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Acute 40 percent exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks

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BACKGROUND: Hemoglobin-vesicles (HbVs; diameter, 251 ± 81 nm) are artificial O_2 carriers. Their efficacy for acute exchange transfusion has been characterized in animal models. However subsequent profiles of recovery involving the degradation of HbV in the reticuloendothelial system (RES) and hematopoiesis remain unknown.
STUDY DESIGN AND METHODS: Isovolemic 40 percent exchange transfusion was performed in 60 male Wistar rats with HbV suspended in 5 g per dL recombinant human serum albumin (rHSA; HbV/rHSA, [Hb] = 8.6 g/dL), stored rat RBCs suspended in rHSA (sRBC/rHSA), or rHSA alone. Hematological and plasma biochemical analyses and histopathological examination focusing on the spleen were conducted for the subsequent 14 days.
RESULTS: The reduced hematocrit (Hct) level (26%) for the HbV/rHSA and rHSA groups returned to its original level (43%) in 7 days. Plasma erythropoietin was elevated in all groups: the rHSA group showed the highest value on Day 1 (321 ± 123 mIU/mL) relating to the anemic conditions (HbV/rHSA, 153 ± 22; sRBC/rHSA, 63 ± 7; baseline, 21 ± 3). Simultaneously, splenomegaly occurred in all the groups as HbV/rHSA > rHSA > sRBC/rHSA. Histopathologically, the accumulated HbV in the spleen was undetectable by Day 14, but hemosiderin was deposited in slight quantities for both the HbV/rHSA and sRBC/rHSA groups. Considerable amounts of erythroblasts were apparent in the spleens of both the rHSA and the HbV/rHSA groups.
CONCLUSION: HbVs were phagocytized and degraded in RES, a physiological compartment for the degradation of RBCs, and the elevated erythropoietic activity resulted in the complete recovery of Hct within 7 days in the rat model.

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed progressively for use as a transfusion alternative. Some are now undergoing clinical trials.[1,2] Advantages of HBOCs include the absence of blood-type antigenicity and infectious pathogens and stability for long-term storage when compared with RBC transfusion.[3] Considerably shorter half-life ($t_{1/2}$) of the HBOCs in the blood stream (2-3 days) limit their use,[4] but they are applicable for shorter periods of use as: 1) a resuscitative fluid for hemorrhagic shock during an emergency situation temporarily or for bridging until RBCs are available;[5] 2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage during elective surgery to avoid or delay alloge-

ABBREVIATIONS: HBOC(s) = hemoglobin-based O_2 carrier(s); HbV(s) = hemoglobin-vesicle(s); MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLP = pyridoxal 5′-phosphate; RES = reticuloendothelial system; rHSA = recombinant human serum albumin; sRBC(s) = stored red blood cell(s).

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neic transfusion;[6] 3) a priming solution for the circuit of an extracorporeal membrane oxygenator during cardiac surgery;[7] and 4) an alternative for use for other potential indications, for example, so-called O_2 therapeutics to oxygenate ischemic tissues.[8,9]

A phospholipid vesicle or liposome-encapsulating concentrated human Hb (Hb-vesicle, HbV) is an HBOC.[10,11] The cellular structure of the HbV (particle diameter, approx. 250 nm) has characteristics that resemble those of natural RBCs because both have lipid bilayer membranes that prevent the direct contact of Hb with blood components and the endothelial lining, thus shielding all side effects of molecular Hb.[12,13] Once in circulation, HbV particles are captured by the phagocytes in the reticuloendothelial system (RES or mononuclear phagocytic system) and are metabolized in the physiologically normal pathway after topload infusions.[14-17]

We tested the efficacy of HbV suspended in plasma-derived and recombinant human serum albumin (rHSA) in extreme normovolemic hemodilution (80-90% blood exchange) and resuscitation from hemorrhagic shock. They have a comparable O_2-transporting capacity with RBCs.[18-21] However, only a few hours of observation after extensive blood exchange has been reported.

This study undertakes, for the first time, a longer period of observation (2 weeks) after moderate and clinically relevant isovolemic exchange transfusion of a 40 percent estimated blood volume with HbV suspended in a 5 g per dL rHSA solution.[21] We analyzed plasma biochemical, hematological, and histopathological examinations, particularly addressing the degradation of HbV in RES and erythropoietic activity after the reduced Hct. Splenomegaly was more dominant than hepatomegaly after single and repeated infusions of HbV in our previous studies.[14,15,17] Senescent RBCs are known to be captured and degraded in the spleen.[22] For that reason, we conducted infusion of stored homologous RBCs to compare the relative impacts on the spleen.

MATERIALS AND METHODS

Preparation of HbVs suspended in rHSA

HbVs were prepared under sterile conditions, as reported in previous studies.[23,24] The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dL) contained 14.7 mmol per L pyridoxal 5'-phosphate (PLP) (Sigma-Aldrich Corp., St. Louis, MO) as an allosteric effector at a molar ratio of PLP/Hb of 2.5. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan), at a molar composition of 5/5/1/0.033. The lipopolysaccharide content, measured with a modified *Limulus* amebocyte lysate test, was less than 0.1 EU per mL.[25] The physicochemical parameters are P_{50}, 27 Torr; 251 ± 81-nm particle diameter; and less than 3 percent MetHb content. Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of rHSA (25 g/dL, 1.4 mL; Nipro Corp. Osaka, Japan) to regulate the rHSA concentration in the suspending medium to 5 g per dL. Consequently, the Hb concentration became 8.6 g/dL.[21] Under these conditions, the colloid osmotic pressure and the viscosity (300/sec, 37°C) of the HbV/rHSA were 20 mmHg and 2.9 cP, respectively.

Preparation of stored homologous RBC suspended in rHSA

Blood was withdrawn from donor Wistar rats via the caudal vena cava during ether anesthesia. This was mixed with an RBC preservation fluid, CPDA-1 (C.A. Karmi, Kawasumi Laboratories Inc., Tokyo, Japan) at the volume ratio of 10 percent. The mixture was stored under sterile conditions at 4°C for 1 week because rat RBCs stored for 1 week are reportedly as fragile as the human RBC stored for 4 weeks.[26] After preservation, the stored blood was centrifuged for 10 min at 4000 × *g*, and then the supernatant and the buffy coat were removed. The sedimented RBCs were resuspended in saline and centrifuged. This procedure was repeated twice. Finally, the RBCs were suspended in a 5 g per dL rHSA solution to prepare stored homologous RBCs suspended in rHSA (sRBC/rHSA). The Hb concentration was regulated at 8.6 g per dL, the same Hb concentration of HbV/rHSA.

Exchange transfusion and 2-week observations

Experiments were conducted with 65 male Wistar rats (223 ± 20 g body weight; Saitama Experimental Animals Supply Co., Kawagoe, Japan). During cannulation and exchange transfusion, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (1 mL/kg; Abbott Laboratories, North Chicago, IL). Polyethylene catheters were introduced into the right common carotid artery. Blood withdrawal and sample injection were repeated through one line at 1 mL per 30 seconds. Samples were HbV/rHSA (n = 20), sRBC/rHSA (n = 20), and rHSA only (n = 20). Five rats were used for baseline measurements.

The systemic blood volume was estimated to be 56 mL per kg of the total body weight.[27] Blood was exchanged under the assumption of normovolemia. Therefore, to estimate the necessary amount of HbV, the exchange was assumed to consist of repeating the number of cycles of 1.0-mL withdrawal and sample infusion. The level of exchange, 40 percent, is therefore given as

$$40\% = 100 \times \{1 - [(0.056 \times \text{body weight} - 1.0)/(0.056 \times \text{body weight})]^n\}. \quad (1)$$

The volume exchanged was calculated as $n \times 1.0$ (mL).[28] The sample volume is calculated as 6.0 mL for a rat body weight of 220 g.

After the blood exchange, the catheter was removed, the artery was ligated, and the neck skin was sutured with a stitch. The rats were housed in cages in a barrier room at the animal experimental facility of Keio University. Rats were provided ad libitum access to food and water in a temperature-controlled environment with a 12-hour dark-light cycle.

Five rats were selected randomly from each group at 1, 3, 7, and 14 days for sequential measurements. At each time point, the rats were anesthetized with a 1.5 percent sevoflurane-mixed air inhalation. After measuring the body weight, approximately 150 μL of blood was withdrawn from the tail vein via an indwelling needle (24-gauge; Nipro Corp.) for Hct measurement with glass capillaries, and blood cell counts with an automatic blood cell counter (Model KX-21, Sysmex Corp., Kobe, Japan). The animals were laparotomized and approximately 6 mL of blood was withdrawn from the caudal vena cava for the plasma biochemical tests. The organs were resected en bloc and fixed in a 10 percent formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Tokyo, Japan) and then embedded in paraffin. Four-micrometer sections were stained with the hematoxylin-eosin, Berlin blue, and Giemsa methods.

The collected blood (approx. 6 mL) was centrifuged (5,000 × *g*, 10 min) to separate the plasma, which was then ultracentrifuged (50,000 × *g*, 20 min) to sediment the HbV particles from the plasma at 1 and 3 days after the exchange transfusion with HbV/rHSA to avoid their interference by HbV particles in the plasma biochemical assays.[29] The obtained transparent serum specimens contained no Hb, indicating that no hemolysis of HbV occurred. They were stored at –80°C until biochemical tests at BML, Inc. (Kawagoe, Japan). Erythropoietin (EPO) was measured with radioimmunoassay. Because the rat EPO shows a high degree of homology with human EPO, the rat EPO cross-reacts in the assay of the antihuman EPO.[30]

The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of School of Medicine, Keio University. It also complied with the *Guide for the Care and Use of Laboratory Animals*.[31]

Statistical analyses

Data are reported as mean ± standard deviation (SD) for all measurements. Differences between the control (baseline) group and a treatment group were analyzed with a one-way analysis of variance followed by Fisher's protected least significant difference test. The changes were considered significant if the p value was less than 0.01.

RESULTS

Body and spleen weights and hematological tests

Rats of all groups tolerated well the 40 percent blood exchange; they survived until their intentional euthanization. The rats survived this intervention because of the normovolemic exchange transfusion while maintaining the blood colloid osmotic pressure with 5 g per dL rHSA as the suspending medium. All rats gained weight until their euthanization (Fig. 1). No noticeable change occurred in their behavior or appearance such as the pilomotor response.

The spleen:body weight ratio increased significantly for the HbV/rHSA group at 1 and 3 days after the exchange. It returned to a level that was comparable to the baseline at 14 days. The rHSA group also showed significant splenomegaly at 3 days, but no splenomegaly at 1 day. At 14 days, the spleen weight reverted to the baseline level. The sRBC/rHSA group also showed moderate, but significant, splenomegaly on Days 1, 3, and 7.

The Hct before the exchange transfusion was approximately 43 percent. It decreased to about 26 percent for the HbV/rHSA and rHSA groups. Both groups showed a monotonic Hct increase; at 7 days, the Hct showed a complete recovery to the baseline level (about 43%) and an overshooting at 14 days (approx. 46%). In the sRBC/rHSA group, the Hct level at 1 day was much higher than that of the other groups because of the sRBC infusion. The Hct level, however, was slightly lower than for the other groups at 7 and 14 days. The mean corpuscular Hb (MCH), mean corpuscular volume (MCV), and mean corpuscular Hb concentration (MCHC) values remained within normal ranges (data not shown); however, MCH and MCHC of the HbV/rHSA group at 1 and 3 days were not measured because of the presence of HbV. The sRBC/rHSA group showed slightly lowered MCV and MCH levels at 1 day. In contrast to Hct, platelet and white blood cell counts showed nonsignificant decreases at 1 day and then maintained rather steady values. The plasma Hb concentration derived from HbV after the exchange transfusion was estimated as 4.4 g per dL, which decreased, respectively, to 1.8 ± 0.1, 1.1 ± 0.1, and 0 g per dL on Days 1, 3, and 7.

Plasma biochemical tests

The plasma EPO level, an indicator of an anemic, hypoxic, or stressed condition, increased significantly from 21 ± 3 IU per L in the normal condition to 312 ± 123 IU per L for the rHSA group at 1 day, which was significantly higher than for the HbV/rHSA group (153 ± 22 IU/L) or the sRBC/rHSA group (63 ± 7 IU/L; Fig. 2). After 3 days, they decreased to less than 100 IU/L; at 7 days, they reverted to the baseline level.

