We made further evaluations of the rHSA-heme solution with 40% blood replacement. In the 40% rHSA group, one rat died after 3 days. Although the reason is not clear, histopathologic data suggest occurrence of an unexpected disorder in the urinary system. No significant difference in the blood biochemical tests and organ weights of the 40% rHSA group in comparison with the control group (except for the decrease in liver weight after 1 day and an increase in spleen weight after 7 days) could be found. The increase in spleen weight was a specific biologic reaction to the administration of rHSA to the rats.

After a 40% blood exchange with the rHSA-heme solution, some liver function markers (*e.g.*, AST, ALT, and LDH) became elevated in three samples, and mild focal necrosis was observed in the liver after 1 day. Because these parameters in the 20% rHSA-heme group were similar or lower than those of the control group, this implies that some disorder in liver function might occur with a 40% blood exchange with the rHSA-heme solution. However, other liver function markers (γ-glutamyltransferase, total bilirubin, and direct bilirubin) remained unaltered after sample infusion, so the "disorder" seems to have been temporary and not serious. Similar to the 20% rHSA-heme group after 7 days, the brown pigment deposit in the Kupffer cells/macrophages, which is presumably heme itself or a decomposition thereof, was also found. The results of the blood biochemical tests and the histopathologic observations returned to normal after 7 days.

In conclusion, the 20 % blood replacement with rHSA-heme in rats did not yield any toxic side effects for 7 days. A mild liver disorder occurred in the 40% rHSA-heme group; however, liver function returned normal within 7 days. Thus, administration of the rHSA-heme solution in a volume of 20% total blood volume did not produce any dysfunction of the vital organs, which allows us to undertake further advanced preclinical testing of this synthetic O_2 carrier as a red blood cell substitute.

Acknowledgment

The authors are grateful to NIPRO Corp. for their supporting the O_2 infusion project.

References

1. Tsuchida E: Perspectives of blood substitutes, in Tsuchida E (ed), *Blood Substitutes: Present and Future Perspectives,* Lausanne: Elsevier Science, 1998, pp. 1–14.
2. Winslow RM: The role of blood substitutes in emerging healthcare systems, in Tsuchida E (ed), *Blood Substitutes: Present and Future Perspectives,* Lausanne: Elsevier Science, 1998, pp. 15–32. .
3. Squires JE: Artificial blood. *Science* 295: 1002–1005, 2002.
4. Greenburg AG, Kim HM: Hemoglobin-based oxygen carriers. *Crit Care* 8: S61–64, 2004 (and references therein).
5. Vandegriff KD, Malavalli A, Wooldridge J, Lohman J, Winslow RM: MP4, a new nonvasoactive PEG-Hb conjugate. *Transfusion* 43: 509–516, 2003.
6. Simoni J, Simoni G, Wesson DE, Griswold JA, Feola M; A novel hemoglobin-adenosine-glutathione based blood substitute: Evaluation of its effect on human blood *ex vivo*. *ASAIO J* 46: 679–692, 2000.
7. Powanda DD, Chang TMS: Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. *Artif Cells Blood Subs Immobil Biotechnol* 30: 23–27, 2002.
8. Komatsu T, Hamamatsu K, Wu J, Tsuchida E: Physicochemical properties and O_2-coordination structure of human serum albumin incorporating tetrakis(*o*- pivamido)phenylporphyrinatoiron(II) derivatives. *Bioconj Chem* 10: 82–86, 1999.
9. Tsuchida E, Komatsu T, Matsukawa Y, Hamamatsu K, Wu J: Human serum albumin incorporating tetrakis(*o*-pivalamido)-phenylporphinatoiron(II) derivative as a totally synthetic O_2-carrying hemoprotein. *Bioconj Chem* 10: 797–802, 1999.
10. Komatsu T, Matsukawa Y, Tsuchida E: Effect of heme structure on O_2-binding properties of human serum albumin–heme hybrids: Intramolecular histidine coordination provides a stable O_2-adduct complex. *Bioconj Chem* 13: 397–402, 2002.
11. Tsuchida E, Komatsu T, Hamamatsu K, *et al:* Exchange transfusion of albumin-heme as an artificial O_2-infusion into anesthetized rats: Physiological responses, O_2 delivery and reduction of the oxidized hemin sites by red blood cells. *Bioconj Chem* 11: 46–50, 2000.
12. Huang Y, Komatsu T, Nakagawa A, Tsuchida E, Kobayashi S: Compatibility *in vitro* of albumin-heme (O_2 carrier) with blood cell components. *J Biomed Mater Res* 66A: 292–297, 2003.
13. Tsuchida E, Komatsu T, Matsukawa Y, *et al:* Human serum albumin incorporating synthetic heme: Red blood cell substitute without hypertension by nitric oxide scavenging. *J Biomed Mater Res* 64A: 257–261, 2003.
14. Komatsu T, Yamamoto H, Huang Y, Horinouchi H, Kobayashi K, Tsuchida E: Exchange transfusion with synthetic oxygen-carrying plasma protein 'albumin-heme' in acute anemia rat model after seventy-percent hemodilution. *J Biomed Mater Res* 71A: 2004, *in press.*
15. Huang Y, Komatsu T, Yamamoto H, Horinouchi H, Kobayashi K, Tsuchida E: Exchange transfusion with entirely synthetic red-cell substitute albumin-heme into rats: physiological responses and blood biochemical tests. *J Biomed Mater Res* 71A: 63–69, 2004.

