001) 34:92–102
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ORIGINAL PAPER

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The protective effect of hepatocyte growth-promoting factor (pHGF) against hydrogen peroxide-induced acute lung injury in rats

Received: May 1, 2001 / Accepted: June 18, 2001

Abstract To examine the protective effect of hepatocyte growth-promoting factor (pHGF) in hydrogen peroxide (H_2O_2)-induced acute lung injury in rats, we observed the pathological changes in lung tissue by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and by light and electron microscopy. We also measured the serum levels of lipid peroxide (LPO). At 6 to 24 h after H_2O_2 injection, the level of LPO was significantly higher in the H_2O_2 group than in the H_2O_2 + pHGF-treated group. This finding indicated that pHGF protected against cell membrane damage in H_2O_2-induced acute lung injury. Positive TUNEL signals were found in capillary endothelial cells, alveolar epithelial cells, and inflammatory cells. In the H_2O_2 + pHGF-treated group, TUNEL-positive signals were reduced compared with those in the H_2O_2 group. This finding indicated that pHGF acts to suppress apoptosis. In the H_2O_2 group, severe pulmonary edema was seen 3 h after H_2O_2 injection, and at 24 h, severe atelectasis was seen. In the H_2O_2 + pHGF-treated group, pulmonary edema was scarcely seen and severe atelectasis was not found. This finding indicated that pHGF acts to suppress both severe pulmonary edema and atelectasis. In the H_2O_2 group, the formation of subendothelial blebs and disruption of endothelial cells was observed. Edema and disruption were seen in type I epithelial cells. In type II lung epithelial cells, mitochondria were swollen and microvilli had disappeared. In the H_2O_2 + pHGF-treated group, the formation of subendothelial blebs was seen, but no severe subendothelial blebs were observed. Disruption of capillary endothelial cells and type I epithelial cells was not evident, nor was there damage to type II lung epithelial cells. These findings indicated that pHGF protects the progression of H_2O_2-induced acute lung injury, and showed that pHGF acts to stabilize the cell membrane in capillary endothelial cells and lung epithelial cells.

Key words Hydrogen peroxide · pHGF · Lung injury · Morphology

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Introduction

Although many studies have investigated the mechanisms that lead to acute lung injury, the mortality rate remains high in patients with acute respiratory distress syndrome.[1,2] Sequestration of neutrophils in lung tissues, intravascular coagulation, disruption of capillary integrity, leading to pulmonary edema, and increased shunt function are major characteristics of this condition.[3] It has been noted that reactive oxygen species and lipid peroxide are involved in many human diseases.[4–9] Although many therapeutic approaches directed at the control of inflammatory responses, such as inflammatory cytokines,[10] adhesion molecules,[11] the complement system,[12] and oxygen radicals,[13] have been evaluated, these approaches have neither attenuated the severity nor decreased the mortality of the disease.[14]

Hepatocyte growth factor (HGF) is a growth factor with multiple biological properties, including mitogenic, motogenic, and morphogenic properties.[15] It has been reported that hepatocyte growth factor is also a mitogenic factor in lung epithelial cells.[16–18] Hepatocyte growth factor prevented respiratory epithelial cell death, as well as promoting the ordered regeneration of the peripheral respiratory tract, and the simultaneous administration of HGF protected against lung injury induced by bleomycin.[19] Hepatocyte growth factor may also have an additional protective activity in damaged respiratory epithelial cells, through an anti-apoptosis mechanism.[19]

However, Zhang et al.[20] reported that hepatocyte growth-promoting factor (pHGF), purified from infant pig liver, showed activity similar to that of HGF. The molecular

weight of pHGF (10.7kDa)[20] differs from that of HGF (82kDa),[21] hepatic stimulator substance (12.4kDa and 17.5kDa),[22] and HGF activator (34kDa).[23] Hepatocyte growth-promoting factor has been used clinically in patients with hepatitis, and the curative effect of pHGF was evident in 1687 patients with subacute fulminant hepatitis treated by Zhang et al.[20] In an experimental study, pHGF acted as a mitogen of hepatocytes, prevented fibrogensis, and stabilized hepatocyte cell membranes, showing effects similar to those of HGF.[24] However, it is not known whether pHGF acts to protect lung injury.

The purpose of the present study was to determine whether pHGF has a protective role in lung epithelial and endothelial injury. To this end, we investigated the effect of pHGF in H_2O_2-induced acute lung injury in rats, by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and light and electron microscopy analyses.

Materials and methods

Hydrogen peroxide and pHGF injections

The rats used in this study were 8-week-old male Wistar rats (weighing 180 to 220g). They were divided into four groups; 30 as the H_2O_2 intoxication alone group, 30 as the H_2O_2 + pHGF-treated group, 30 as the pHGF alone group, and 30 as the control group. The rats were injected with 0.4ml of 220mM H_2O_2 through the tail vein, according to a procedure described by Sato et al.[25] A single pHGF (300μg/kg)[20] (Yang Jiang, Guang Dong, China) injection was administered immediately after the H_2O_2 injection. Control rats were injected with saline at the same volume as the H_2O_2 injection. The animals were maintained on standard chow and water.

Serum lipid peroxide (LPO) levels

The concentration of LPO in the serum in each of the four groups was measured 30min, and 3, 6, 12, 24, and 48h after the H_2O_2 or saline injection. Blood was collected from the heart. LPO was determined according to a method previously described by Yaji.[26]

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

Apoptosis in vivo was assessed by TUNEL assay. Samples of lung tissue were fixed overnight at 4°C in 10% buffer red formalin, and then embedded in paraffin. An in-situ Apoptosis Detection Kit (Takara, Otau, Japan) was used to carry out TUNEL staining on 5-μm-thick sections, according to the manufacturer's instructions.[27] Color revelation was realized with diaminobenzidine. Ten fields, at ×200, were randomly selected, and the number of TUNEL-positive cells was calculated.