Regarding the other routine analytes, apartase aminotransferase showed slight increases on Day 1 for all

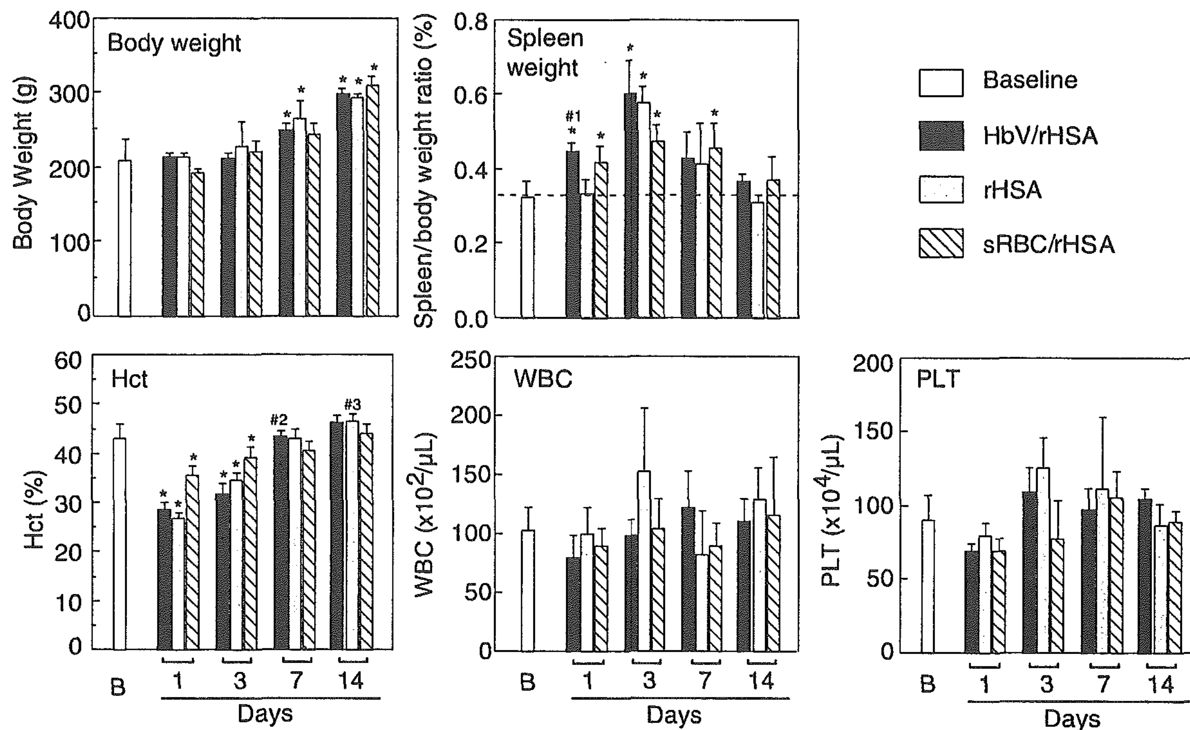

Fig. 1. Changes in body weight, spleen:body weight ratio, and hematological parameters after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The spleen:body weight ratio (baseline, 0.32 ± 0.04%) increased significantly for the HbV/rHSA group at 1 day (0.45 ± 0.03%) and 3 days (0.60 ± 0.09%). It returned to the baseline at 14 days (0.37 ± 0.02%). The rHSA group also showed significant splenomegaly at 3 days (0.58 ± 0.05%) and returned to 0.31 ± 0.02 percent at 14 days. The sRBC/rHSA group also showed splenomegaly at 1, 3, and 7 days (0.42 ± 0.04, 0.48 ± 0.04, and 0.46 ± 0.06%, respectively). The baseline Hct level was 43 percent; it decreased to about 26 percent for the HbV/rHSA and rHSA groups. At 7 days, they showed complete recovery to approximately 43 percent and then further increased to approximately 46 percent at 14 days. The values are means ± SD. The broken line indicates the baseline value. *Significantly different from the baseline ($p < 0.01$); #1 significantly different from the rHSA group ($p < 0.01$); #2 $p = 0.0288$ versus sRBC/rHSA; #3 $p = 0.0353$ versus sRBC/rHSA. B = baseline.

groups (HbV/rHSA, 70 ± 5 U/L; rHSA, 69 ± 12; sRBC/rHSA, 72 ± 9; baseline, 60 ± 7), but it reverted to the original level, whereas alanine aminotransferase was stable. Alkaline phosphatase and γ-glutamyltransferase showed significant or nonsignificant reductions for all groups throughout the experiment. Creatine phosphokinase was stable for 14 days. For all groups, creatinine and uric acid were maintained at low levels for 14 days (data not shown). Amylase showed some significant reduction, but did not change markedly for 14 days (Fig. 3). In contrast, lipase showed significant and marked increases for the HbV/rHSA group for 3 days, but it tended to decrease after 7 days.

Regarding plasma lipid components in the HbV/rHSA group, the total cholesterol and free cholesterol showed significant increases with maximum values at 3 days (Fig. 3). Nevertheless, they returned to their original levels at 7 days. The β-lipoprotein tended to decrease after the exchange transfusion, showing significant reductions at 3 and 7 days for the rHSA group. The high-density lipoprotein cholesterol also tended to decrease with a significant reduction at 3 days for the rHSA group. Triglyceride tended to decrease for all groups with a significant difference in the HbV/rHSA group at 1 and 3 days, partly because of ultracentrifugation of the plasma fractions, and in the rHSA group at 7 days. At 14 days, they generally recovered to the baseline level. The phospholipid tended to decrease with significant differences for all groups. Free fatty acid tended to increase at 14 days. The serum bilirubin (<0.1 mg/dL) remained at a low level throughout the experiment. Fe^{3+} showed significant reductions at 1 and 3 days for the HbV/rHSA group, at 3 and 7 days for the rHSA group, and at 3 days for the sRBC/rHSA group, but they returned to the original level at 14 days (Fig. 3).

Histopathological study

Histopathological examination revealed no significant changes in the lung, heart, and kidney in all groups. At 1 and 3 days after infusion, significant amounts of HbV phagocytized by macrophages in the marrow and Kupffer

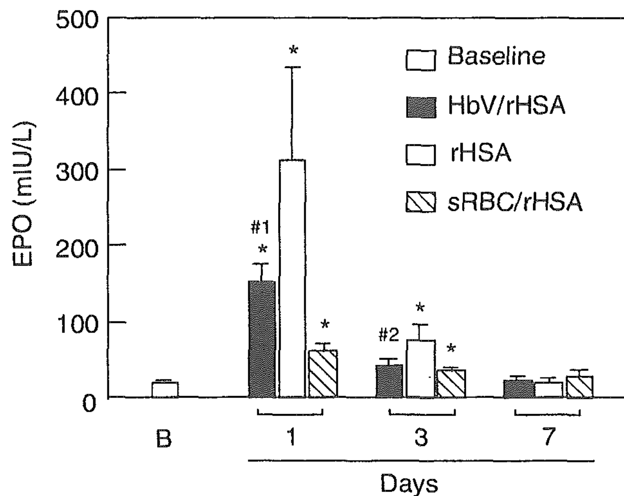

Fig. 2. Plasma EPO activity after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. All groups showed significant increases at 1 day. However, the HbV/rHSA groups showed a lower level than the rHSA group. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$); #1p = 0.0222 versus rHSA; #2p = 0.0195 versus rHSA. B = baseline.

cells in the liver were observed. However, HbV decreased significantly at 7 days and was undetectable at 14 days. At 3 days after infusion, the pancreas in the HbV/rHSA group showed no significant morphological changes in spite of the increased lipase activity.

Sections of the spleen of the HbV/rHSA group, which is stained with Giemsa method, revealed the accumulation of HbV particles in the red pulp zone at 1 and 3 days after the exchange transfusion. The amount of the accumulated HbV decreased at 7 days and then became undetectable at 14 days (Fig. 4). Throughout the period examined in this study, nests composed of erythroblasts and proerythroblasts were formed in the splenic cord, especially at 3 and 7 days, indicating extramedullar erythropoiesis. Nest formation was remarkable for the rHSA group at 3 days. Hematopoietic activity was also observed at 3 days in the marrow of the HbV/rHSA group that contained erythroblastic islets.

The Berlin blue method indicated the presence of hemosiderin in macrophages of the spleen in the HbV/rHSA group at 7 days. This hemosiderin deposition increased until 14 days (Fig. 5). A small amount of hemosiderin was confirmed in the Kupffer cells of the liver at 14 days. Hemosiderin deposition, however, was undetected in the marrow. In addition, in the sRBC/rHSA group, hemosiderin deposition was present in the spleen macrophages at 14 days.

DISCUSSION

A main finding of this study is that the reduced Hct level after the 40 percent exchange transfusion with HbV/rHSA returned to the original level after 7 days; furthermore, the accumulated HbVs in RES became undetectable within 14 days. Significant splenomegaly is attributable to the combination of the accumulation of HbV in the red pulp zone and the considerable presence of nests of erythroblasts in the splenic cord in response to the EPO secretion, but these observations subsided within 14 days.

Extensive studies of circulation kinetics and organ distribution of isotope-labeled HbV clarified that HbV accumulates preferentially in the RES.[11,16] One cause of the splenomegaly is the accumulation of HbV particles in the red pulp zone, as shown in Fig. 4 but this subsided completely within 14 days. Gradual increases in the plasma cholesterol levels by 3 days after infusion and lack of disruption of the HbV in the plasma suggest that the cholesterols are liberated from the RES after the HbVs are captured by the RES and destroyed in the phagosomes of the macrophages.[14,15] In our previous studies of topload HbV infusions, significant increases in the high-density lipoprotein cholesterol, β-lipoprotein, and phospholipids were observed as surplus amounts.[15,17] In contrast, we observed no such significant increases after the 40 percent blood exchange, only decreases. A large demand of nutrients should pertain for hematopoiesis and so on; also, the lipid components from HbV might be utilized efficiently for proliferation.

During the metabolism of Hb, we would expect a release of bilirubin and iron. But they did not increase in the plasma within 14 days. The released heme from Hb in HbV might be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages.[15,32] Bilirubin would normally be excreted in the bile as a normal pathway, and no obstruction or stasis of the bile should occur in the biliary tree. Berlin blue staining revealed considerable deposition of hemosiderin in the liver and spleen, even after 14 days. Normally, iron from a heme is stored in the ferritin molecule.[33] Both ferritin and hemosiderin release iron. They are anticipated to induce hydroxyl radical production followed by lipid peroxidation.[34] The iron release rate from hemosiderin, however, is substantially less than that from ferritin.[35] Consequently, the excess amount of iron would then normally be stored in an insoluble and less toxic form as hemosiderin. Hemosiderosis is often observed in patients who have received repeated blood transfusions because of the shorter $t_{1/2}$ of the stored RBCs. Moderate splenomegaly and hemosiderin deposition were also confirmed in the spleen in the sRBS/rHSA groups of this study, partly because of the accumulation and degradation of stored RBCs with the lowered membrane deformability and shortened circulation $t_{1/2}$.[26] These results indicate that the metabolism of heme from HbV and the iron storage is within the physiological capacity that has been well characterized for the metabolism of senescent RBCs.[36]