Hemorrhagic Shock Resuscitation With an Artificial Oxygen Carrier, Hemoglobin Vesicle, Maintains Intestinal Perfusion and Suppresses the Increase in Plasma Tumor Necrosis Factor-α

Akira Yoshizu,* Yotaro Izumi,* Sungick Park,‡ Hiromi Sakai,‡ Shinji Takeoka,‡ Hirohisa Horinouchi,* Eiji Ikeda,† Eishun Tsuchida,‡ and Koichi Kobayashi*

It is known that damage to the intestinal mucosa followed by systemic inflammatory response is one of the leading causes of shock related morbidity and mortality. In this study, we examined the ability of an artificial oxygen carrier hemoglobin vesicle (HbV) to sustain systemic and intestinal perfusion during hemorrhagic shock. In rabbits, hemorrhagic shock (40% of the estimated blood volume) was resuscitated with 5% albumin (alb group), HbV suspended in 5% albumin (HbValb group), or washed red blood cells suspended in 5% albumin (RBCalb group). Plasma tumor necrosis factor (TNF)-α level was measured in rats under the same experimental protocol. No significant intergroup differences were seen in systemic hemodynamics. In contrast, parameters of intestinal perfusion significantly deteriorated in the alb group but were equally well sustained in the HbValb and RBCalb groups. Also, a significant increase in plasma TNF-α level was seen in the alb group but not in the RBCalb or HbValb groups. These results indicate the proficient oxygen transporting capability of HbV and its potential efficacy in shock resuscitation. *ASAIO Journal* 2004; 50:458–463.

Blood replacement is the basic therapeutic modality when a considerable amount of blood is lost because of trauma or major surgery. Despite the recent progress in transfusion medicine, enormous investments are still necessary to establish and sustain the systems from blood donation to transfusion. Donated blood inspections to avoid the side effects of homologous blood transfusion, such as transfusion associated infectious disease, alloimmunization, and graft *versus* host diseases are still essential.[1,2] To overcome these problems associated with transfusion, development of artificial blood substitutes is important. To this end, we have developed several types of artificial oxygen carriers and have evaluated the efficacy of these compounds in various animal models.[1] Among these compounds, hemoglobin vesicle (HbV), a form of liposome encapsulated hemoglobin, is rapidly approaching clinical trials. The cellular structure of HbV, similar to red blood cells, shields all of the physiologic effects of acellular Hb solutions.[3–5] We have studied the oxygen transporting capabilities of HbV, using several exchange transfusion and hemorrhagic shock models.[6–10] In these studies, we have shown that HbV effectively restores the systemic circulation in hemorrhagic shock.

It is known that gastrointestinal perfusion is compromised at a relatively early stage in hypovolemic shock to sustain the systemic circulation to other vital organs.[11] This, however, causes damage to the intestinal mucosa followed by systemic inflammatory response syndrome (SIRS) or sepsis, which is one of the leading causes of shock related morbidity and mortality.[12,13] In the present study, we examine the ability of HbV to sustain not only systemic but also intestinal perfusion to further evaluate the efficacy of HbV in hemorrhagic shock.

Materials and Methods

Animal Care

The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of Keio University, School of Medicine. It also complies with Guidelines for the Care and Use of Laboratory Animals of Keio University, School of Medicine. All rabbits and rats were housed in groups of two in standard cages and were provided with food and water in a temperature controlled room on a 12 hour dark/light cycle.

Preparation of Hemoglobin Vesicle Suspended in 5% Albumin

HbV suspension was prepared in a similar manner as previously reported in the literature.[14,15] In brief, a purified and concentrated human hemoglobin solution (40 g/dl) was obtained from outdated red blood cells.[16] Added to this purified hemoglobin solution were pyridoxal 5'-phosphate (18 mM, Merck Co., Frankfurter, Germany) as an allosteric effector and homocysteine (Aldrich Co., Milwaukee, WI) as a reductant of methemoglobin. The lipid bilayer of HbV was composed of Presome PPG-I (Nippon Fine Chem. Co., Osaka, Japan) containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, (DPPC), cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phos-

From the *Department of Surgery and the †Department of Pathology, Keio University School of Medicine, Tokyo, Japan; and the ‡Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, Japan.

Submitted for consideration November 2003; accepted for publication in revised form June 2004.

Correspondence: Koichi Kobayashi, Director and Chief, Division of Thoracic Surgery, Department of Surgery, Keio University School of Medicine, Tokyo 160–8582, Japan.

DOI: 10.1097/01.MAT.0000136508.51676.EF

Table 1. Physicochemical Properties of PEG Modified HbV Suspended in Human Serum Albumin

Hb (g/dl)	10
Lipid (g/dl)	6.2
Hb/lipid (g/g)	1.61
Diameter (nm)	251 ± 87
P_{50} (torr)	32
Hill number	2.2
Viscosity (cP at 358 s^{-1})	3.7
HbCO (%)	2
MetHb (%)	3

PEG,.

phatidylglycerol (DPPG), which were purchased from Nippon Fine Chem. Co. (Osaka, Japan), and α-tocopherol was added to these at the composition so that the molar ratios for DPPC: cholesterol:DPPG: α-tocopherol became 5:5:1:0.1. The surface of the HbV was modified with poly(ethylene glycol) (Mw: 5 kDa, 0.3 mol% of the lipids in the outer surface of vesicles) using 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (Sunbright DSPE-50H, H-form, NOF Co., Tokyo, Japan). HbVs were suspended in 5% human serum albumin (alb) containing 160 mEq/L sodium and 107 mEq/L chloride (Albumin 5%-cutter, Bayer) and filtered through sterilizable filters (Dismic, Toyo Roshi Co., Tokyo, Japan, pore size: 0.45 micrometer). The whole procedure was performed at temperatures below 10°C in a sterile environment.

The properties of HbV suspended in alb (HbValb) are summarized in **Table 1**. The amount of oxygen release was calculated to be 6.2/100 ml. This is close to 7.0/100 ml of human blood (hemoglobin concentration 15 g/dl) because of, theoretically, the increased oxygen transporting efficiency (the difference in oxygen saturation between 40 and 110 mm Hg PO_2) of HbV compared with human red blood cells (37% to 28%, respectively).