Light and electron microscopy

Five rats in each group were killed at each time point of 30min, and 3, 6, 12, 24, and 48h after the administration of H_2O_2. Lung specimens were taken from each rat (n = 5 rats × six specimens = 30 specimens for each group). Specimens were cut into 2-mm^3 blocks, immersion-fixed with 2.5% glutaraldehyde, and postfixed with 1% osmium tetroxide. The tissue samples were dehydrated through a graded ethyl alcohol series and embedded in Epok 812 (Oken, Tokyo, Japan). For light microscopy, semithin sections were cut on a Porter MT 5000 ultramicrotome (Dupon, Switzerland) with a glass knife, and stained with 0.1% toluidine blue. For electron microscopy, after the semithin section had been observed, two specimens from each group were selected. Thin sections were cut on an ultramicrotome with a diamond knife and stained with uranyl acetate and lead citrate. The sections were examined under a JEL-1010 transmission electron microscope (JEOL, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed with Student's t-test. P values of less than 0.05 were considered statistically significant.

Results

Serum lipid peroxide (LPO) levels

The results of the LPO assay are shown in Fig. 1. LPO levels in the H_2O_2 group were higher at 30min and 3 to 48h than those in the control group ($P < 0.01$). The H_2O_2 group and the H_2O_2 + pHGF-treated group showed similar LPO levels at 30min and at 3h ($P > 0.05$). However, at 6 to 48h, the LPO level in the H_2O_2 group was higher than that in the H_2O_2 + pHGF-treated group ($P < 0.01$).

There was a significant difference between the H_2O_2 + pHGF-treated group, the control group, and the pHGF alone group at all times ($P < 0.05$).

TUNEL signals in lung tissues

Positive TUNEL signals were found in endothelial cells, alveolar epithelial cells, and inflammatory cells (Fig. 2A,B). In the control and pHGF alone groups, there were a few TUNEL-positive cells at all times (1.1 ± 0.6/×200 field). In the H_2O_2 group, TUNEL-positive cells appeared at 6h, (22.2 ± 4.35/×200 field) at 12h (23.0 ± 3.24/×200 field); and at 24h (4.6 ± 1.29/×200 field) (Fig. 3).

In the H_2O_2 + pHGF-treated group, the TUNEL-positive cells appeared at 6h (12.0 ± 2.17/×200 field), at 12h (12.4 ± 3.14/×200 field), and at 24h (1.2 ± 0.49/×200 field) (Fig. 3).

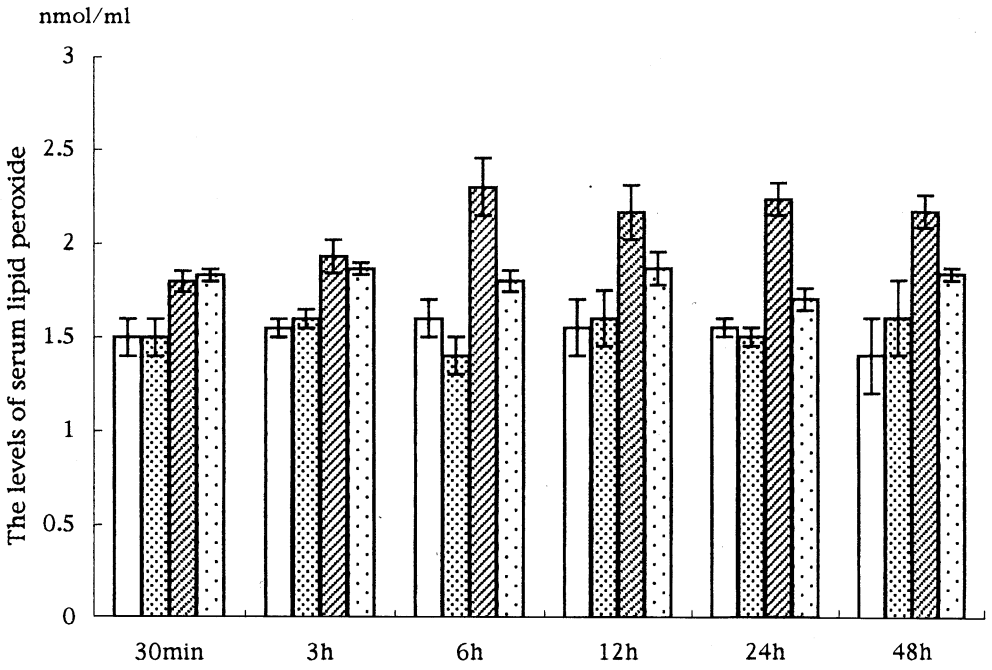

Fig. 1. Levels of serum lipid peroxide in the control group (*white bars*), hepatocyte growth-promoting factor (pHGF) alone group (*closely dotted bars*), H_2O_2 group (*striped bars*), and H_2O_2 + pHGF-treated group (*sparsely dotted bars*). Values are means ± SE; $n = 5$ rats/group. There was a significant difference between the H_2O_2 group and the H_2O_2 + pHGF-treated group at 6 to 48 h ($P < 0.05$), and there was a significant difference between the control group and the H_2O_2 + pHGF-treated group at each time point ($P < 0.05$). There was a significant difference between the H_2O_2 group and the control group at each time point ($P < 0.01$)