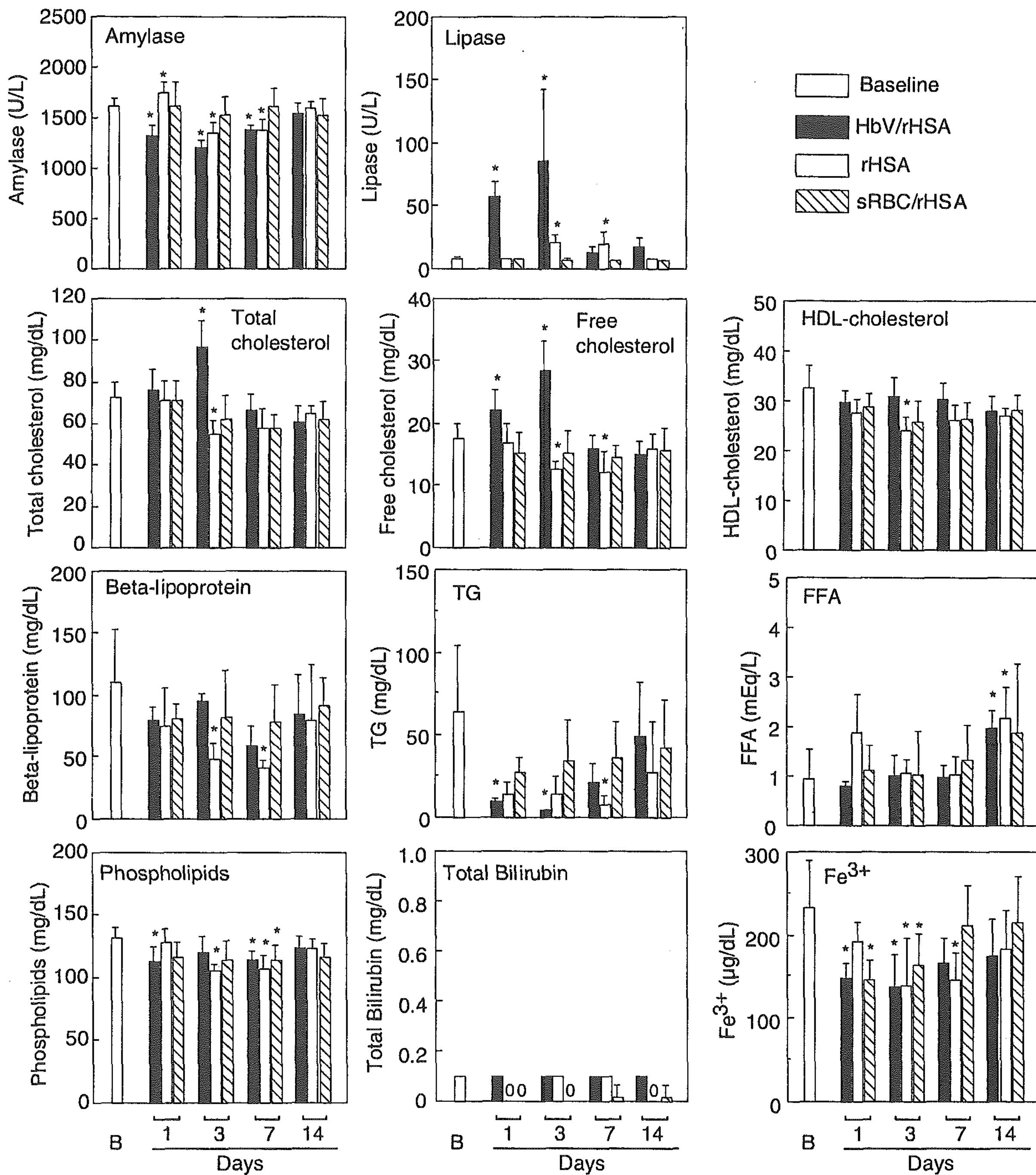

Fig. 3. Plasma biochemical tests representing the metabolism of the components of HbV (lipids and Hb) and pancreatic function after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$). TG = triglyceride; FFA = free fatty acid; B = baseline.

Interestingly, not only the HbV/rHSA and sRBC/rHSA groups, but also the rHSA group showed a significant splenomegaly at 3 days, even though the rHSA group showed no symptoms on Day 1. In rats, extramedullary hematopoiesis induced by hypoxia is localized predominantly in the spleen.[37,38] We observed extensive nests of erythroblasts in the splenic cords, especially at 3 days. It is not plausible that the rHSA as an xenogeneic protein accumulates in the spleen macrophages, according to the fact the ^{125}I-labeled rHSA in a rat showed no specific distribution to the spleen.[39,40] Therefore, the splenomegaly for the rHSA group is attributed to the erythropoiesis stimulated by the significant increase in the plasma EPO level.

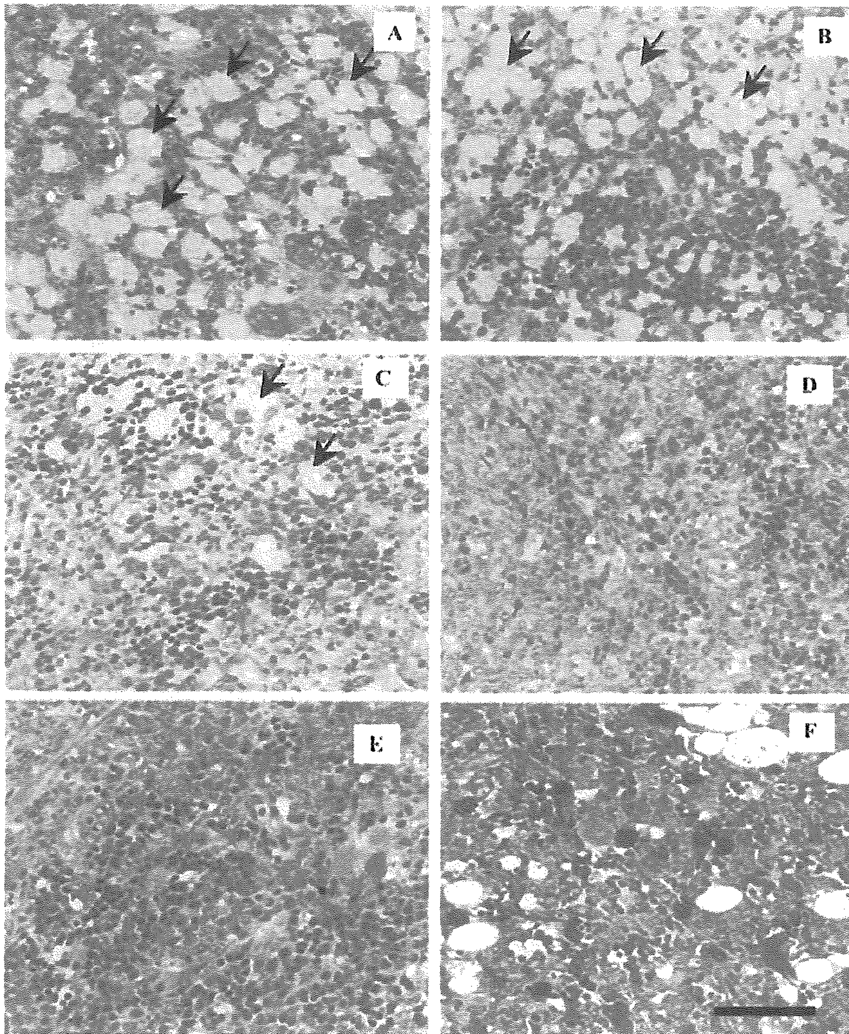

Fig. 4. Histology of rat spleen and marrow after exchange transfusion with HbV/rHSA or rHSA alone. (A-C) Respective images of the spleen of the HbV/rHSA group at 1, 3, and 7 days. Accumulated HbV particles are visible as light-blue areas (black arrows). Nests of erythroblasts are visible as dark blue cells (red arrows). The domain of the HbV particles decreased significantly at 7 days. (D) Spleen of the HbV/rHSA group at 14 days. HbV particles had disappeared, whereas the erythroblast nests remained, as indicated by the red arrows. (E) Spleen of the rHSA group at 3 days. The erythroblast nest formation is remarkable. (F) Marrow of the HbV/rHSA group at 3 days. Hematopoietic activity is visible. Bar = 50 μm (Giemsa method).

Plasma EPO release from the kidney strongly reflects an anemic condition, depending on the O_2-carrying capacity of the circulating blood.[41,42] The highest EPO level was seen in the rHSA group, indicating that its anemic condition was the most severe. Because of the short $t_{1/2}$ and MetHb formation,[43] the HbV/rHSA also showed a significant increase in the EPO level. However, it was considerably lower than that of the rHSA group. The sRBC/rHSA group also showed a moderate increase in the EPO level probably caused by the reduced Hct by the exchange transfusion. Accordingly, the splenomegaly for the HbV/rHSA and sRBC/rHSA groups is also partly attributable to the nests of erythroblasts for erythropoiesis that was sufficient for recovery from the reduced Hct. Interestingly, both HbV/rHSA and rHSA groups tended to show higher Hct values than the sRBC/rHSA group at 7 and 14 days, probably because of the enhanced erythropoiesis caused by the higher levels of EPO excretions than for the sRBC/rHSA group. The MCH, MCV, and MCHC levels were normal overall, supporting our inference of normal erythropoiesis.

Routine plasma biochemical tests showed that the hepatic function was maintained despite the large amount of HbV that were captured and degraded by Kupffer cells. Significant reductions were seen in the amylase activity, whereas a transient increase in lipase activity was observed consistently in our previous topload infusion experiments; this should be due to the up regulation of lipase in response to the infusion of phospholipid vesicles.[15,17,44]

In conclusion, all rats tolerated the 40 percent exchange transfusion with HbV/rHSA and showed complete Hct recovery within 7 days. Although transient splenomegaly and the hemosiderin deposition were confirmed, no excess iron was found in the blood. The recycling or excretion of iron as well as lipid components should be on the physiological pathway that is known for the degradation of senescent RBCs. Although some aspects remain unresolved, the present results offer important information on the safety and handling of HbV during preoperational or perioperational infusion in a clinical setting.

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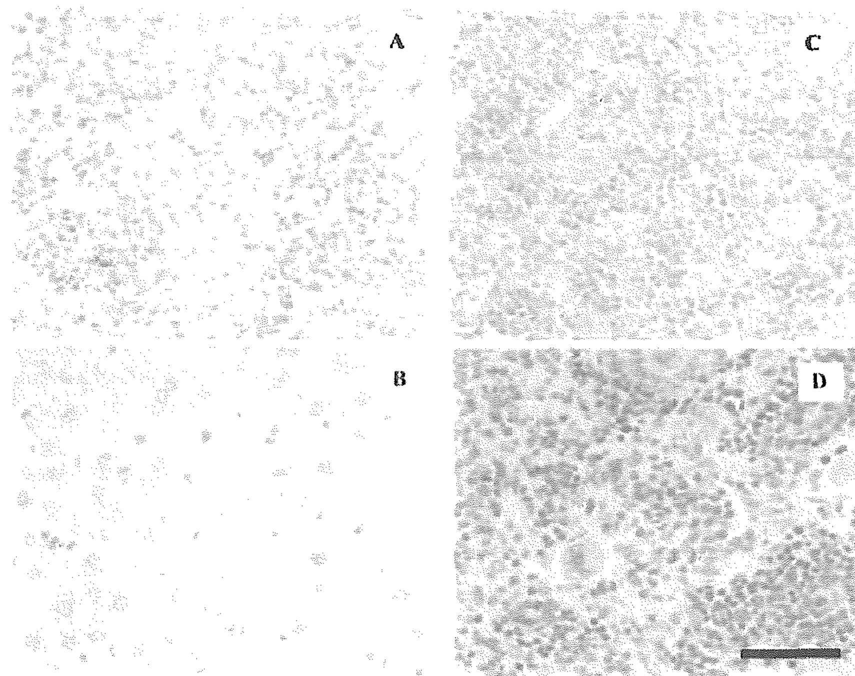

Fig. 5. Histology of rat spleen, liver, and marrow 14 days after exchange transfusion with HbV/rHSA or sRBC/rHSA. Spleen (A), liver (B), and marrow (C) of the HbV/rHSA group. The spleen and liver contained slight hemosiderin deposition, but not the marrow. The spleen of the sRBC/rHSA group (D) also contained slight hemosiderin deposition. Bar = 50 μm (Berlin blue method).

ful discussion of phagocytic and hematopoietic activities. The rHSA was obtained from Nipro Corp.

REFERENCES

1. Chang TM. Hemoglobin based red blood cells substitutes. Artif Organs 2004;28:789-994.
2. Buehler PW, Alayash AI. Toxicities of hemoglobin solutions. in search of in-vitro and in-vivo model systems. Transfusion 2004;44:1516-30.
3. Sakai H, Tomiyama K, Sou K, et al. Polyethyleneglycol-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. Bioconjugate Chem 2000;11:425-32.
4. Lee R, Neya K, Svizzero TA, Vlahakes GJ. Limitations of the efficacy of hemoglobin-based oxygen-carrying solutions. J Appl Physiol 1995;79:236-42.
5. Johnson JL, Moore EE, Offner PJ, et al. Resuscitation with a blood substitute abrogates pathologic postinjury neutrophil cytotoxic function. J Trauma 2001;50:449-56.
6. Standl T, Burmeister MA, Horn EP, et al. Bovine haemoglobin-based oxygen carrier for patients undergoing haemodilution before liver section. Br J Anesth 1998;80:189-94.
7. York GB, DiGeronimo RJ, Wilson BJ, et al. Extracorporeal membrane oxygenation in piglets using a polymerized bovine hemoglobin-based oxygen-carrying solution (HBOC-201). J Pediatr Surg 2002;37:1387-92.
8. Contaldo C, Plock J, Sakai H, et al. Hemodilution with polymerized and encapsulated hemoglobins improves oxidative energy metabolism in collateralized hamster flap tissue. Crit Care Med 2005;33:806-12.
9. Nozue M, Lee I, Manning JM, et al. Oxygenation in tumors by modified hemoglobins. J Surg Oncol 1996;62:109-14.
10. Djordjevich L, Mayoral J, Miller IF, Ivankovich AD. Cardiorespiratory effects of exchanging transfusions with synthetic erythrocytes in rats. Crit Care Med 1987;15:318-23.
11. Awasthi VD, Garcia D, Klipper R, et al. Neutral and anionic liposome-encapsulated hemoglobin: effect of postinserted poly(ethylene glycol)-distearoyl-phosphatidylethanolamine on distribution and circulation kinetics. J Pharmacol Exp Ther 2004;309:241-8.
12. D'Agnillo F, Alayash AI. Redox cycling of diaspirin cross-linked hemoglobin induces G2/M arrest and apoptosis in cultured endothelial cells. Blood 2001;98:3315-23.
13. Sakai H, Hara H, Yuasa M, et al. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension in conscious hamster model. Am J Physiol Heart Circ Physiol 2000;279:H908-H915.
14. Sakai H, Horinouchi H, Tomiyama K, et al. Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in metabolism. Am J Pathol 2001;159:1079-88.
15. Sakai H, Masada Y, Horinouchi H, et al. Physiologic capacity of reticuloendothelial system for degradation of hemoglobin-vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. J Pharmacol Exp Ther 2004;311:874-84.
16. Sou K, Klipper R, Goins B, et al. WT. Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute. J Pharmacol Exp Ther 2005;312:702-9.
17. Sakai H, Horinouchi H, Masada Y, et al. Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. Biomaterials 2004;25:4317-25.
18. Cabrales P, Sakai H, Tsai AG, et al. Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. Am J Physiol Heart Circ Physiol 2005;288:H1885-H1892.
19. Yoshizu A, Izumi Y, Park S, et al. Hemorrhagic shock resuscitation with an artificial oxygen carrier, hemoglobin

vesicle, maintains intestinal perfusion and suppresses the increase in plasma tumor necrosis factor-alpha. ASAIO J 2004;50:458-63.