Preparation of Washed Rabbit (Rat) Red Blood Cells Suspended in 5% Albumin

Blood samples were withdrawn from rabbits/rats into heparinized syringes and centrifuged to obtain a red blood cell concentrate. This was washed twice to remove plasma components and buffy coat by resuspension in 5% human serum albumin and centrifugation (4,300 rpm, 10 min). The hemoglobin concentration was adjusted to 10 g/dl, equivalent to that of HbValb.

Hemorrhagic Shock Resuscitation

Animal preparation was performed as follows (**Figure 1**). Male Japanese white rabbits (3.0 ± 0.4 kg) were anesthetized with intramuscular injection of ketamine hydrochloride (50 mg/kg) and intravenous injection of pentobarbital sodium (20 mg/kg) through the marginal ear vein. The body temperatures of the animals were maintained between 36 and 37°C by a heating lamp during the experiment.

Tracheostomy tubes were placed to secure the airway. The animals breathed spontaneously during the experiment. A polyethylene tube (outer diameter 1.7 mm, ATOM Japan) was introduced into the right carotid artery for blood withdrawal and connected to a pressure transducer (Polygraph System,

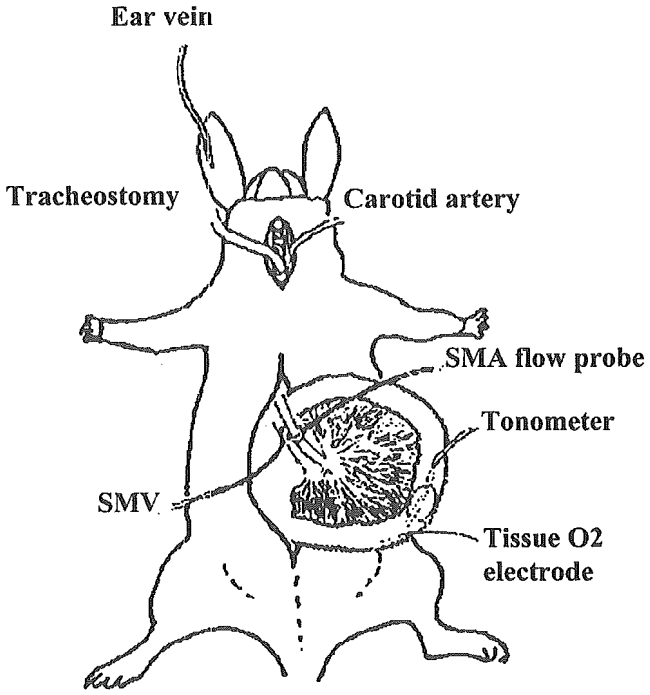


Figure 1. Schematic representation of the shock resuscitation experiment in the rabbit is shown. SMA, superior mesenteric artery; SMV, superior mesenteric vein.

Nihon Koden, Tokyo Japan) for continuous mean arterial pressure (MAP) monitoring. A median abdominal incision was made, and the superior mesenteric artery (SMA) was identified and dissected from surrounding tissue close to its origin from the aorta. A 2 mm ultrasonic flow probe (20 MHz, Crystal Biotech, Hopkinton, MA) was placed around the root of SMA and connected to a blood flow meter for measurement of SMA flow and heart rate. A small vein in the mesentery was ligated distally and cannulated with polyethylene catheter (PE-20). The catheter was advanced 5–10 cm proximally until the tip was located in the superior mesentric vein (SMV) for sampling of venous blood. For arterial and venous blood gas measurements, Corning 170 pH/blood gas analyzer (Corning Medical, Medfield, MA) was used. Hemoglobin concentration was determined by hemoglobin analyzer, Sysmex E-400 (Toa Medical Electronics Co, LTD, Kobe, Japan).

A sigmoid tonometor (Tonomiter Tonometorics) was positioned in the duodenum 2–3 cm from the pylorus for intestinal mucosal pH (pHi) measurements. The pHi was determined from partial carbon dioxide pressure (PCO_2) in the tonometer saline, the bicarbonate concentration, and the Henderson-Hasselbalch equation (l):

$$pHi = 6.1 + \log_{10}(HCO_3^-/(0.22 \cdot PCO_2 \cdot k)) \quad (1)$$

A needle type polarographic oxygen electrode (Intermedical, Tokyo, Japan) was inserted into the submucosa of the small intestine for continuous intestinal submucosal tissue oxygen tension measurements.

Approximately 20 minutes was allowed for MAP, SMA blood flow, and tissue oxygen tension measurements to stabilize. Hemorrhagic shock was induced by withdrawal of 40% of the estimated total blood volume of the rabbit from the right

carotid artery at a rate of 10 ml/min (3 ml/kg/min). Approximately 10 minutes after bleeding, they were infused with the lost volume *via* the marginal ear vein at the same rate with 5% albumin (alb group, n = 6), HbValb (HbValb group, n = 6), or washed rabbit red blood cell (RBCalb group, n = 6). This procedure was repeated twice. Arterial (carotid artery) and SMV blood samples were drawn before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30); pHi was measured at BASAL, BL2, IN2, and AFTER30.

Histologic Examination

After completion of the experiment, the animals were killed by pentobarbital overdose. The heart, lung, kidney, liver, spleen, and small intestine were removed and fixed in 10% formalin. The tissues were embedded in paraffin, and the sections were stained with hematoxylin and eosin for light microscopic examinations.