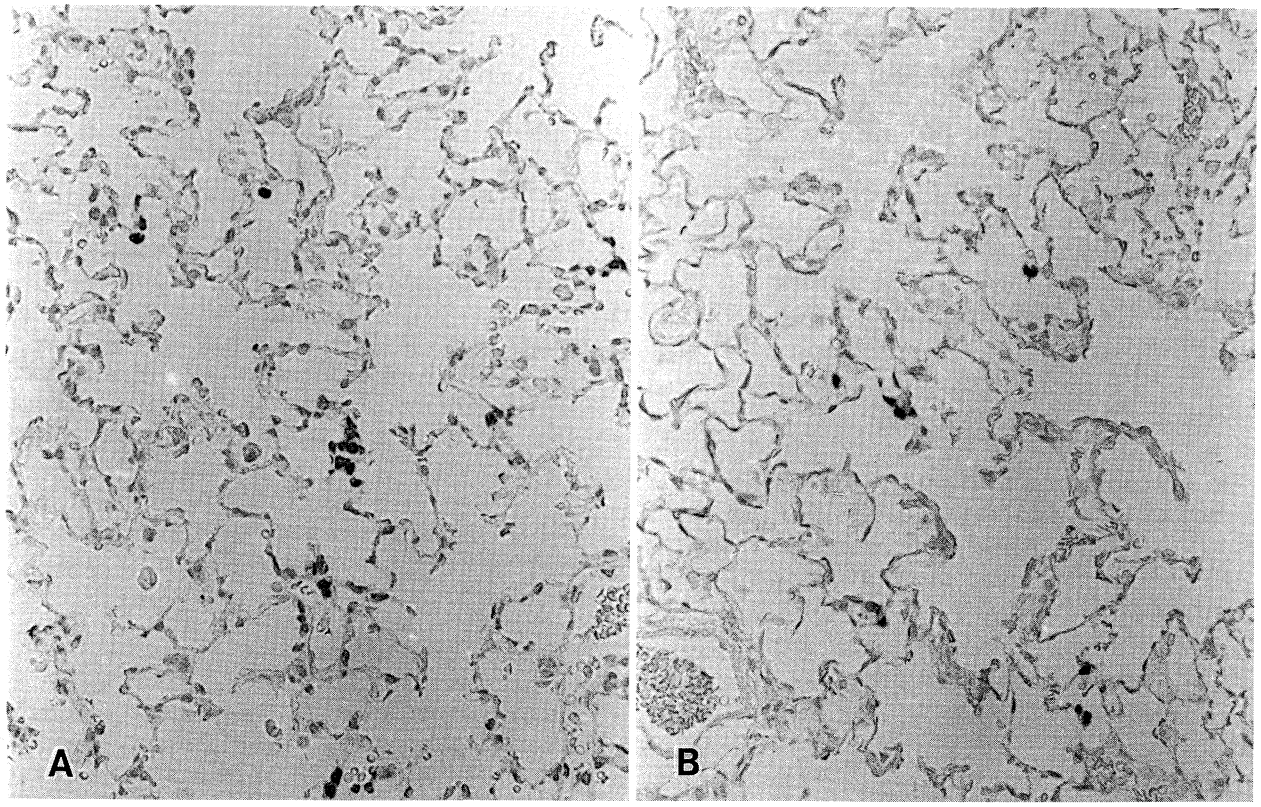

Fig. 2A,B. Light micrographs show terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) in lung tissues; **A** At 6 h, positive TUNEL signals are seen in endothelial cells, alveolar epithelial cells, and inflammatory cells in the interstitium in the H_2O_2 group rats. **B** At 6 h, in the H_2O_2 + pHGF-treated group, positive TUNEL signals are observed in epithelial and endothelial cells, but the number of TUNEL-positive cells is decreased compared with that in the H_2O_2 group. **A** and **B** ×100

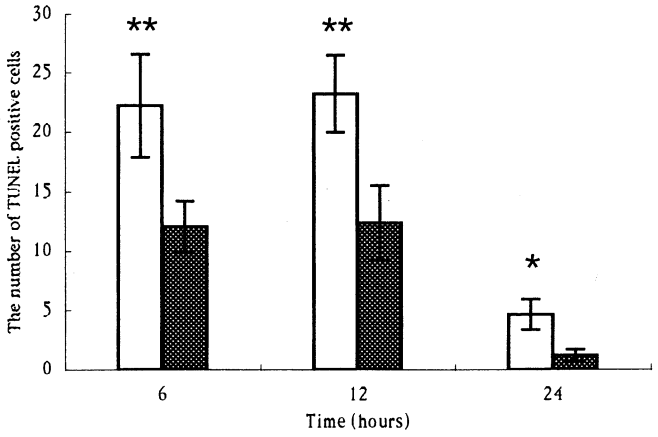

Fig. 3. Quantitative analysis of apoptotic cells. Cells were counted in ten random fields (×200) of two slides and expressed as the number of apoptotic cells per field. Values represent means ± SE for five animals. $^{**}P < 0.01$ compared with the H_2O_2 group (*white bars*) at 6 and 12 h. $^{*}P < 0.05$ at 24 h. *Stippled bars*, pHGF + H_2O_2 group

Light microscopic observations

The pathological changes observed in the pHGF alone and control groups were not seen at all times. In the H_2O_2 group, pulmonary edema was seen at 30 min, and at 3 to 12 h. Severe pulmonary edema was seen at 3 h (Fig. 4A). Three to 48 h after the H_2O_2 injection, atelectasis was seen. At 24 h, severe atelectasis was seen (Fig. 4B). Most pathological changes had recovered at 48 h.

In the H_2O_2 + pHGF-treated group, pulmonary edema was seen at 30 min but at 3 h (Fig. 4C) and 6 h, pulmonary edema was not observed. Slight atelectasis was seen at 3 and 12 h, but no atelectasis was seen at 24 h (Fig. 4D).

Ultrastructural observations

Air-blood barrier damage

In the H_2O_2 group, severe pulmonary edema was seen in the alveoli and dilated capillaries at 3 h. Congestion was also observed in the capillary lumen (Fig. 5).

The formation of subendothelial blebs, and interstitial edema, were seen at 30 min, and at 3 to 24 h after H_2O_2 injection (Fig. 6A,B,C,E). At 30 min and 24 h after H_2O_2 administration, the formation of subendothelial blebs was slight (Fig. 6A); however, at 3 and 6 h, the formation of subendothelial blebs was markedly evident (Fig. 6B,C), with notable elevation of the endothelial plasma membrane from the capillary basement membrane. This appeared to precede the stripping of endothelial cells from the basement membrane (Fig. 6B,C), together with the fragmentation of endothelial plasma membranes, resulting in discontinuities of the capillary endothelial lining (Fig. 6B,C). At 6 to 24 h, endothelial cell disruptions were noted (Fig. 6D). Type I lung epithelial cell edema was seen at 30 min, and at 3 to 24 h, and was severe at 6 h (Fig. 6B). Type I lung epithelial cell disruption was seen at 12 to 24 h (Fig. 6E).