20. Sakai H, Takeoka S, Park SI, et al. Surface-modification of hemoglobin vesicles with polyethyleneglycol and effects on aggregation, viscosity, and blood flow during 90%-exchange transfusion in anesthetized rats. Bioconjugate Chem 1997; 8:15-22.
21. Sakai H, Masada Y, Horinouchi H, et al. Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. Crit Care Med 2004;32:539-45.
22. Landaw SA. Factors that accelerate or retard red blood cell senescence. Blood Cells 1988;14:47-59.
23. Sou K, Endo T, Naito Y, et al. Efficient up-scale production of hemoglobin-vesicles (HbV) using the freeze-thawing and rapid extrusion. Biotechnol Progr 2003;19:1547-52.
24. Sakai H, Yuasa M, Onuma H, et al. Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. Bioconjugate Chem 2000;11: 56-64.
25. Sakai H, Hisamoto S, Fukutomi I, et al. Detection of lipopolysaccharide in hemoglobin-vesicles by limulus amebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment with a surfactant. J Pharm Sci 2004;93:310-21.
26. d'Almeida MS, Jagger J, Duggan M, et al. A comparison of biochemical and functional alterations of rat and human erythrocytes stored in CPDA-1 for 29 days: implications for animal models of transfusion. Transfus Med 2000;10:291-303.
27. Izumi Y, Sakai H, Hamada K, et al. Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical tissue oxygen tension. Crit Care Med 1996;24:1869-73.
28. Sakai H, Tsai AG, Kerger H, et al. Subcutaneous micro-vascular responses to hemodilution with a red cell substitute consisting of polyethyleneglycol-modified vesicles encapsulating hemoglobin. J Biomed Mater Res 1998;40: 66-78.
29. Sakai H, Tomiyama K, Masada Y, et al. Pretreatment of serum containing Hb-vesicles (oxygen carriers) to avoid interference in clinical laboratory tests. Clin Chem Lab Med 2003;41:222-31.
30. Wen D, Boissel JP, Tracy TE, et al. Erythropoietin structure-function relationships: high degree of sequence homology among mammals. Blood 1993;82:1507-16.
31. Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council-National Academy of Sciences. Guide for the care and use of laboratory animals. Washington (DC): National Academy Press; 1996.
32. Braggins PE, Trakshel GM, Kutty RK, Maines MD. Characterization of two heme oxygenase isoforms in rat spleen: comparison with the hematin-induced and constitutive isoforms of the liver. Biochem Biophys Res Commun 1986;141:528-33.
33. Finch CA, Huebers H. Perspectives in iron metabolism. N Engl J Med 1982;306:1520-8.
34. Grady JK, Chen Y, Chasteen ND, Harris DC. Hydroxyl radical production during oxidative deposition of iron in ferritin. J Biol Chem 1989;264:20224-9.
35. O'Connell MJ, Ward RJ, Baum H, Peters TJ. Iron release from haemosiderin and ferritin by therapeutic and physiological chelators. Biochem J 1989;260:903-7.
36. Bennett GD, Kay MM. Homeostatic removal of senescent murine erythrocytes by splenic macrophages. Exp Hematol 1981;9:297-307.
37. Ou LC, Kim D, Layton WM Jr, Smith RP. Splenic erythropoiesis in polycythemic response of the rat to high-altitude exposure. J Appl Physiol 1980;48:857-61.
38. Stutte HJ, Sakuma T, Falk S, Schneider M. Splenic erythropoiesis in rats under hypoxic and post-hypoxic conditions. Virchows Arch A Pathol Anat Histopathol 1986;409:251-61.
39. Okano K, Sogame Y, Ohkubo M, et al. Metabolic fate of recombinant human serum albumin (rHSA) (2). Jpn Pharmacol Ther (Yakuri tou Chiryou) 1997;25(Suppl): 2007-18.
40. Baynes JW, Thorpe S. Identification of the sites of albumin catabolism in the rat. Arch Biochem Biophys 1981;206:372-9.
41. Eckardt KU, Koury ST, Tan CC, et al. Distribution of erythropoietin producing cells in rat kidneys during hypoxic hypoxia. Kidney Int 1993;43:815-23.
42. Hughes GS Jr, DeSmith VL, Locker PK, Francom SF. Phlebotomy of 500 or 750 milliliters of whole blood followed by isovolemic hemodilution or autologous transfusion yields similar hemodynamic, hematologic, and biochemical effects. J Lab Clin Med 1994;123:290-8.
43. Teramura Y, Kanazawa H, Sakai H, et al. Prolonged oxygen-carrying ability of hemoglobin vesicles by coencapsulation of catalase in vivo. Bioconjugate Chem 2003;14:1171-6.
44. Stuecklin-Utsch A, Hasan C, Bode U, Fleischhack G. Pancreatic toxicity after liposomal amphotericin B. Mycoses 2002;45:170-3.

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The sealing effect of fibrin glue against alveolar air leakage evaluated up to 48 h; comparison between different methods of application

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Abstract

Objective: There is little experimental evidence to show how much positive airway pressure fibrin sealants can actually withstand, and in particular, how this effect changes over time. In the present study, we experimentally evaluated the sealing effect of fibrin glue against alveolar air leakage up to 48 h after application. **Methods:** Beagles were used ($n=48$). Under thoracotomy, approximately 5×10 mm defects (2 mm depth) were made on the lung surface. Fibrin glue sealants were applied to this defect in three ways. In rubbing and spray method, fibrinogen was rubbed, followed by spraying of both fibrinogen and thrombin solutions. In double layer method, fibrinogen was dripped, followed by thrombin. Collagen fleece, coated with fibrinogen and thrombin (TachoComb) was also tested. The minimum positive airway pressure which produced air leakage was measured for each sealed defect (seal breaking pressure, cmH_2O) at 0, 3, 6, 12, 24, and 48 h after application ($n=6$ at each time point). **Results:** The seal-breaking pressure increased over time in all of the application methods. At 6 h, differences between methods were not significant but three defects in RS reached 70 cmH_2O, the maximum pressure tested, compared with none in other two methods. At 12 h, the seal-breaking pressure was significantly higher in RS compared with the other two methods (rubbing and spray method vs TachoComb; 66 ± 3 vs 47 ± 17, $P=0.047$, rubbing and spray method vs double layer method; 66 ± 3 vs 42 ± 18, $P=0.024$). Beyond 24 h, sealing pressure reached close to 70 cmH_2O in all the methods. **Conclusions:** The results show that the sealing effect of fibrin glue is relatively unstable up to 12 h after its application. Rubbing and spray method may help the fibrin seal to reach its full strength faster compared with the other two methods.
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Keywords: Fibrin glue; Air leakage; Pulmonary resection; Sealing effect

1. Introduction

Alveolar air leakage is a very common complication in lung surgery. Along with inadequate control of postoperative pain, persistence of air leakage was identified as the most common cause of delay in hospital discharge after thoracic surgery [1]. Many tissue sealants are being applied to prevent air leakage after surgery [2-8]. Among them, fibrin glue is a popular sealant with a variety of application methods [9,10]. However, there are also reports that indicate that the use of fibrin glue does not reduce the duration of chest-tube drainage or hospital stay [11-13]. This implies that, air leakage often restarts shortly after surgery despite the application of fibrin glue.

Intraoperatively, we test the efficacy of fibrin glue by applying positive airway pressure. But we usually do not apply pressure beyond 20-25 cmH_2O, since it defeats the purpose to break the seal at this point. Clinically, it is not rare that air leakage becomes apparent, for example through the chest tube, shortly after surgery. While this may be air leakage from lesions that were missed during surgery, it is also true that airway pressure often spikes beyond the pressure tested, 25 cmH_2O, as the patient recovers spontaneous breathing. The fibrin seal may be broken at this point. To our knowledge, there is little experimental evidence to show how much positive airway pressure fibrin sealants can actually withstand, and in particular, how this effect changes over time. In the present study, we experimentally evaluated the sealing effect of fibrin glue against alveolar air leakage up to 48 h after application. We also compared three different methods of application.

2. Materials and methods

2.1. Animals

Adult male beagles, 10-12 months of age, weighing 8-11 kg were used for this study (Toyota Trading Co., Kumamoto, Japan) ($n=36$). Animals were housed individually and provided

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food and water ad libitum. All animal studies were approved by the School of Medicine, Keio University Institutional Animal Care and Use Committee. All animals received humane care in accordance with the Japanese Government Animal Protection and management law

2.2. Fibrin glue application

The fibrin glue used in this study was Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). We also compared, fibrinogen-based collagen fleece, TachoComb (ZLB Behring Co., USA). The mechanism of fibrin glue formation is well described [14]. The fibrin glue product consists of two components. Solution A is a protein concentrate consisting of fibrinogen, plasma fibronectin, factor XIII, and plasminogen, reconstituted in aprotinin solution. Solution B is thrombin reconstituted in calcium chloride solution. TachoComb is a collagen fleece coated with dry fibrinogen and thrombin on one side.

We applied fibrin glue in two different ways, rubbing and spray method and double layer method. In rubbing and spray method, solution A was dripped and gently rubbed onto the air leakage area. Then both solutions were sprayed simultaneously onto the rubbed surface as a mixed aerosol using Bolheal Spray Set (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). In double-layer method, solution A was dripped onto the air leakage surface after which solution B was dripped. To apply TachoComb, the fibrinogen-coated side was first soaked in saline, and then was attached to the air leakage surface. The sheet was gently pressed with dry gauze for about 5 min so that the collagen fleece was attached to the lung surface with fibrin glue.

2.3. Experiment

The dogs were anesthetized with an intravenous injection of pentobarbital sodium (25-30 mg/kg). The dogs were placed in left decubitus position, intubated, and mechanically ventilated. The right chest wall was shaved, and disinfected. Through a thoracotomy, defects were created on the right lung surface using scalpels, one on each of the three lobes (anterior, middle, and posterior). The defect was created with the lung fully inflated at a positive airway pressure of approximately 20 cmH_2O. The defect size was adjusted to be approximately 5×10 mm, and approximately 2 mm in depth. Hemostasis was obtained when necessary with the minimum use of electrocautery. In each animal, each of the three defects was sealed with one of three methods, rubbing and spray method, double-layer method, or TachoComb. Randomization was performed to allot these three methods to each lobe equally. The chest was closed, and the animals were allowed to recover, except for time 0. Xylazine (2 mg/kg) was administered as needed as analgesics. The minimum positive airway pressure which produced air leakage was measured for each sealed defect (seal-breaking pressure) at 0, 3, 6, 12, 24, and 48 h after the application of the fibrin sealant ($n=6$ at each time point). Except for time 0, thoracotomy was performed again under anesthesia. Air leakage pressure for each defect was evaluated separately by clamping the remaining two lobar bronchi with forceps. The maximum positive airway pressure applied was 70 cmH_2O, since higher pressure induced air leakage from uninjured lung around the hilum. After the completion of seal-breaking pressure measurement at each time point, each animal was sacrificed with intracardiac injections of pentobarbital (1000 mg/body).