Tumor Necrosis Factor-α Measurements

Male Wistar rats (364 ± 15 g) were used for the experiment. They were anesthetized with intraperitoneal injections of pentobarbital (50 mg/kg). A longitudinal midline ventral cervical incision was made, and catheters (PE-20 tubing, outer diameter 0.8 mm, inner diameter 0.5 mm) were introduced into the right jugular vein for infusion and into the right common carotid artery for blood withdrawal. Shock resuscitation was performed following the protocol in the rabbit. Forty percent of the estimated total blood volume was drawn from the right carotid artery at a rate of 1 ml/min (3 ml/kg/min). After bleeding, they were infused *via* the jugular vein with the same rate and volume of 5% albumin (n = 6), HbValb (n = 6), or washed rat red blood cell (n = 6). This procedure was repeated twice. Thirty minutes after the second infusion, corresponding to AFTER30, blood was sampled from the carotid artery. After centrifuging the blood at 4,300 rpm for 10 minutes, the plasma component was separated and stored at −80°C until measurement. TNF-α was measured by enzyme linked immunosolvent assay (ELISA) using Genzyme-Technic rat TNF-α determination kit.

Data Analysis

Data are shown as mean ± SD, as percentage changes or differences from basal values. The error bars in the figures indicate SD. Data were compared between groups at corresponding time points by Mann-Whitney *U* test (StatView, Institute Inc., Cary, NC). The level of confidence was placed at 95% for all experiments.

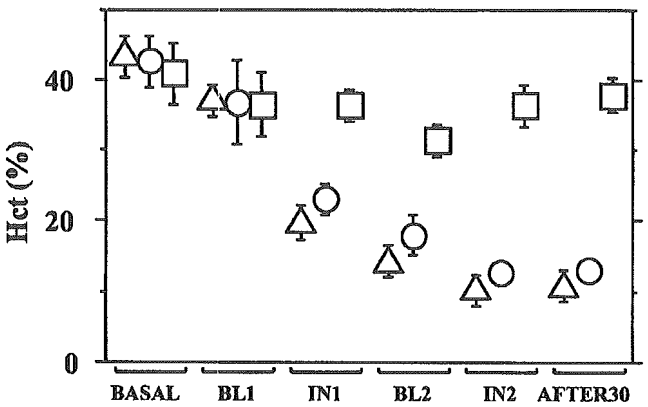


Figure 2. Changes in hematocrit in rabbits. Time points: before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30). Triangle, alb group; circle, HbValb group; square, RBCalb group.

Results

Hemorrhagic Shock Resuscitation

Hemodynamic, blood gas, and intestinal measurements were performed in rabbits. After IN2, the hematocrit (Hct) (**Figure 2**) decreased from approximately 40% to 10% in both alb and HbValb groups. This indicated that approximately 40% of the circulating blood volume was actually replaced twice.

In **Table 2**, values at BASAL are shown for parameters representing hemodynamics, arterial blood gas, and intestinal perfusion. No significant intergroup differences were observed regarding these parameters. Therefore, the subsequent data changes are shown as percentage changes or differences from values at BASAL.

Mean arterial pressure (MAP) (**Figure 3a**) declined sharply after bleeding but rapidly recovered after infusion. There were no significant differences between groups. Heart rate (HR) (**Figure 3b**) tended to decrease slightly during the course of the experiment, but there were no significant differences between groups. Superior mesenteric aortic (SMA) blood flow (**Figure 3c**) declined sharply after bleeding but rapidly recovered after

Table 2. Basal Values of Measured Parameters

Basal Values	Alb Group	HbValb Group	RBCalb Group
Mean arterial pressure (mm Hg)	120 ± 13	110 ± 21	132 ± 19
Heart rate (beats/min)	233 ± 23	250 ± 33	240 ± 45
PaO_2 (Torr)	97.1 ± 11.0	92.3 ± 7.8	99.0 ± 9.7
$PaCO_2$ (Torr)	31.0 ± 4.7	30.1 ± 2.9	30.4 ± 2.6
Arterial base excess (mmol/L)	−3.2 ± 3.6	−2.9 ± 4.2	−4.8 ± 2.0
Superior mesenteric arterial flow (ml/min/kg)	22.0 ± 10.0	32.2 ± 11.2	36.9 ± 21.1
Intestinal mucosal pH	7.4 ± 0.1	7.4 ± 0.3	7.4 ± 0.1
Intestinal tissue PO_2 (Torr)	21.4 ± 3.4	18.4 ± 4.8	19.8 ± 6.0
Superior mesenteric venous PO_2 (Torr)	45.8 ± 3.1	42.2 ± 9.4	51.4 ± 8.9