In the H_2O_2 + pHGF-treated group, the formation of subendothelial blebs and the attenuation of endothelial cells were seen at 30 min, and at 3 to 24 h (Fig. 7A,B,C,D). The ultrastructural features of the H_2O_2 + pHGF-treated group were similar to those of the H_2O_2 group at 30 min (Fig. 7A). At 3 and 6 h, endothelial cell blebs were rarely seen. Disruption of endothelial cells was rarely seen at 12 h. Disruption of type I pulmonary epithelial cells was not seen (Fig. 7A,B,C,D).

Type II epithelial cell damage

In the H_2O_2 group, mitochondria were swollen and microvilli had disappeared in type II epithelial cells at 3 to 24 h after H_2O_2 administration (Fig. 8A). In the H_2O_2 + pHGF-treated group, there were no ultrastructural changes in the type II epithelial cells (Fig. 8B). These light microscopic and ultrastructural findings in the two groups (H_2O_2 group and H_2O_2 + pHGF-treated group) are summarized in Table 1.

Discussion

The present study was performed to examine the protective effect of pHGF against H_2O_2-induced lung epithelial and endothelial cell injury in rats; using TUNEL assay, and light and electron microscopy analyses. The serum lipid peroxide levels were also measured.

Serum lipid peroxide levels (LPO)

Hydrogen peroxide (H_2O_2) is one of the factors that induces acute lung injury, and it can cross membranes almost as readily as can water.[4] The changed O_2^- molecule can cross membranes and enter cells via transmembrane anion channels.[4,28] The unsaturated bonds of membrane cholesterol and fatty acids can readily react with free radicals and undergo peroxidation. The loss of cell membrane unsaturated fatty acids, the formation of lipid peroxides, and oxygen uptake by lipid preparations all indicate peroxidation.[4] Some studies have indicated that cellular membranes are the primary sites of injury after the administration of peroxides.[29–31]

In the present study, the LPO level was higher 30 min to 48 h after H_2O_2 injection than levels at the same time points after saline injection in the control group. These findings indicate that cell membrane damage occurs after H_2O_2 injection. The LPO level in the H_2O_2 + pHGF-treated group was also higher than that in the control group at 30 min to 48 h, but, at 6 to 48 h, the level in the H_2O_2 + pHGF-treated group was lower than that in the H_2O_2 group. This finding indicates that pHGF acts to decrease the formation of LPO. Thus, pHGF may protect the cell membrane against damage in H_2O_2-induced lung injury.

Apoptosis

Apoptosis, or programmed cell death, plays a major role in cellular homeostasis, maintaining the delicate balance