2.4. Histological examinations

A separate group of animals was used to obtain histological specimens because the fibrin seal is broken by the seal breaking pressure measurements. Two specimens for each method and time points were prepared ($n=12$). The animals were sacrificed and the whole right lung was fixed in 10% neutral formaline. After fixation, each defect site was resected, embedded in paraffin, and processed in 3 μm sections for hematoxylin-eosin staining. Specimens were analyzed at Clinicopathological Division of Keio University Hospital in a blinded manner by M.M.

2.5. Statistical analyses

The results are presented as the mean $\pm$SD. Seal-breaking pressure per time point was compared between different methods using unpaired T-test. Differences within each method were tested using paired T-test. Significance was assumed at $P<0.05$.

3. Results

3.1. Seal breaking pressure measurements

The seal-breaking pressure increased over time in all the application methods (Fig. 1). At 0 h, seal-breaking pressure was significantly higher in rubbing and spray method compared with TachoComb (54 ± 5 vs 36 ± 6, $P<0.001$), and in TachoComb significantly higher compared with double layer method (36 ± 6 vs 27 ± 3, $P=0.007$). Seal breaking

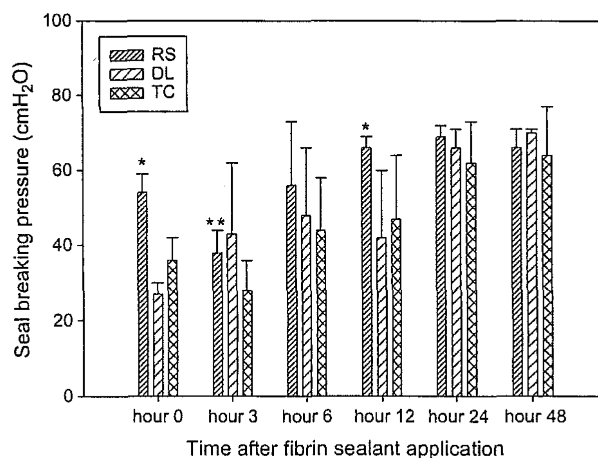

Fig. 1. The time interval changes in seal-breaking pressure after application of the fibrin sealants is shown. RS, rubbing and spraying method; DL, double layer method, TC, TacoComb. *$P<0.05$ vs other two groups, **$P<0.05$ vs RS at 0 h.

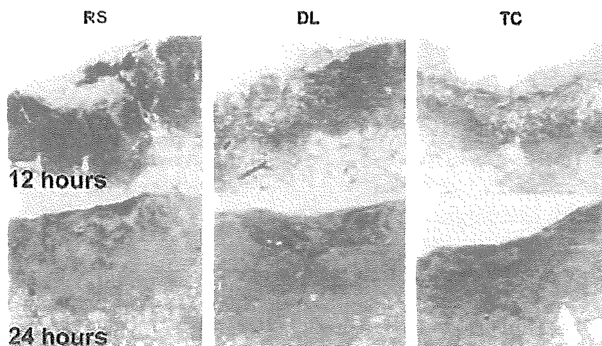

Fig. 2. Hematoxylin-eosin staining of the injured lung sealed by different fibrin sealants. At 12 h, deeper penetration of fibrin into the injured lung parenchyma was seen in RS compared with the other two methods. This difference was not apparent between application methods beyond 24 h. Upper and lower panels correspond, respectively, to 12, and 24 h after sealant application RS, rubbing and spraying method; DL, double layer method; TC, TacoComb, magnification 2×.

pressure in rubbing and spray method declined significantly from 0 to 3 h (from 54 ± 5 to 38 ± 6, $P<0.001$). At 3 h, seal-breaking pressure in double layer method tended to increase, and in TachoComb tended to decline compared with 0 h, but these changes were not significant. At 6 h, differences between methods were not significant but three defects in rubbing and spray method reached 70 cmH_2O, the maximum pressure tested, compared with none in other two methods. At 12 h, the seal-breaking pressure was significantly higher in rubbing and spray method compared with the other two methods (rubbing and spray method vs TachoComb; 66 ± 3 vs 47 ± 17, $P=0.047$, rubbing and spray method vs double layer method; 66 ± 3 vs 42 ± 18, $P=0.024$). Beyond 24 h, sealing pressure reached close to 70 cmH_2O in all the methods, with no significant differences between methods.

3.2. Histological examinations

The layer of fibrin covering the lung surface could be observed in all three methods by hematoxylin-eosin staining (Fig. 2). Up to 12 h, deeper penetration of fibrin into the injured lung parenchyma was seen in rubbing and spray method compared with the other two methods. This difference was not apparent between application methods beyond 24 h. Also, at 3 h, hemorrhage was more evident underneath the fibrin layer in rubbing and spray method compared with other two methods.

4. Discussion

Fibrin glue is derived from human, or in some products like bovine plasma, and hence, carry the same risks as blood transfusion. We have reported the possibility of viral transmission by clinical use of fibrin glue [16]. Despite these potential drawbacks, fibrin glue is widely used in order to reduce postoperative alveolar air leakage, but questions remain regarding its clinical efficacy [5,12,13,15]. The results of this study show that the sealing effect of fibrin glue is relatively unstable up to 12 h after its application. Clinically, this result suggests that coughing or positive pressure ventilation should be kept to a minimum for 12 h in order to fully exploit the sealing effect of fibrin glue.

The sealing effect of fibrin glue is affected by the concentration of fibrin, and how well it attaches to tissue. The concentration of fibrin depends primarily on how well the thrombin and fibrinogen solutions are mixed on application. The attachment of fibrin may be affected at least in part by its penetration into tissue. Rubbing and spray method is a method that we have recently devised. Our intention was to improve tissue penetration by rubbing fibrinogen into the lung parenchyma. We also utilized the effective mixing of the two solutions by aerosol to form a more even layer of fibrin in continuity with the penetrated fibrinogen, which is converted to fibrin by the spray. The present study suggests that rubbing and spray method may help the fibrin seal to reach its full strength faster compared with the other two methods. Histological findings, at least in part suggest that this may be due to the initial deeper penetration of fibrin into the lung parenchyma. We speculate that because of this, the attached surface area of fibrin was initially greater in rubbing and spray method compared with the other two methods. Presumably, this difference became insignificant with the formation of tissue-derived fibrin. We evaluated TachoComb and double layer method as the most widely used methods. Double layer method is the application method recommended by most manufacturers, and is therefore, presumably most often used. It is encouraging that both these methods provided satisfactory sealing effect beyond 24 h. Control experiments, in which no sealant was used, was not performed due to ethical reasons. In our preliminary studies, the alveolar leakage created in this experiment did not stop spontaneously, and respiratory distress was unavoidable even with the use of chest tubes. Regarding rubbing and spray method, there was haemorrhage underneath the fibrin layer at 3 h, which resolved at 6 h. Presumably this was caused by rubbing. This may in part explain the significant decrease in seal-breaking pressure in rubbing and spray method at 3 h. A less invasive way to infiltrate the fibrinogen solution, for instance the use of a soft sponge, is currently being studied.

References

[1] Wright CD, Wain JC, Grillo HC, Moncure AC, Macaluso SM, Mathisen DJ. Pulmonary lobectomy patients care pathway: a model to control cost and maintain quality. Ann Thorac Surg 1997;64:299-302.

[2] Kanno S, Yamazaki H, Kashiwabara S, Uchiyama H, Maekawa Y, Ito G, Muto T, Kariya K, Kojima T, Koshiyama Y, Oda M, Kurumi M. Adhesive and sealing effects of TO-193 on tissues and organs in various experimental models. Folia Pharmacol Jpn 1999;113:269-76.

[3] Otani Y, Tabata Y, Ikada Y. Sealing effect of rapidly curable gelatin-poly (L-glutamic acid) hydrogel glue on lung air leak. Ann Thorac Surg 1999; 67:922-6.

[4] Tsuda T, Nakamura T, Yamamoto Y, Teramachi M, Kiyotani T, Lee YH, Shimizu Y. Prevention of postoperative air leakage from lungs using a purified human collagen membrane—polyglycolic acid sheet. Ann Thorac Surg 1999;68:339-42.

[5] Porte HL, Jany T, Conti M, Gillet PA, Guidat A, Wurtz AJ. Randomized controlled trial of a synthetic sealant for preventing alveolar air leaks after lobectomy. Ann Thorac Surg 2001;71:1618-22.

[6] Ranger WR, Halpin D, Sawhney AS, Lyman M, Locicero J. Pneumostasis of experimental air leaks with a new photopolymerized synthetic tissue sealant. Am Surg 1997;63:788-95.
[7] Macchiarini P, Wain J, Almy S, Dartevelle P. Experimental and clinical evaluation of a new synthetic, absorbable sealant to reduce air leaks in thoracic operations. J Thorac Cardiovasc Surg 1999;117:751-8.
[8] Herget GW, Kassa M, Riede UN, Lu Y, Brethner L, Hasse J. Experimental use of an albumin-glutaraldehyde tissue adhesive for sealing pulmonary parenchyma and bronchial anastomoses. Eur J Cardiothoracic Surg 2001; 19:4-9.
[9] Yuasa K, Shimizu T, Matsubara J, Toyoda T. Sealing effect of fibrin adhesive by various method on protection of air leakage in lung surgery. Kyobu Geka 1998;51:1001-5.
[10] Morikawa T, Katoh H. Improved techniques of applying fibrin glue in lung surgery. Eur Surg Res 1999;31:180-6.
[11] Fleisher AG, Evans KG, Nelems B, Finley RJ. Effect of routine fibrin glue use on the duration of air leaks after lobectomy. Ann Thorac Surg 1990; 49:133-4.
[12] Wong K, Goldstraw P. Effects of fibrin glue in the reduction of postthoracotomy alveolar air leak. Ann Thorac Surg 1997;64: 979-81.
[13] Wain JC, Kaiser LR, Johnstone DW, Yang SC, Wright CD, Freidberg JS, Feins RH, Heitmiller RF, Mathisen DJ, selwyn MR. Trial of a novel synthetic sealant in preventing air leaks after lung resection. Ann Thorac Surg 2001;71:1623-8.
[14] Sierre D. Fibrin sealant adhesive systems: a review of their chemistry, material properties and clinical applications. J Biomater Appl 1993;7: 309-51.
[15] Fleisher AG, Evans KG, Nelems B, Finley RJ. Effect of routine fibrin glue use on the duration of alveolar air leaks after lobectomy. Ann Thorac Surg 1990;49:133-4.
[16] Kawamura M, Sawafuji M, Watanabe M, Horinouchi H, Kobayashi K. Frequency of transmission of human parvovirus B19 infection by fibrin sealant used during thoracic surgery. Ann Thorac Surg 2002;73: 1098-100.

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Role of Rho-kinase in reexpansion pulmonary edema in rabbits

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Sawafuji, Makoto, Akitoshi Ishizaka, Mitsutomo Kohno, Hidefumi Koh, Sadatomo Tasaka, Yoshiki Ishii, and Koichi Kobayashi. Role of Rho-kinase in reexpansion pulmonary edema in rabbits. *Am J Physiol Lung Cell Mol Physiol* 289: L946–L953, 2005. First published July 8, 2005; doi:10.1152/ajplung.00188.2004.—Reexpansion of a collapsed lung increases the microvascular permeability and causes reexpansion pulmonary edema. Neutrophils and their products have been implicated in the development of this phenomenon. The small GTP-binding proteins Rho and its target Rho-kinase (ROCK) regulate endothelial permeability, although their roles in reexpansion pulmonary edema remain unclear. We studied the contribution of ROCK to pulmonary endothelial and epithelial permeability in a rabbit model of this disorder. Endothelial and epithelial permeability was assessed by measuring the tissue-to-plasma (T/P) and bronchoalveolar lavage (BAL) fluid-to-plasma (B/P) ratios with ^{125}I-labeled albumin. After intratracheal instillation of ^{125}I-albumin, epithelial permeability was also assessed from the plasma leak (PL) index, the ratio of ^{125}I-albumin in plasma/total amount of instilled ^{125}I-albumin. T/P, B/P, and PL index were significantly increased in the reexpanded lung. These increases were attenuated by pretreatment with Y-27632, a specific ROCK inhibitor. However, neutrophil influx, neutrophil elastase activity, and malondialdehyde concentrations in BAL fluid collected from the reexpanded lung were not changed by Y-27632. In endothelial monolayers, Y-27632 significantly attenuated the H_2O_2-induced increase in permeability and mitigated the morphological changes in the actin microfilament cytoskeleton of endothelial cells. These in vivo and in vitro observations suggest that the Rho/ROCK pathway contributes to the increase in alveolar barrier permeability associated with reexpansion pulmonary edema.

acute lung injury; acute respiratory distress syndrome; reexpansion pulmonary edema; Rho; Rho-kinase

REEXPANSION OF A COLLAPSED lung occasionally causes acute lung injury (ALI) known as reexpansion pulmonary edema (3, 17, 18). The main characteristic of this disorder is an increase in pulmonary microvascular permeability (24, 40). Vascular endothelial permeability is related to the cellular cytoskeleton. An increase in vascular permeability is accompanied by a reorganization of the actin-based cytoskeleton and contraction of endothelial cells, resulting in the formation of an intercellular gap (7, 28). A calmodulin-dependent myosin light chain kinase (MLCK) contributes to the regulation of cell contraction and endothelial cell permeability. Activation of MLCK causes the phosphorylation of the myosin light chains, resulting in the contraction of actomyosin, in the formation of stress fibers and in subsequent hyperpermeability of endothelial cells (8). A signaling pathway of the small GTPase Rho and its target protein, Rho-kinase (Rho-associated coiled-coil-forming protein kinase; ROCK), also regulates the contraction of endothelial cells (5). The activation of Rho leads to the phosphorylation of the myosin binding subunit of myosin light chain phosphatase via the action of ROCK, inactivating the myosin light chain phosphatase and blocking the dephosphorylation of myosin light chains (16). Continued phosphorylation of myosin light chains results in sustained cell contraction.