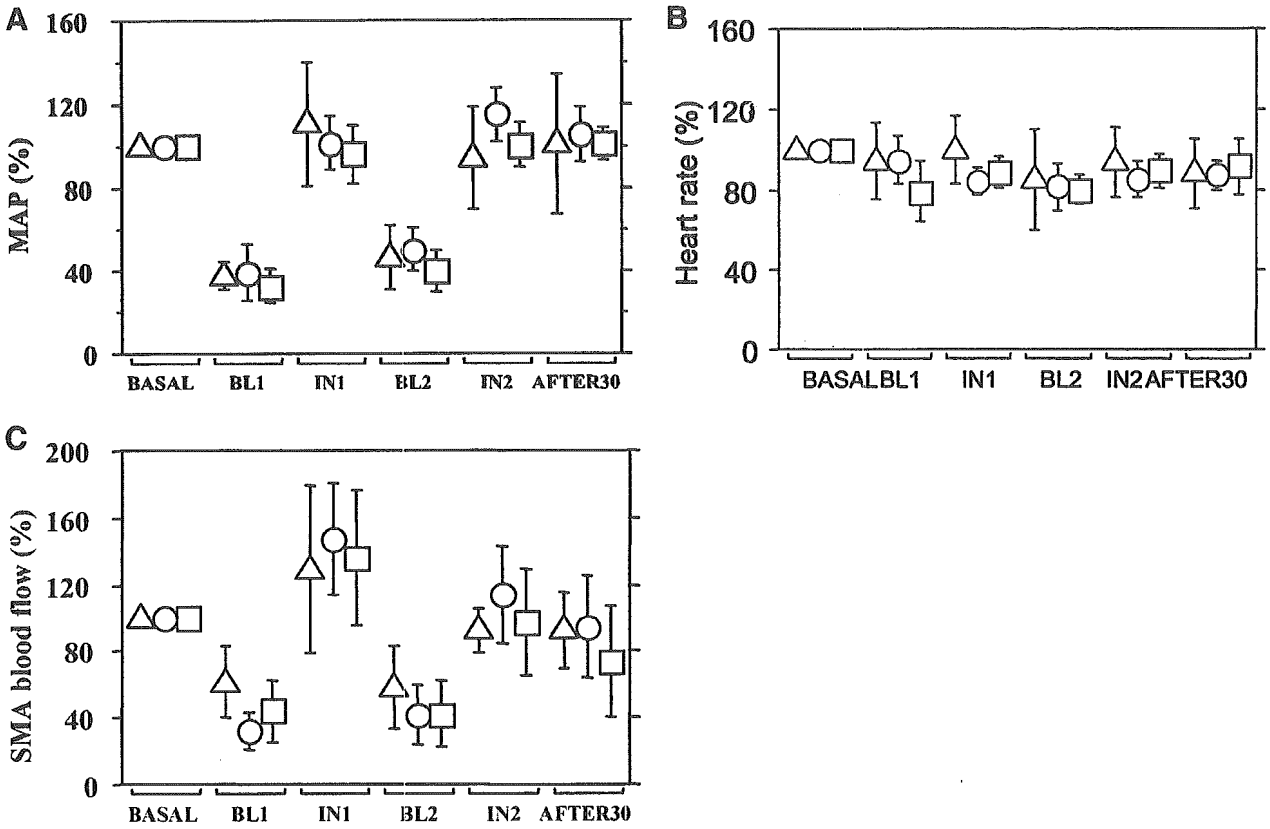


Figure 3. Changes in hemodynamic parameters from basal values in rabbits. Time points: before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30). Triangle, alb group; circle, HbValb group; square, RBCalb group.

infusion. There were no significant differences between groups.

Arterial oxygen tension (PaO_2) (**Figure 4a**) tended to increase slightly during bleeding and infusion in all the groups. There were no significant differences between groups. Arterial carbon dioxide tension ($PaCO_2$) (**Figure 4b**) remained stable throughout the study in all the groups. Systemic base excess (BE) (**Figure 4c**) declined significantly in the alb group compared with the RBCalb group at BL2. At IN2 and AFTER30, BE in the alb group was significantly lower compared with both HbValb and RBCalb groups.

In the alb group, pHi (**Figure 5a**) declined significantly compared with both HbValb and RBCalb groups beyond BL2. Intestinal tissue oxygen tension (**Figure 5b**) declined after bleeding but recovered to baseline by infusion in the HbValb and RBCalb groups but not in the alb group. The differences were significant beyond IN2. Superior mesenteric venous (SMV) oxygen tension (**Figure 5c**) declined sharply after bleeding but rapidly recovered close to baseline after infusion in all the groups. However, at AFTER30, it significantly increased in the alb group compared with both HbValb and RBCalb groups.

Histologic Examination

No significant abnormalities or differences among groups were observed in any of the organs examined in the rabbits.

Plasma Level of Tumor Necrosis Factor-α

In the rats, TNF-α concentration in plasma (pg/ml) was increased approximately 40-fold in the alb group (4,634 ± 4,276) compared with the HbValb group (124 ± 65). In the RBCalb group, it was below detection limit (<25).

Discussion

Peripheral tissue perfusion is controlled in response to changes in systemic hemodynamics. Intestinal perfusion is known to be one of the first to decline in hemorrhagic shock when the redistribution of systemic blood flow occurs to other vital organs such as the heart and the brain. However, it is also known that the loss of adequate intestinal function caused by insufficient perfusion leads to serious complications such as bacterial translocation and cytokine production,[17] which can eventually lead to mortality even when other vital organs are initially well sustained. It has been shown that indices such as intestinal mucosal pH are valid in assessing the severity of shock, as well as predicting prognosis.[18] To this end, in this study, we observed parameters of intestinal perfusion in addition to systemic hemodynamic parameters to evaluate the applicability of HbV in hemorrhagic shock resuscitation.

In the present study, in the rabbits, shock resuscitation with albumin satisfactorily restored parameters such as MAP and HR. Lung function was also maintained as shown by the