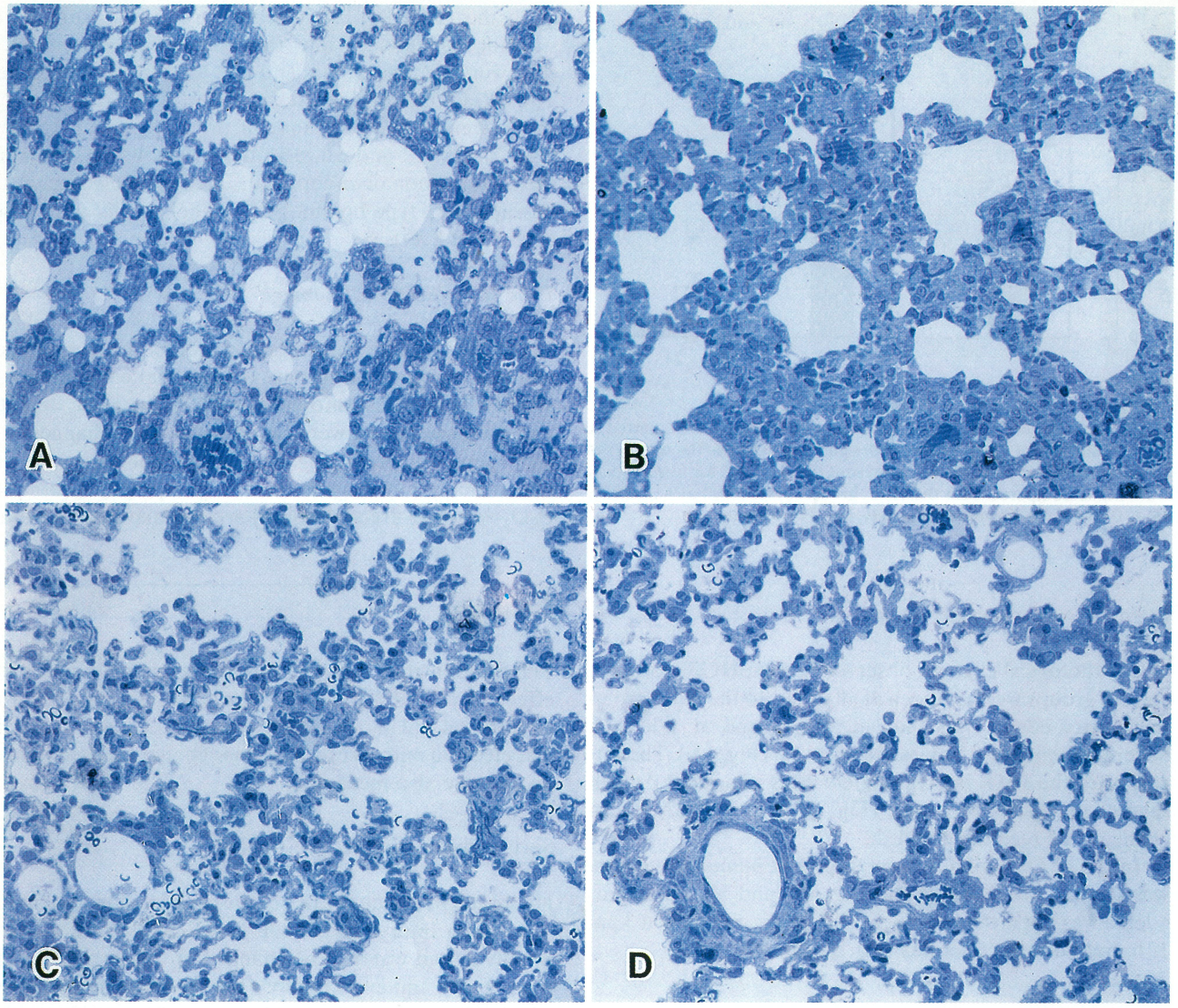

Fig. 4A–D. Light micrographs. **A** At 3 h after administration of H_2O_2, pulmonary edema is seen. **B** At 24 h after administration of H_2O_2, marked focal atelectasis is seen. **C** At 3 h after administration of H_2O_2 in rats treated concomitantly with pHGF, pulmonary edema is not observed. **D** At 24 h after administration of H_2O_2 in rats treated concomitantly with pHGF, an atelectatic area is not observed. Toluidine blue, ×100

Table 1. Summary of light microscopic and ultrastructural findings in H_2O_2 alone group and H_2O_2 + pHGF-treated group

	H_2O_2 group						H_2O_2 + pHGF-treated group					
	30 min	3	6	12	24	48 h	30 min	3	6	12	24	48 h
Light microscopy												
Pulmonary edema	+	3+	+	+	−	−	+	±	±	−	−	−
At electasis	−	±	+	+	3+	+	−	±	±	+	−	−
Electron microscopy												
Subendothelial bleb	+	2+	2+	+	±	−	+	+	+	+	+	−
Endothelial cell disruption	−	−	±	2+	±	−	−	−	−	±	−	−
type I cell edema	±	+	2+	+	±	−	±	±	±	±	−	−
type I cell disruption	−	−	−	±	+	−	−	−	−	−	−	−

Light microscopy, graded on a scale of − to 3 +: −, 0%; ±, 0%–5%; +, 5%–10%; 2+, 10%–20%; 3+, 30%–50% (percentage of the area of the specimen)
Electron microscopy, graded on a scale of − to 2+: −, Absent or normal; ±, absent or present but not evident; +, present; 2+, abundantly present
pHGF, Hepatocyte growth-promoting factor

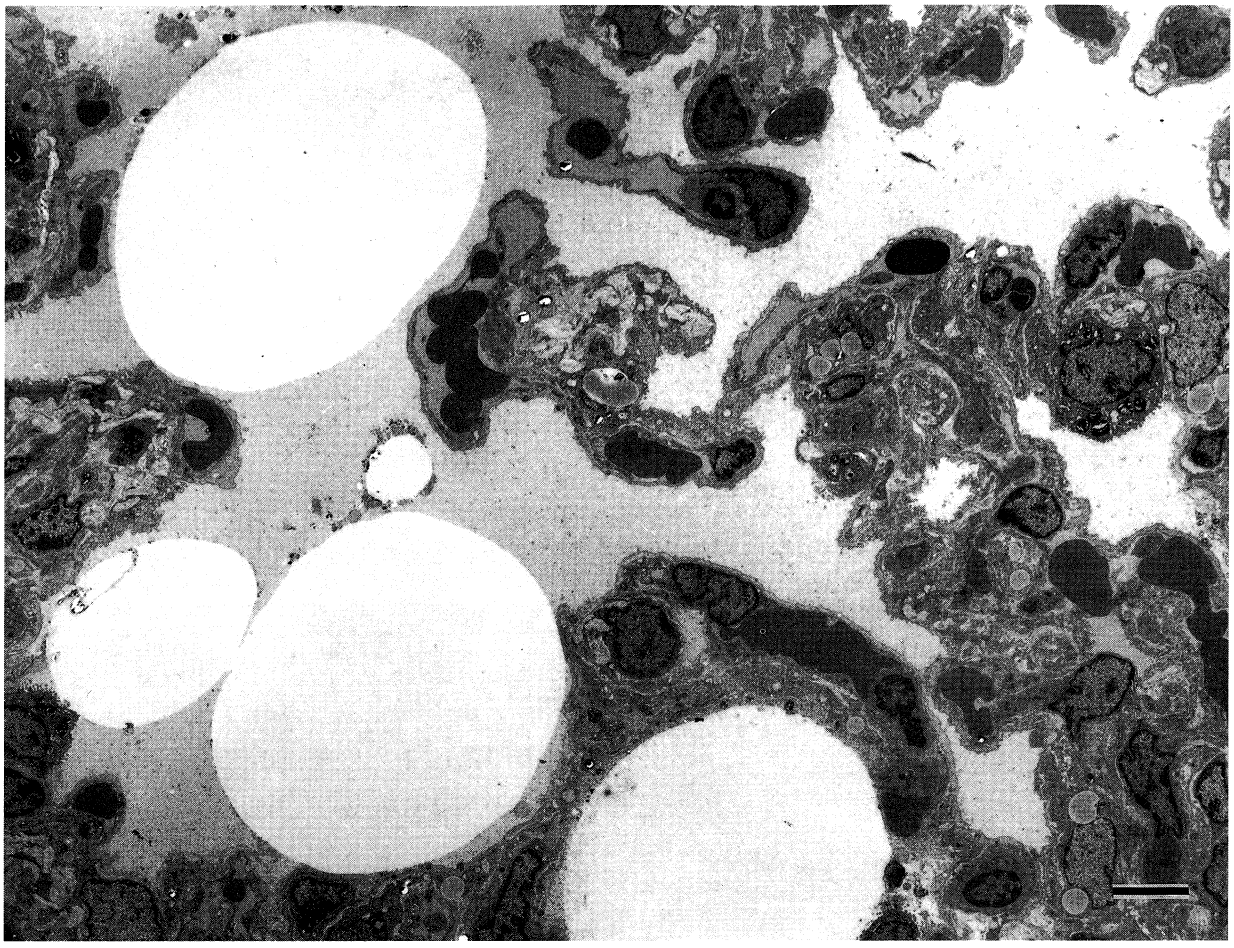

Fig. 5. Electron micrograph shows pulmonary edema after administration of H_2O_2. At 3 h, marked pulmonary edema and dilated capillaries are seen; congestion is also seen in the capillary lumen. *Bar*, 5 μm