In studies in isolated rat lungs, a MLCK inhibitor attenuated the injury associated with ischemia-reperfusion (15) and that induced by ventilators (23). These observations suggest that the intracellular signal transduction that regulates the cellular cytoskeleton is involved in the pathophysiology of some forms of ALI. However, the role of the Rho/ROCK-mediated pathway in reexpansion pulmonary edema in vivo remains unclear, although more attention has been paid to the role of alveolar epithelial injury in the pathophysiology of ALI (38). The Rho/ROCK-mediated pathway has been implicated in the organization of perijunctional actin and in the regulation of permeability of the tight junction of the epithelial cells (6, 22). Therefore, we hypothesized that the Rho/ROCK-mediated pathway is involved in the changes in alveolar endothelial and epithelial barrier permeability in reexpansion pulmonary edema.

Y-27632, a highly selective inhibitor of ROCK in vitro and in vivo (36), was found useful to examine the role of the Rho/ROCK-mediated pathway in vivo (11, 29). To test our hypothesis, we examined the inhibitory effect of Y-27632 in our rabbit model of reexpansion lung injury (20). We measured the flux of transvascular albumin as an indicator of endothelial permeability (10). Alveolar epithelial permeability was assessed by measuring the bidirectional flux of albumin across the alveolar epithelial barrier (10, 30, 39). We also performed in vitro experiments in human pulmonary artery endothelial cell monolayers to study the effects of Y-27632 on hyperpermeability and endothelial cytoskeletal rearrangement induced by H_2O_2. These experiments were performed since reactive oxygen species appear to be involved in the injury to pulmonary endothelial cells observed in reexpansion pulmonary edema (12). We observed morphological changes in the microfilament cytoskeleton using a rhodamine-phalloidin stain (25). The main objective of this study was to ascertain the role of the Rho/ROCK pathway in the increased alveolar endothelial and epithelial barrier permeability present in reexpanded lungs.

METHODS

The experiments were performed in 45 male Japanese White rabbits, weighing between 900 and 1,800 g. All procedures were

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reviewed and approved by the Laboratory Animal Care Panel of Keio University. The animals were divided into the following three groups: *1*) a reexpansion group (RE), *2*) a Rho-kinase inhibitor pretreatment group (RE + Y), which received 10 mg/kg Y-27632 intraperitoneally before reexpansion of the left lung, and *3*) a sham-operated group (sham) that underwent thoracotomy only. The surgical techniques have been previously reported in detail (20). Briefly, general anesthesia was induced with intramuscular ketamine (75 mg/kg) and xylazine (5 mg/kg). A tracheostomy was performed, and a 3.5-mm-diameter Portex LTD tracheal cannula (Hythe, Kent, UK) was inserted. The rabbits were mechanically ventilated (SN-480–7; Shinano, Tokyo, Japan) with room air at 30 breaths/min and a tidal volume of 10 ml/kg.

In the RE and RE + Y groups, after completion of the left posterolateral thoracotomy, the left main bronchus was clamped with an atraumatic vascular clip (Vascu-statt; Scanlan International, St. Paul, MN) to produce complete collapse of the left lung by absorption of the alveolar gas in the pulmonary circulation. A 4–0 nylon string attached to the clip was exteriorized for later retraction. In the sham group, a left posterolateral thoracotomy was performed, and the left main bronchus was dissected from the surrounding tissue. The chest wall was closed. The rabbits were allowed to recover for 36 h before the experiments began. The tracheal cannula was removed after the recovery of spontaneous breathing.

Experimental Protocol

Experiment 1. Twenty-one animals were divided into three groups listed above to measure endothelial and epithelial permeability. The protocol for *experiment 1* is provided in Fig. 1. Endothelial permeability was assessed from the transvascular flux of ^{125}I-labeled albumin administered intravenously. Epithelial permeability was measured as the flux of ^{125}I-albumin from intravascular space to airspace. To correct blood contamination in the lung tissue or bronchoalveolar lavage (BAL) fluid samples, ^{131}I-albumin was injected intravenously. On the day of experiments, the rabbits were sedated with intramuscular ketamine (100 mg/kg) 30 min before the experiment and placed in the supine position, and a 24-gauge catheter was inserted in an ear vein. ^{125}I-labeled BSA (2 ml of 37 kBq/ml; Life Science Products, Boston, MA) was injected intravenously to assess the pulmonary extravasated albumin in all experimental groups. In the RE and RE + Y groups, 10 min after ^{125}I-albumin injection, the left main bronchus was reopened by retracting the string. At this time, a 9-Fr drainage chest tube (Sumitomo Bakelite, Tokyo, Japan) was inserted in the left pleural cavity, and continuous suction at −10 cmH_2O was applied through a chest tube to reinflate the collapsed lung. In the RE + Y group, 10 mg/kg Y-27632 were administered in the peritoneal cavity 30 min before reopening the left main bronchus. An identical volume of saline was injected intraperitoneally in the sham and RE groups. Room air breathing resumed spontaneously. To prevent hypotension and dehydration, 50 ml/kg normal saline were infused intravenously throughout the period of continuous suction. ^{131}I-labeled human serum albumin (2 ml of 37 kBq/ml; Daiichi Radioisotope Laboratories, Tokyo, Japan) was injected intravenously 115 min after reopening the left main bronchus. We used ^{131}I-albumin as a plasma volume marker to correct for residual blood content in excised lung tissue samples. Heparin (2,000 IU iv) was injected simultaneously. The animals were killed 120 min after reopening the left main bronchus by injection of 100 mg pentobarbital intravenously. The chest was immediately opened, 5 ml blood were collected by cardiac puncture, each pulmonary hilus was clamped at the end of expiration, and the lungs were removed and divided into superior and inferior lobes. Blood and superior lobe lung samples were placed in a preweighed glass tube for measurements of weight and radioactivity.

Total blood cells were counted with a Sysmex K-1000 counter adapted for rabbit cells (Sysmex, Kobe, Japan). Differential counts of 200 cells were performed on glass slide smears stained by a modified Wright's stain (Diff-Quick; American Scientific, McGraw Park, IL).

BAL was performed with 10 ml normal saline through a catheter secured within the bronchus of the excised inferior lobe. The volume of fluid recovered from the left lung of the sham, RE, and RE + Y groups were 7.9 ± 0.3, 8.1 ± 0.4, and 8.0 ± 0.5 ml, respectively. The corresponding volumes recovered from the right lung were 7.9 ± 0.4, 8.3 ± 0.3, and 8.3 ± 0.5 ml, respectively. There was no significant difference in the recovery of BAL fluid among the groups. The fluid was centrifuged at 400 *g* and 4°C for 10 min. The supernatant was used for gamma counting of ^{125}I and ^{131}I to measure the pulmonary epithelial permeability, and for the analysis of neutrophil elastase activity and malondialdehyde (MDA) concentration. The cell pellet was resuspended in 1 ml saline, and the cells were counted by a modified hemocytometer method (Unopet Microcollection System; Becton-Dickson, Rutherford, NJ). Cell differential in BAL was counted in smears obtained by cytocentrifugation. Differential counts were made on 200 cells from smears stained with a modified Wright's stain.

In nine additional animals, divided into the three experimental groups and treated similarly, the mean arterial pressure was measured with a polygraph (Nihon Koden, Tokyo, Japan) via a catheter inserted in the carotid artery.

Experiment 2. Twelve animals were divided among the three experimental groups described earlier to measure the epithelial permeability. Epithelial permeability was measured as the flux from the airspace to intravascular space of ^{125}I-albumin instilled in the left lung. On the day of experiments, 5 ml blood were collected from a catheter inserted in the right carotid artery, heparinized, and centrifuged to obtain plasma. The blood was replaced with an equal volume of Ringer lactate. After the onset of continuous suction (5 min), the rabbits were placed in the left lateral decubitus position to facilitate the collection of liquid in the left lung. Autologous plasma (2 ml/kg) containing 3 mg Evan's blue dye and 212 kBq ^{125}I-labeled BSA were instilled in the left inferior lobe over 5 min with an 18-gauge tube advanced through the tracheostomy, as previously described (30, 39)

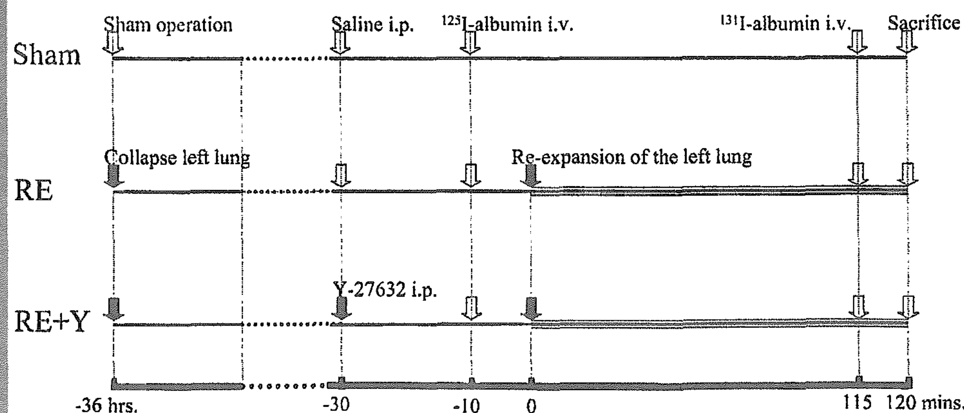

Fig. 1. Protocol for *experiment 1*. The experimental groups consist of the following: sham-operated group (Sham), reexpansion group (RE), and Rho-kinase inhibitor pretreatment group (RE + Y). Sham group, only thoracotomy was performed. RE group, left main bronchus was declamped after 36 h of clamping, and the collapsed lung was reexpanded for 2 h. RE + Y group, 10 mg/kg specific Rho-kinase inhibitor (Y-27632) was administered in the peritoneal cavity 30 min before reopening the left main bronchus. ^{125}I-albumin was injected iv 10 min before reopening the left main bronchus to assess transvascular permeability. ^{131}I-albumin was injected iv 5 min before death to correct blood contamination.

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We confirmed that most of the dye was distributed in the left lung after death of the animal. Blood samples were obtained for measurements of ^{125}I-albumin activity at baseline and 10, 30, 60, 90, and 120 min after instillation. Because, clinically, the onset of reexpansion pulmonary edema takes place immediately or within a few hours (17), we chose these time points of observation to examine the early changes in flux of albumin from lung airspace to intravascular space.

Pulmonary Endothelial Permeability

Pulmonary endothelial permeability was calculated as the ratio of ^{125}I-albumin per unit weight of lung tissue extravascular space over that in plasma (T/P ratio) in the left and right superior lobes. The activities of ^{125}I and ^{131}I in superior lobe parenchyma and blood were counted with a gamma counter (ARC-300; Aloca, Tokyo, Japan), with appropriate corrections for crossover between radionuclides. The extravascular ^{125}I-albumin in the superior lobe was calculated by subtracting the amount of intravascular ^{125}I-albumin from the total ^{125}I-albumin in the lung tissue sample. The intravascular ^{125}I-albumin in the superior lobe was corrected by calculating the ratio of ^{125}I to ^{131}I counts in the unit weight of blood and ^{131}I-albumin in the unit weight of superior lobe parenchyma. We assumed that all of the ^{131}I-albumin was confined to the circulation, since the effect of leakage of the plasma volume marker in the extravascular space is negligible when calculating T/P (13). Therefore, the ^{131}I counts must reflect the residual blood in the lung tissue sample. The following formula was used to calculate the T/P ratio (10)

$$(^{125}\text{I tissue} - {}^{125}\text{I blood} \times {}^{131}\text{I tissue}/{}^{131}\text{I blood})/[{}^{125}\text{I blood}/(1 - \text{Hct})] \quad (1)$$

where ^{125}I tissue is ^{125}I cpm from a unit weight of superior lobe lung tissue, ^{125}I blood is ^{125}I cpm from a unit weight of blood, ^{131}I tissue is ^{131}I cpm from a unit weight of superior lobe lung tissue, ^{131}I blood is ^{131}I cpm from a unit weight of blood, and Hct is hematocrit.