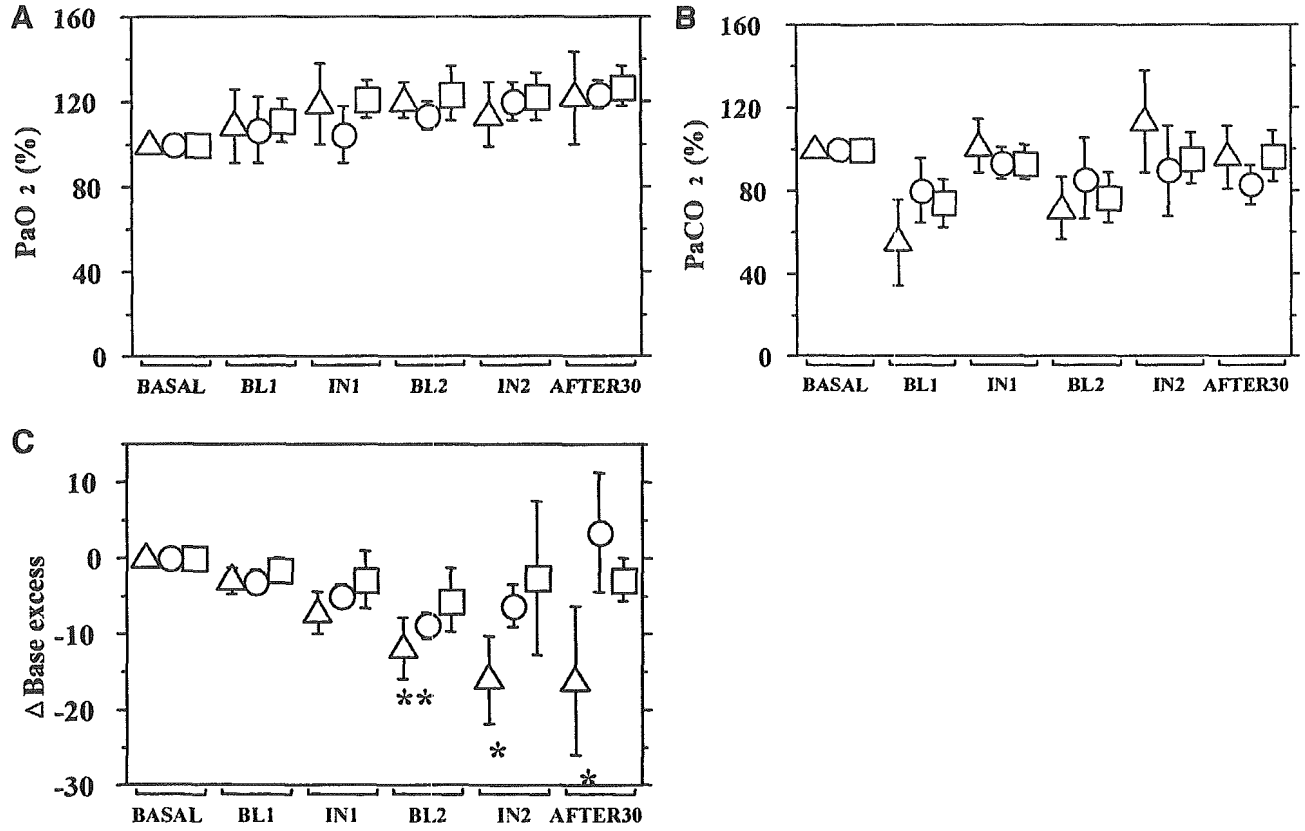


Figure 4. Changes in arterial blood gas parameters from basal values in rabbits. Time points: before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30). Triangle, alb group; circle, HbValb group; square, RBCalb group. *$p < 0.05$ *vs.* HbValb and RBCalb groups; **$p < 0.05$ *vs.* RBCalb group.

systemic oxygen and carbon dioxide tension. However, systemic base excess significantly declined in the alb group indicating peripheral hypoperfusion, and our data show that one such organ is the intestine. Even though SMA blood flow was maintained, lack of peripheral perfusion in the alb group was depicted by the significant decline in pHi and intestinal tissue oxygen tension. The subsequent significant increase in SMV oxygen tension was most likely brought about by the shutdown of peripheral circulation leading to shunting of SMA blood. We consider that all of these changes resulted from the impairment in cardiac function caused by decreased oxygen content in the alb group, which subsequently limited the oxygen delivery to the cardiac muscles. It is likely that a longer observation period was required for these changes to become morphologically apparent on histology. However, most of the animals in the alb group could not survive beyond 30 minutes after the second infusion in this study design.

TNF-α is believed to be an important mediator of SIRS. It has been reported that the intestine is a major source of TNF-α production during hemorrhagic shock.[19] We used rats for the measurement of plasma TNF-α. Ideally, the assay for TNF-α should have been performed in rabbits. However, we were not able to find an appropriate TNF-α antibody to perform ELISA in rabbits. On the other hand, we had previously performed TNF-α assay in the rat, and the assay technique was well established. In our preliminary shock resuscitation experiments in rats, we found that withdrawal of 40% of estimated circulating blood volume reduced MAP to approximately 40% of baseline (data not shown). Also, we have previously reported that withdrawal of 50% of estimated circulating blood volume in rats reduced MAP to approximately 20% of baseline, and base excess declined from 0 to approximately −6.[9] From these data, we extrapolated that the hemodynamic changes would be similar in rats compared with rabbits under the same shock resuscitation protocol. Therefore, we decided to perform the TNF-α measurements in rats. Under the same experimental protocol, we saw a significant increase in the plasma levels of TNF-α in the alb group. This was effectively suppressed in the HbValb group, although not quite to the level of RBCalb group. In this particular experiment, there is no evidence to show that the hemodynamic changes or the changes in the intestinal parameters were the same in the rats compared with the rabbits. However, we believe that the substantial intergroup differences in TNF-α in the rats, although not directly, provide support that intestinal, and possibly other organ damage, was reduced by shock resuscitation with HbV.

These data show that significant covert damage to the intestine is present in the alb group despite seemingly adequate systemic hemodynamics. This was because of the deficiency of blood oxygen content despite sufficient volume. In contrast, systemic, as well as intestinal, perfusion in the HbValb group were well sustained and were comparable with the RBCalb group. Plasma TNF-α level was also effectively reduced in the