between cell proliferation and cell death.[32] It has been reported that apoptosis is important in the development of pulmonary disease.[33,34] The endothelium is not considered to be a rapidly renewing tissue, and, therefore, apoptosis in endothelial cells can lead to lung injury.[35] It has been reported that apoptosis in lung endothelial and epithelial cells, and inflammatory cells, was induced 12 to 24 h after lipopolysaccharide injection in mice.[14] In vitro, apoptosis in tumor cells can be induced by exposing the cells to exogenous oxidants.[36–38] In the present study, apoptosis in lung endothelial and epithelial cells, and inflammatory cells in animals treated with H_2O_2 alone was identified, using TUNEL, 6 to 24 h after the H_2O_2 injection.

Hepatocyte growth factor may be involved in the prevention of apoptosis, and it has been reported that HGF may have protective activity in damaged respiratory epithelial cells, through an anti-apoptosis mechanism.[19] We also demonstrated, using the TUNEL assay, that pHGF prevented apoptosis in epithelial and endothelial cells with H_2O_2-induced lung injury.

Morphology

In the present study, the administration of pHGF suppressed the severe pulmonary edema and atelectasis seen in H_2O_2-induced lung injury. In addition, pHGF acted to inhibit type I lung epithelial cell edema and type II lung epithelial cell damage.

Pulmonary edema and subendothelial edema were already evident 30 min after H_2O_2 injection. This finding suggested that oxygen-derived free radicals induced an increase in capillary permeability. In studies of brain[39] and myocardium,[25,40] no ultrastructural damage to capillaries was found with exogenous free radicals. In the present study, endothelial cell disruption was not shown by electron microscopy 30 min or 3 h after H_2O_2 injection. It may be that the increasing capillary permeability could not be detected by transmission electron microscopy. In both the H_2O_2 group and the H_2O_2 + pHGF-treated group, slight pulmonary edema and few subendothelial blebs, as well as slight interstitial edema, were found 30 min after the H_2O_2 injection. Thereafter, in the H_2O_2 group, severe pulmonary

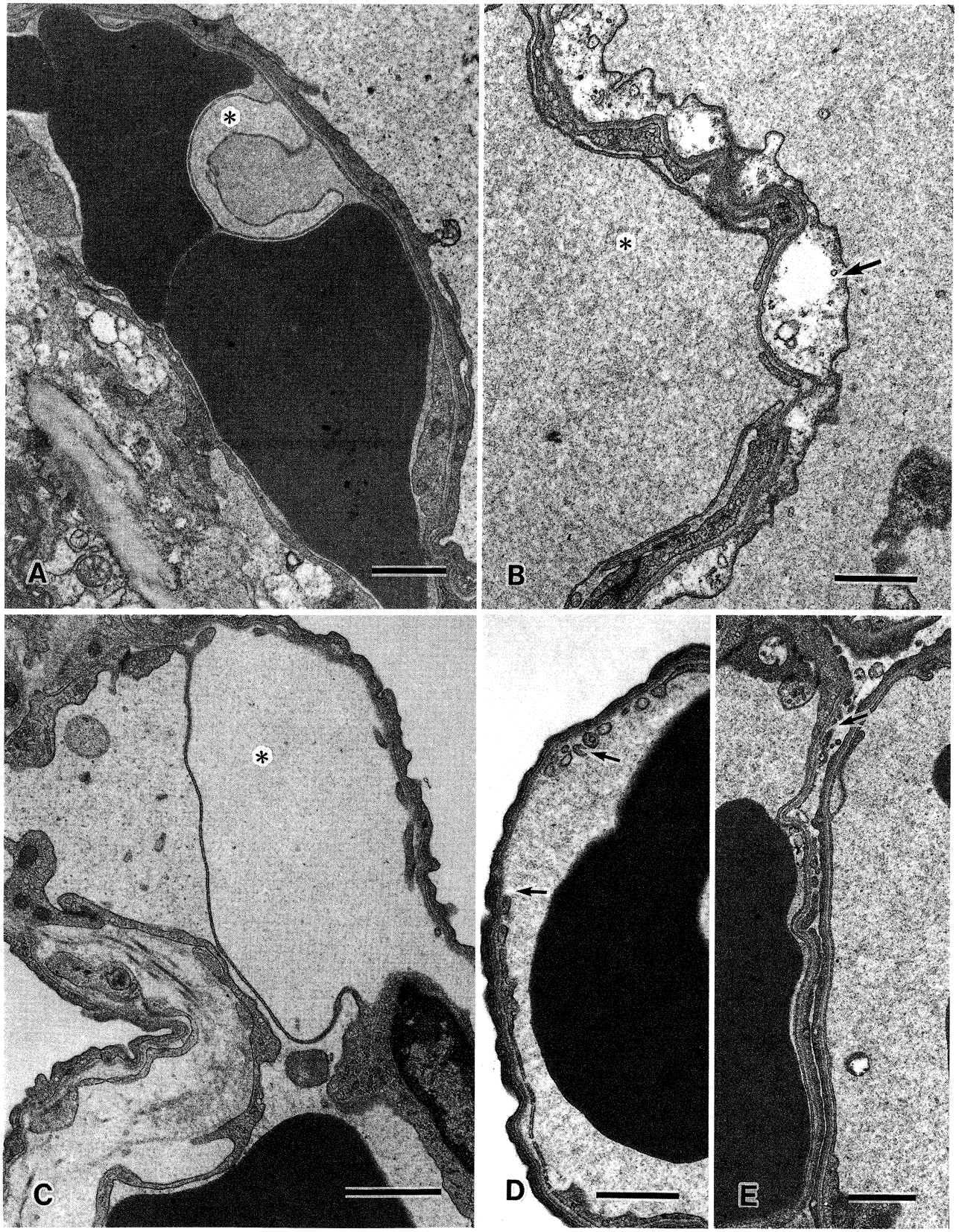

Fig. 6A–E. Electron micrographs show lung damage after administration of H_2O_2. **A** At 30 min, subendothelial blebs (*asterisk*) are seen. **B** At 3 h, type I lung epithelial cell edema (*arrow*) and subendothelial blebs (*asterisk*) are observed. **C** At 6 h, subendothelial blebs (*asterisk*) are seen. **D** At 12 h, endothelial cell disruption (*arrows*) is seen. **E** At 24 h, type I lung epithelial cell disruption (*arrow*) is seen. *Bars*, 1 μm