Pulmonary Epithelial Permeability

Pulmonary epithelial permeability was ascertained by two separate methods used to measure the bidirectional flux of albumin across the alveolar epithelial barrier. *1*) In *experiment 1*, the flux of albumin from intravascular space to lung airspace was assessed by measuring the BAL fluid-to-plasma ^{125}I-albumin concentration ratio (B/P ratio). We hypothesized that, if epithelial permeability was increased, the amount of ^{125}I-albumin entering the airspace from the circulation would increase, thus increasing the amount of ^{125}I-albumin collected in BAL fluid. Blood contamination in BAL fluid was also corrected by ^{131}I activity of BAL fluid (10). The following formula was used to calculate the B/P ratio (10)

$$(^{125}\text{I BAL} - {}^{125}\text{I blood} \times {}^{131}\text{I BAL}/{}^{131}\text{I blood})/[{}^{125}\text{I blood}/(1 - \text{Hct})] \quad (2)$$

where ^{125}I BAL is ^{125}I cpm from a unit weight of BAL fluid and ^{131}I BAL is ^{131}I cpm from a unit weight of BAL fluid.

2) In *experiment 2*, the flux of albumin from airspace to intravascular space, the plasma leak (PL) index, was calculated as the ratio of ^{125}I-albumin in circulating plasma to that instilled in the left lung, a modification of a previously described method (30, 39). The amount of ^{125}I-albumin in circulating plasma was calculated by assuming the plasma volume as volume (liter) = body weight (kg) × 0.07(1 − hematocrit) (30, 39).

Extravascular Lung Water

Extravascular lung water was measured as the blood-free wet-to-dry lung weight ratio (W/D). The superior lobe parenchyma and blood samples were weighed immediately after excision, dried for 72 h in a vacuum oven (DP22; Yamato, Tokyo, Japan) at 90°C and 200 mmHg, and then weighed again. We estimated the weight of residual blood contained in the tissue samples by counting ^{131}I in the tissue and blood samples obtained at death. Because we assumed that all of the ^{131}I-albumin was confined to the circulation, we were able to correct for the wet weight of the blood-free tissue sample. In addition, using the W/D ratio of the blood sample, we were also able to correct for the dry weight of the residual blood in the lung tissue sample. We calculated the dry weight of blood-free lung tissue by subtracting the weight of the residual blood from that of lung tissue sample. This allowed us to calculate the blood-free W/D ratio of each superior lobe lung tissue sample.

Neutrophil Elastase Activity and MDA Concentration in the BAL Fluid

Neutrophil elastase activity was determined with the highly specific synthetic substrate *N*-methoxysuccinli-Ala-Ala-Pro-Val *p*-nitroanilline by the method of Yoshimura et al. (42). MDA was measured with a BIOXYTECH LPO-586 kit (Oxis International, Portland, OR) by the method of Gerard-Monnier et al. (9). Briefly, *N*-methyl-2-phenylindole was added to the BAL fluid, followed by the addition of 12 N HCl, and incubated at 45°C for 60 min. After centrifugation at 2,500 *g* at 4°C for 10 min, the absorbance of the clear supernatant was measured at 586 nm. The standard curve was constructed with an MDA standard, and the MDA concentration was expressed in micromoles per liter.

Histopathological Examination

Histopathological examinations were performed to assess pulmonary neutrophil sequestration. Twelve animals were divided among the three experimental groups described earlier, and treated similarly. The right and left lungs were fixed by inflation with formalin, gravimetrically instilled at 25 cmH_2O. The lung was fixed for ≥48 h before the preparation of 2- to 5-mm sagittal sections embedded in paraffin, from which 5-μm sections were sliced and stained with hematoxylin and eosin. The number of neutrophils was counted under oil at ×1,000 magnification among 60 randomly selected fields per rabbit. The neutrophil count was divided by the number of alveoli in the corresponding field to compensate for variable lung inflation (34).

In Vitro Permeability Study

Human pulmonary artery endothelial cells in their fourth passage were obtained from KURABO Industries (Osaka, Japan) and were

Table 1. *^{125}I and ^{131}I activities in blood, lung, and BAL fluid*

	Sham (*n* = 7)	RE (*n* = 7)	RE + Y (*n* = 7)
Blood			
^{125}I, cpm/g	13,696±9,094	4,411±395	10,897±5,032
^{131}I, cpm/g	8,225±952	7,351±1,021	10,650±1,290
R-lung			
^{125}I, cpm/g	7,367±4,746	3,091±291	6,996±2,936
^{131}I, cpm/g	2,200±211	2,264±418	3,095±526
L-lung			
^{125}I, cpm/g	9,208±6,330	3,851±261	7,553±3,224
^{131}I, cpm/g	1,959±232	1,945±436	3,488±378
R-BAL			
^{125}I, cpm/ml	227±90	110±24	300±176
^{131}I, cpm/ml	84±43	22±7	210±202
L-BAL			
^{125}I, cpm/ml	523±241	618±135	605±251
^{131}I, cpm/ml	93±43	279±164	161±55

Values are means ± SE. R, right; L, left; lung, superior lobe lung tissue; BAL, bronchoalveolar lavage; RE, reexpansion; RE + Y, Y-27632 treatment and reexpansion.

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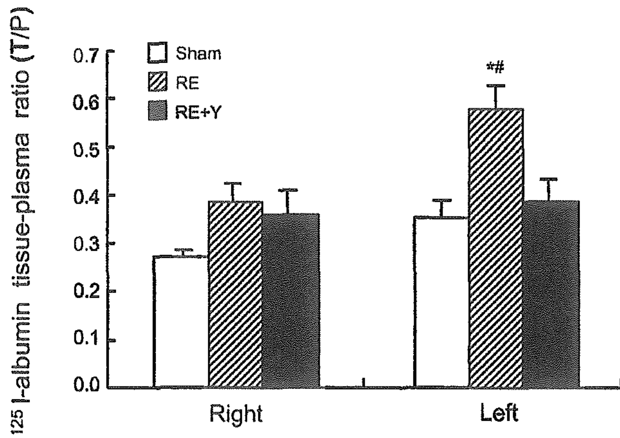

Fig. 2. The tissue-to-plasma (T/P) ratio, as an index of pulmonary endothelial permeability. The left lung is the test lung, and the right lung is the control. Values are means + SE; $n = 7$ rabbits in each group. $P < 0.01$ vs. sham group (*) and vs. RE + Y group (#).

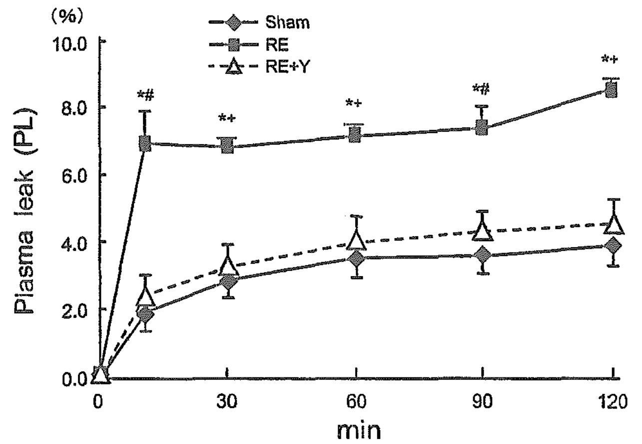

Fig. 4. Changes in plasma leak (PL) index up to 2 h. Values are means ± SE; $n = 4$ in each group. $P < 0.01$ vs. sham group (*) and vs. RE + Y groups (+). #$P < 0.05$ vs. RE + Y group.

cultured in a humidified 5% CO_2 atmosphere with RPMI-1640 medium supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated, endotoxin free FBS (GIBCO-BRL Life Technologies, Grand Island, NY). Monolayers of endothelial cells were prepared on filters as previously described (41). In brief, 12-mm diameter Millicell-HA tissue culture plate well inserts were obtained from Millipore (Bedford, MA). The inserts consist of a surfactant-free 0.45-μm-pore size microporous cellulose membrane filter sealed to a cylindrical polystyrene holder. They were incubated for 3 h with 7 μg/cm^2 human fibronectin (Sigma Chemical. St. Louis, MO) at 37°C to facilitate the cell attachment. The fibronectin solution was aspirated, and the human pulmonary artery endothelial cells suspended in the culture medium were seeded on the membrane filter at a density of 4×10^5 cells/insert (upper chamber). The inserts were placed in a six-well culture plate (Falcon; Becton-Dickinson, Lincoln Park, NJ), with each well filled with 2 ml culture medium, and incubated at 37°C in a humidified 5% CO_2 atmosphere for 2 wk before the measurements of permeability.

To assess the permeability, we measured the albumin transferred across the monolayers of human pulmonary artery endothelial cells cultured on a porous filter. These monolayers were incubated at 37°C for 30 min in the culture medium containing the Y-27632 solution in concentrations of 1 and 10 μM, in a humidified 5% CO_2 atmosphere. A 0.50-mM H_2O_2 solution was then added to the upper chamber. The culture medium was aspirated 1 h later, and 500 μl PBS containing 0.1% BSA were added to the upper chamber. The insert was placed in 1 well of a 24-well culture plate (Falcon; Becton-Dickinson), where each well was filled with 0.7 ml PBS alone. After incubation for 20 min, the insert was removed from the well, and the albumin concentration of the lower chamber was measured with a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA).

Morphological Observations Using Rhodamine-Phalloidin Stain

The changes in the actin microfilament cytoskeleton of the monolayers of human pulmonary artery endothelial cells grown on filters were examined with a rhodamine-phalloidin stain (Molecular Probes, Eugene, OR), as described previously (25), and photographed with a fluorescence microscope equipped with epi-illumination (Nikon Optiphot; Nikon, Garden City, NY).

Statistical Analysis

All data are expressed as means ± SE. One-way ANOVA followed by Fisher's least significant difference test was used for among-groups and between-lung comparisons. One-way ANOVA with repeated-measurements analysis was used to estimate the significance of PL and mean arterial pressures. Differences with P values <0.05 were considered statistically significant.

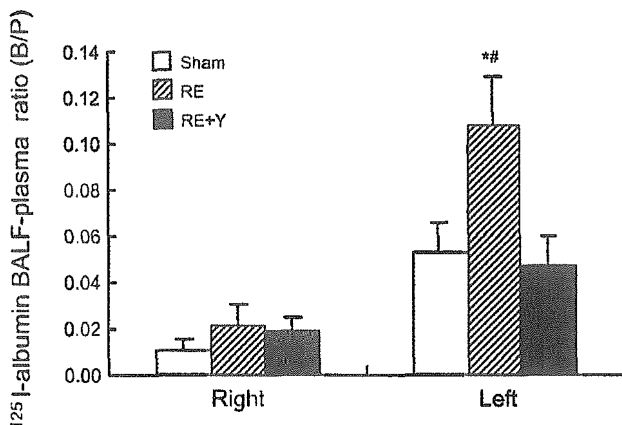

Fig. 3. The bronchoalveolar lavage fluid (BALF)-to-plasma (B/P) ratio, as an index of pulmonary epithelial permeability. The left lung is the test lung, and the right lung is the control. Values are means + SE; $n = 7$ in each group. $P < 0.05$ vs. sham group (*) and vs. RE + Y group (#).

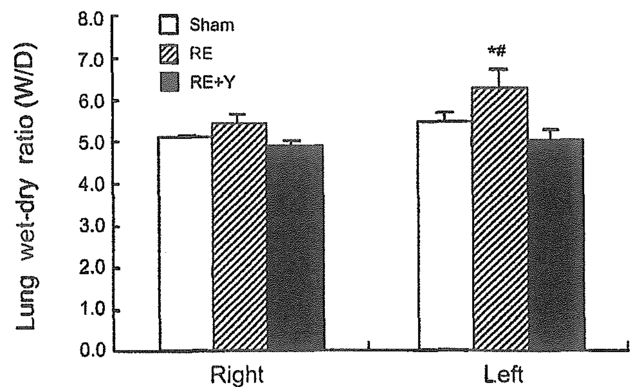

Fig. 5. Lung wet-to-dry (W/D) ratio, an index of pulmonary edema. The left lung is the test lung, and the right lung is the control. Values are means + SE; $n = 7$ in each group. $P < 0.05$ vs. the sham group (*) and vs. the RE + Y group (#).