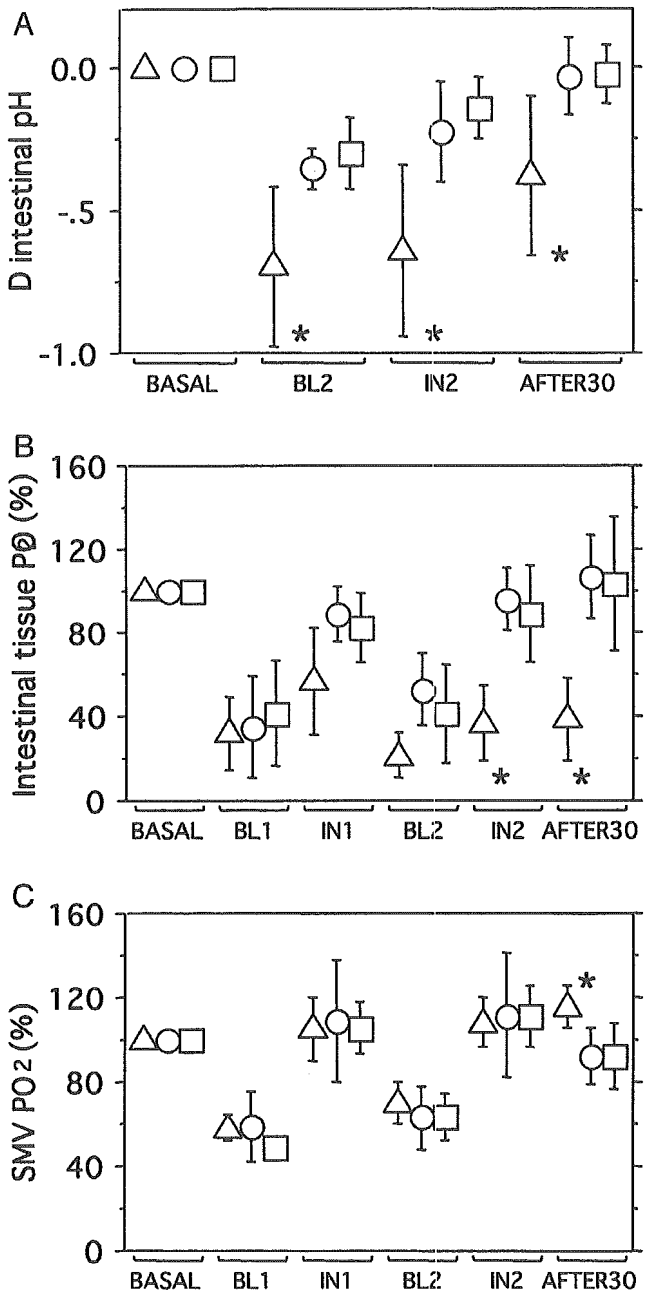


Figure 5. Parameters representing intestinal perfusion are shown as percentage changes or difference from basal values in rabbits. Time points: before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30). Triangle, alb group; circle, HbValb group; square, RBCalb group. *$p < 0.05$ *vs.* HbValb and RBCalb groups.

HbValb group, close to the RBCalb group. These data collectively indicate the proficient oxygen transporting capability of HbV and its potential efficacy in shock resuscitation. One of the powerful advantages of HbV is that its properties, such as oxygen binding and release, viscosity, and colloid osmotic pressure, can be manipulated by changing the amount of allosteric effector in HbV and the plasma expander in which to suspend HbV. We believe that currently ongoing optimization of these properties will further improve the efficacy of HbV in shock resuscitation.

Acknowledgement

This work was supported in part by a grant from the Ministry of Health, Labor and Welfare, Japan (15141201) and Grants in Aid for Scientific Research from the Ministry of Education, Japan (10470247). The authors thank BML Inc. (Kawagoe, Japan) for the cytokine measurement.

References

1. Tsuchida E: *Blood Substitutes: Present and Future Perspectives.* Amsterdam: Elsevier, 1998.
2. Chang TMS: *Blood Substitutes: Principles, Methods, Products, and Clinical Trials.* Basel: Karger, 1997.
3. Sakai H, Hara H, Yuasa M, *et al*: Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am J Physiol Heart Circ Physiol* 279: H908–915, 2000.
4. Goda N, Suzuki K, Naito M, *et al*: Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. *J Clin Invest* 101: 604–612, 1998.
5. Takeoka S, Teramura Y, Atoji T, Tsuchida E: Effect of Hb-encapsulation with vesicles on H_2O_2 reaction and lipid peroxidation. *Bioconjug Chem* 13: 1302–1308, 2002.
6. 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 oxygen tension. *Crit Care Med* 24: 1869–1873, 1996.
7. Izumi Y, Sakai H, Takeoka S, *et al*: Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *ASAIO J* 43: 289–297, 1997.
8. Kobayashi K, Izumi Y, Yoshizu A, *et al*: The oxygen carrying capability of hemoglobin vesicles evaluated in rat exchange transfusion models. *Artif Cells Blood Substit Immobil Biotechnol* 25: 357–366, 1997.
9. Yoshizu A, Yamahata T, Izumi Y, *et al*: The O_2 transporting capability of hemoglobin vesicle, an artificial O_2 carrier, evaluated in a rat hemorrhagic shock model. *Artif Blood* 5: 18–22, 1997.
10. Sakai H, Takeoka S, Wettstein R, Tsai AG, Intaglietta M, Tsuchida E: Systemic and microvascular responses to the hemorrhagic shock and resuscitation with Hb-vesicles. *Am J Physiol Heart Circ Physiol* 283: H1191–H1199, 2002.
11. Nelson DP, King CE, Dodd SL, Schumacker PT, Cain SM: Systemic and intestinal limits of O_2 extraction in the dog. *J Appl Physiol* 63: 387–394, 1987.
12. Carrico CJ, Meakins JL, Marshall JC, Fry D, Maier RV: Multiple-organ-failure syndrome. *Arch Surg* 121: 196–208, 1986.
13. Sori AJ, Rush BF Jr, Lysz TW, Smith S, Machiedo GW: The gut as source of sepsis after hemorrhagic shock. *Am J Surg* 155: 187–192, 1988.
14. Sakai H, Takeoka S, Park SI: Surface-modification of hemoglobin vesicles with polyethyleneglycol and effects on aggregation, viscosity, and blood flow during 90%-exchange transfusion in anesthetized rats. *Bioconjugate Chem* 8: 15–22, 1997.
15. Sakai H, Masada Y, Takeoka S, Tsuchida E: Characteristics of bovine hemoglobin for the potential source of hemoglobin-vesicles as an artificial oxygen carrier. *J Biochem* 131: 611–617, 2002.
16. Sakai H, Takeoka S, Yokohama H, Seino Y, Nishide H, Tsuchida E: Purufication of concentrated Hb using organic solvent and heat treatment. *Protein Expression Purif* 4: 563–569, 1993.
17. Tamion F, Richard V, Lyoumi S: Gut ischemia and mesenteric synthesis of inflammatory cytokines after hemorrhagic or endotoxic shock. *Am J Physiol* 273: G314–321, 1997.
18. Nordin A, Makisalo H, Mildh L, Hockerstedt K: Gut intramucosal pH as an early indicator of effectiveness of therapy for hemorrhagic shock. *Crit Care Med* 26: 1110–1117, 1998.
19. Tani T, Fujino M, Hanasawa K, Shimizu T, Endo Y, Kodama M: Bacterial translocation and tumor necrosis factor-alpha gene expression in experimental hemorrhagic shock. *Crit Care Med* 28: 3705–3709, 2000.