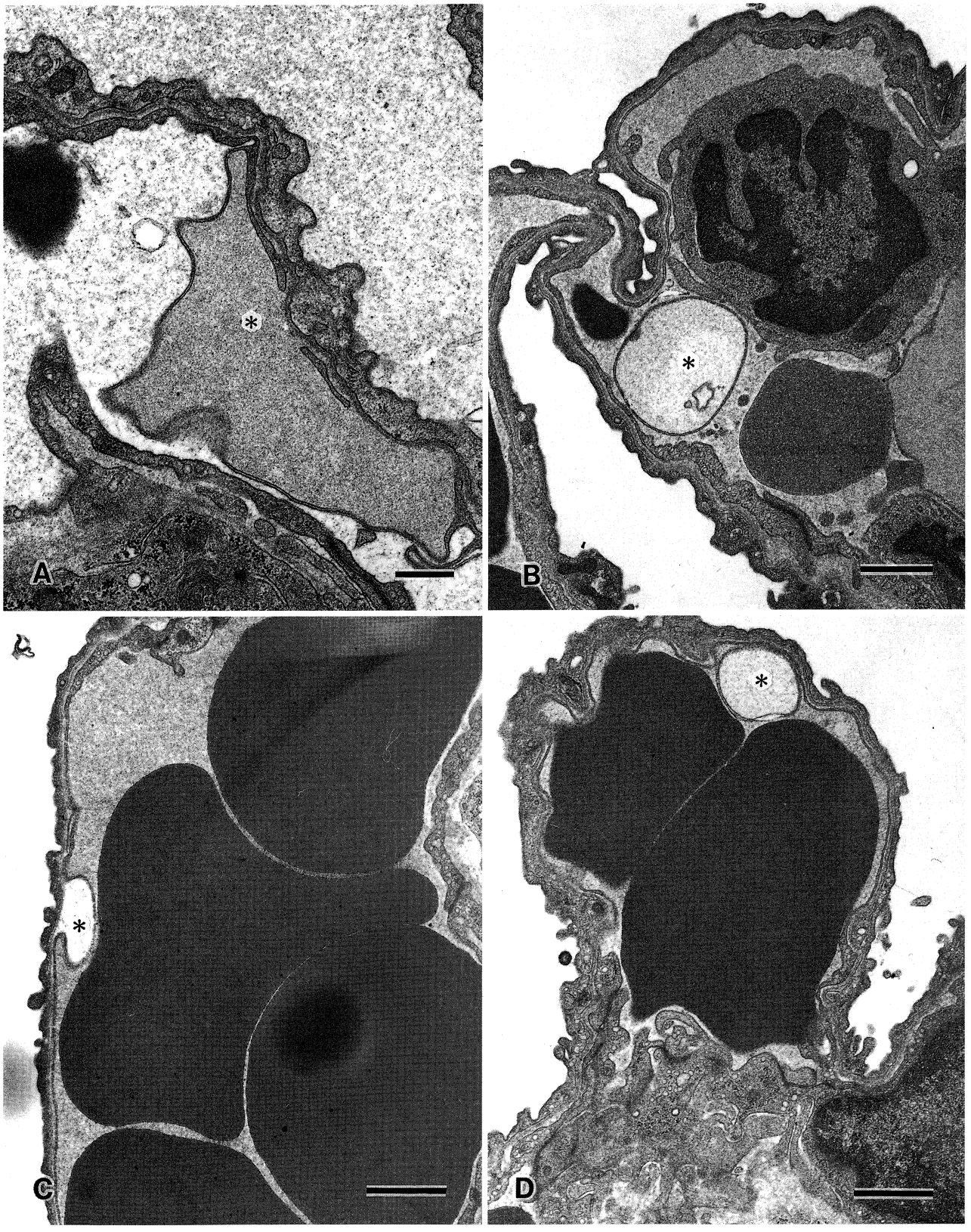

Fig. 7A–D. Electron micrographs show lung tissue after the administration of H_2O_2 in rats treated concomitantly with pHGF. **A** At 30 min, subendothelial blebs (*asterisk*) are clearly seen. *Bar*, 0.5 μm. **B** At 6 h, subendothelial blebs (*asterisk*) are seen. *Bar*, 1 μm. **C** At 12 h, subendothelial blebs (*asterisk*) are seen, and there are no ultrastructural changes in type I lung epithelial cells. *Bar*, 1 μm. **D** At 24 h, subendothelial blebs (*asterisk*) are rarely seen, and attenuation of endothelial cells is observed. *Bar*, 1 μm