Table 2. *Total and differential white blood cell counts at time of animal death*

	Total	Neutrophil	Lymphocyte	Monocyte
Sham	31.7±10.5	17.2±6.0	13.2±4.1	1.2±0.5
RE	52.0±10.0	22.3±7.1	26.5±5.8	1.2±0.3
RE + Y	41.4±14.8	24.3±5.4	16.5±6.7	2.2±1.3

Values ($\times 10^2$/mm^3) are means ± SE; n = 7 rabbits in each group.

RESULTS

In Vivo Study

The measurements of ^{125}I and ^{131}I activities in blood, superior lobe lung tissue, and BAL fluid are shown in Table 1. The T/P ratios are shown in Fig. 2. In the left or reexpanded lung, pretreatment with Y-27632 inhibited the increase in T/P ratio after reexpansion. Endothelial permeability in the RE group was significantly higher than in the sham and RE + Y groups. In contrast, in the right lung, there was no significant difference in the T/P ratio among the three groups.

Figure 3 shows the B/P ratio measured in each lung. In the left lung of the RE group, the albumin flux in the airspace was significantly higher than in the left lung of the sham and the RE + Y groups. There was no difference among groups in the B/P ratio in the right lung.

The PL from airspace to intravascular space up to 120 min after instillation of ^{125}I-albumin in the left lung is shown in Fig. 4. PL was significantly higher at 10 min after instillation of ^{125}I-albumin in the RE group than in the other two groups. Furthermore, in the RE group, the W/D ratio was significantly greater than in the RE + Y group (Fig. 5).

The mean total peripheral neutrophil counts at the end of experiments are shown in Table 2. There were no significant differences among the groups. On light microscopy, the number of neutrophils per alveolus in the left lung of both the RE and RE + Y groups was significantly greater than in the sham group (Fig. 6). There was no difference, in the right lung, in neutrophil counts among the groups. The neutrophil count in the left lung BAL fluid in the RE and RE + Y groups was more than twofold greater than in the sham group, although the difference did not reach statistical significance (Fig. 7).

The BAL fluid neutrophil elastase activity in the left lung of the RE and RE + Y groups was significantly higher than in the left lung of the sham group, whereas there was no significant difference in neutrophil elastase activity among the three groups in the right lung (Table 3). In the left lung, the MDA concentration of BAL fluid was significantly higher in the RE than in the sham group, although it was similar in the RE and the RE + Y groups (Table 3).

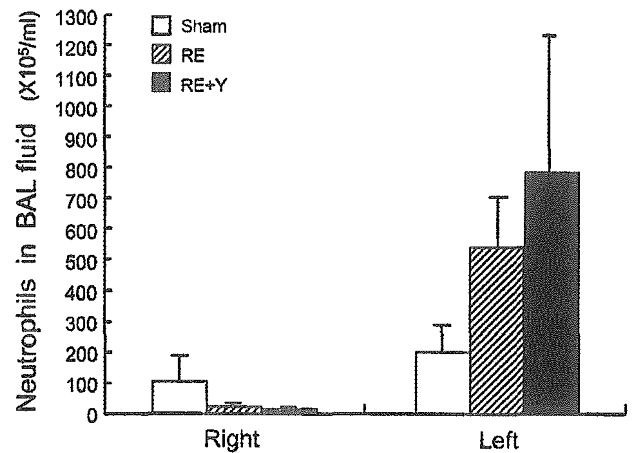

Fig. 7. Neutrophil counts ($\times 10^5$/ml) in bronchoalveolar lavage (BAL) fluid. Values are means ± SE; n = 7 in each group.

Mean arterial pressure did not differ significantly among the experimental groups (Table 4).

Permeability in Pulmonary Endothelial Cell Monolayers

Figure 8 shows the endothelial permeability index estimated from the albumin transfer across human pulmonary endothelial cell monolayers. H_2O_2 increased the permeability of the monolayer above control levels, and treatment with Y-27632 in concentrations of 1 and 10 μM significantly attenuated the increase induced by H_2O_2.

Morphological Changes

We used four monolayers in each group and show representative findings in Fig. 9. Close cell-to-cell contact was present in the control preparations of human pulmonary artery endothelial cells (Fig. 9*A*). Treatment with H_2O_2 caused the development of randomly oriented stress fibers, cell contraction, and intercellular gaps (Fig. 9*B*), and treatment with Y-27632 mitigated these changes (Fig. 9*C*).

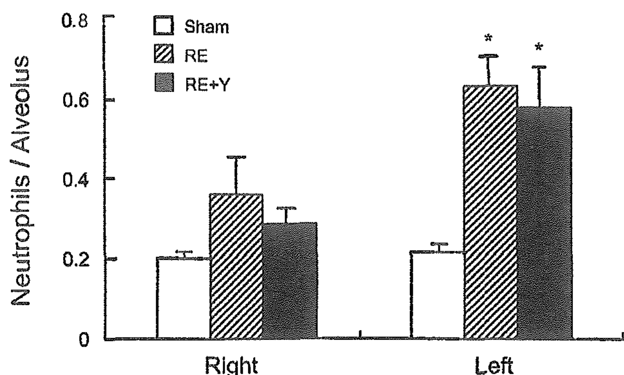

Fig. 6. Tissue neutrophil counts per alveolus in lung. Values are means ± SE; n = 4 in each group. *P < 0.01 vs. sham group.

Table 3. *Malondialdehyde concentration and neutrophil elastase activity in BAL fluid*

	NE Activity, nmol *p*-NA/ml	MDA, μmol/l
Sham		
R	0.28±0.03	0.18±0.04
L	0.40±0.27	0.34±0.03
RE		
R	0.11±0.07	0.56±0.11*
L	1.95±0.49*	2.40±0.88*
RE + Y		
R	0.02±0.02	0.27±0.04
L	2.63±0.41*	1.49±0.04

Values are means ± SE. NE, neutrophil elastase; MDA, malondialdehyde; *p*-NA, *p*-nitroanilline. *P < 0.05 vs. sham group; n = 4 in each group.

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Table 4. *Mean arterial pressures*

	Baseline	Time After Continuous Suction, min			
		30	60	90	120
Sham	65±2	67±3	68±2	68±1	73±6
RE	68±3	67±1	73±2	73±5	71±2
RE + Y	67±1	70±1	72±2	67±2	73±4

Values are means ± SEM; $n = 3$ rabbits in each group. Units are Torr.

DISCUSSION

We examined the role of the Rho/ROCK-mediated pathway in the changes in vascular endothelial and alveolar epithelial permeability, in a new rabbit model of reexpansion pulmonary edema. Treatment with Y-27632, a ROCK-specific inhibitor, attenuated ALI after reexpansion of a lung collapsed for 36 h. We used the ^{125}I-albumin T/P ratio as an index of vascular endothelial permeability, measured the bidirectional flux of albumin across the alveolar epithelium by a modification of the method described by Smedira et al. (30) and Wiener-Kronish et al. (39), and calculated the ^{125}I-albumin B/P ratio and the PL index as indexes of epithelial permeability. These indexes were all increased in the reexpanded lung, increases that were suppressed by pretreatment with Y-27632 (Figs. 2–4). However, Y-27632 neither prevented the accumulation of neutrophils nor inhibited the effects of neutrophil elastase activity and MDA production in the reexpanded lung (Figs. 6 and 7 and Table 2).

The T/P ratio represents the amount of ^{125}I-albumin that traverses the pulmonary vascular endothelium per unit of time. The transendothelial flux of albumin is mainly determined by endothelial permeability, capillary surface area, and capillary pressure (4, 35). Although the administration of Y-27632 did not lower the systemic blood pressure in this study, it may have lowered the pulmonary artery and capillary pressures and increased the capillary surface area, since it relaxes smooth muscles by its ROCK inhibitory action (2). However, if the intraperitoneal administration of Y-27632 had changed the T/P ratio by relaxing the smooth muscle in this model, the ratio in the right lung should have differed among the groups. Therefore, the suppression of the increased T/P by Y-27632 pretreatment in the reexpanded lung was mainly the result of suppression of an increased pulmonary vascular permeability to albumin. These findings confirm that the Rho/ROCK pathway may

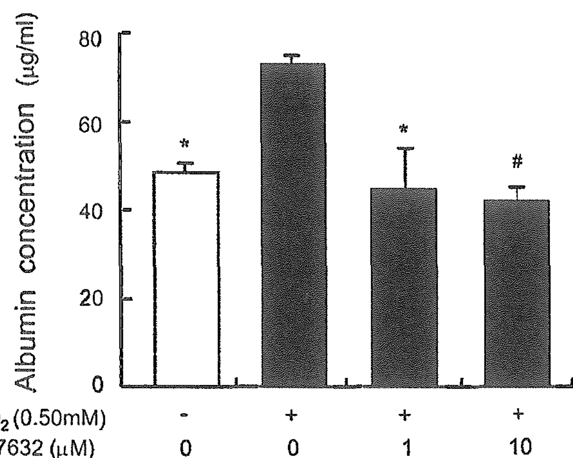

Fig. 8. Effect of Y-27632 on the H_2O_2-induced (0.50 mM) increase in permeability in pulmonary endothelial cell monolayers. Values are means ± SE; $n = 5$ in each group. *$P < 0.005$ vs. H_2O_2 + Y-27632 (0 μM). #$P < 0.0001$ vs. H_2O_2 + Y-27632 (0 μM).

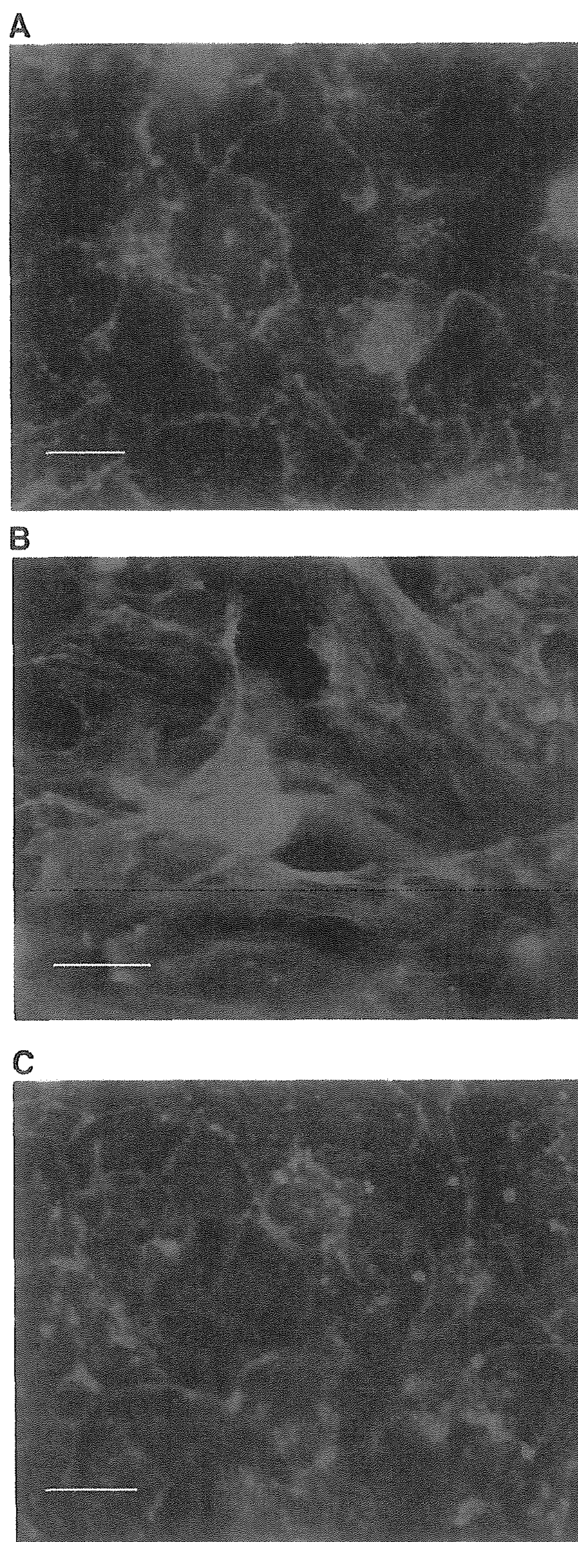

Fig. 9. Morphological changes in pulmonary endothelial cells. Rhodamine-phalloidin stain of monolayers. *A*: control; *B*: monolayers 60 min after treatment with 0.50 mM H_2O_2; *C*: pretreatment with Y-27632 (10 μM) for 30 min before H_2O_2 treatment. Scale bar = 20 μm.