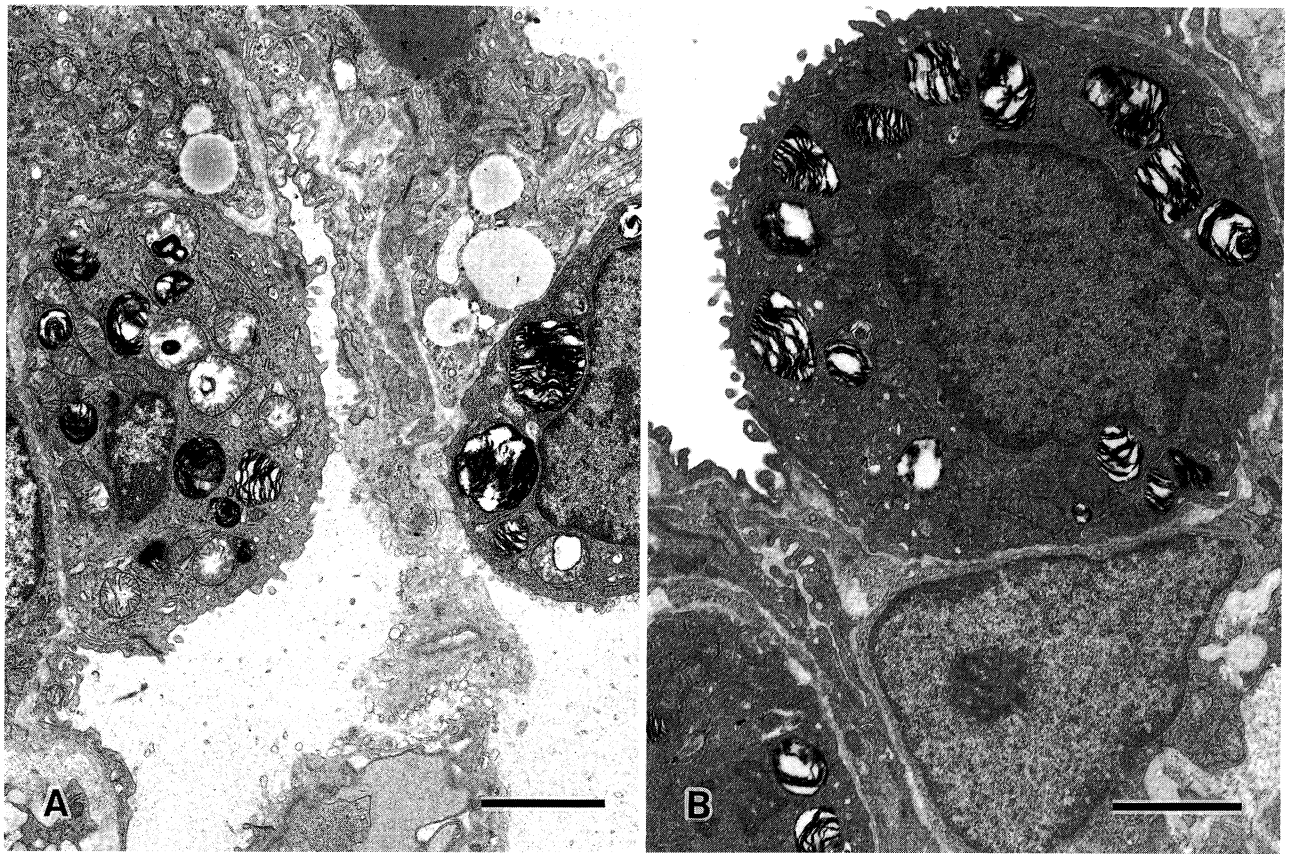

Fig. 8A,B. Electron micrographs show type II lung epithelial cells. **A** In the H_2O_2 group, at 3 h, mitochondria are swollen and microvilli have disappeared in type II epithelial cells. **B** In the H_2O_2 + pHGF-treated group, type II lung epithelial cells have normal lamellar inclusion bodies, mitochondria, and microvilli. *Bars*, 2 μm

edema and many subendothelial blebs were seen. However, in the H_2O_2 + pHGF-treated group, the area of pulmonary edema was decreased, and the number of subendothelial blebs did not increase. This finding suggested that pHGF acted to prevent an increase in capillary permeability. It may be that pHGF acted to stabilize endothelial cell membrane injury.

Severe pulmonary edema appeared at 3 h, and then severe atelectasis was observed at 24 h after H_2O_2 injection. This finding indicated that when pulmonary edema disappeared, severe ateletasis appeared. It may be that the development of atelectasis was caused by the pulmonary edema. Blood filled the alveolar space, so that surfactant, and the alveolar surface layer, could have been broken down. In the H_2O_2 group, atelectasis was found at 3 to 48 h after the H_2O_2 injection, but in the H_2O_2 + pHGF-treated group, it was seen at 3 to 12 h. In addition, the area of atelectasis in the H_2O_2 + pHGF-treated group was decreased compared with that in the H_2O_2 group.

In the present study, lung epithelial cell and endothelial cell disruption was seen 12 to 24 h after the H_2O_2 injection. Tamura[41] reported that, in oleic acid-induced lung injury, electron microscopic findings of lesions with the most severe damage showed complete cell necrosis and disruption of both capillary endothelial cells and alveolar epithelial cells. In the H_2O_2 group in our study, both type I epithelial cell disruption and endothelial cell disruption were seen. However, in the H_2O_2 + pHGF-treated group, type I epithelial cell disruption and endothelial cell disruption were rarely observed.

It was reported that the lung was the primary target of the toxic effects of free radicals.[42–44] The target cells in H_2O_2-induced lung injury appeared to be the capillary endothelial cells.[44,45] Gardner et al.[46] reported that, at high concentrations of H_2O_2, target cells ($P388D_1$ cells in vitro) appeared necrotic, while at lower H_2O_2 concentrations, they showed apoptosis. We found apoptosis and disruption of capillary endothelial cells after H_2O_2 injection. In endothelial cells, pHGF inhibited not only apoptosis but also the disruption of the cells.

In conclusion, we examined the protective effect of pHGF against H_2O_2-induced acute lung injury. Our findings indicated that pHGF was effective in suppressing pulmonary edema, atelectasis, and disruption to capillary endothelial and type I lung epithelial cells. pHGF also suppressed endothelial and lung epithelial cell apoptosis. In addition, pHGF acted to decrease the formation of lipid peroxide. Thus, it may be that pHGF acted to stabilize the cell membranes of capillary endothelial and lung epithelial cells. In China, at presently, pHGF is used clinically only in patients with liver disease. Further long-term studies will be necessary to confirm the clinical efficacy of this therapy in lung

diseases such as the early stage of respiratory distress syndrome.

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