Importance of Tumor Necrosis Factor-α Cleavage Process in Post-Transplantation Lung Injury in Rats

Taichiro Goto, Akitoshi Ishizaka, Fujio Kobayashi, Mitsutomo Kohno, Makoto Sawafuji, Sadatomo Tasaka, Eiji Ikeda, Yasunori Okada, Ikuro Maruyama, and Koichi Kobayashi

Departments of Surgery, Medicine, and Pathology, School of Medicine, Keio University, Tokyo; Research & Development Division, Mitsubishi Pharma Corporation, Yokohama; and Department of Laboratory and Molecular Medicine, Faculty of Medicine, Kagoshima University, Kagoshima, Japan

Tumor necrosis factor-α (TNF-α) has two forms with apparently different biological activities: a membrane-associated form and a soluble form. TNF-α–converting enzyme (TACE) mediates a cleavage of membrane-associated TNF-α to induce its bioactive soluble form. We hypothesized that inhibition of TACE might prevent TNF-α–induced tissue injury while preserving the benefits of TNF-α. In this study, we evaluated the role of TACE in acute inflammation using an inhibitor of the enzyme in a rat model of lung transplantation. Inbred Lewis rats underwent left lung isotransplantation, and the donor lungs were kept in Euro-Collins solution with or without the inhibitor. After 6 hours of ischemia, the left lung was transplanted into the recipient rat and reperfused for 4 hours. Inhibition of TACE significantly attenuated endothelial and alveolar septal damage, as assessed by radiolabeled albumin leakage after transplantation. The inhibition also attenuated neutrophil accumulation in the alveolar space and other histopathologic findings, including intercellular adhesion molecule-1 expression. In addition, significantly lower levels of monocyte chemotactic protein-1, cytokine-induced neutrophil chemoattractant-1, high mobility group box-1, and soluble epithelial cadherin and decreased neutrophil elastase activity were observed in bronchoalveolar lavage fluid from the rats treated with the inhibitor. We conclude that TACE mediates a critical step in the development of post-transplantation lung injury.

Keywords: acute inflammation; epithelial cadherin; high mobility group box-1; lung transplantation; TNF-α–converting enzyme

Although much progress has been made in transplantation immunology in recent years, reimplantation injury is still a serious problem. Indeed, severe acute injury has been reported to occur in 15 to 30% of patients after lung transplantation (1–4). It has also been reported that acute lung injury in the early stage after lung transplantation aggravates rejection via expression of various cytokines and adhesion molecules (5, 6). Despite the well-known proinflammatory effects of tumor necrosis factor-α (TNF-α), its role in the pathogenesis of lung injury after lung transplantation remains unclear. Much experimental work has addressed this issue with a view to develop procurement techniques, allowing such effective organ preservation as that achieved in liver and kidney transplantation. TNF-α plays a critical role in certain physiologic defensive response, but when produced in excess, it causes severe cellular and tissue damage in the host (7). TNF-α has two forms with apparently different biological activities: a membrane-associated form and a soluble form generated from the membrane-bound protein by proteolytic cleavage mediated by TNF-α–converting enzyme (TACE). TACE is present on the surface of macrophages, the major TNF-α–producing cells (8). TNF-α produced in response to various stimuli, such as bacterial challenge and tumor burden, is transported by the trans-Golgi network, expressed on the membrane surface, and transiently present as membranous TNF-α (memTNF-α). Within 1 hour, memTNF-α is processed by TACE and converted to soluble TNF-α, which is incorporated into tissues and plasma in the form generally called TNF-α (9–11). In the pathologic state of systemic inflammatory response syndrome, autotissue injury may be caused by excess expression of soluble TNF-α and other proinflammatory cytokines (12). When TACE is inhibited, a number of memTNF-α molecules remain on the macrophage surface because of blockade of memTNF-α processing. It has been suggested in recent years that these remaining memTNF-α molecules serve as a protection function such as defense against infection, tumor cell cytotoxicity, productive T cell–B cell interactions, and thymocyte proliferation (13–18). Thus, inhibition of TACE preserves beneficial memTNF-α and blocks the production of soluble TNF-α.

In this study, we performed orthotopic left lung transplantation in an inbred rat strain and investigated the involvement of soluble TNF-α in post-transplantation acute lung inflammation. We focused on chemokines and high mobility group box-1 (HMGB-1) as downstream mediators of TNF-α and examined their involvement in lung injury. To investigate the involvement of alveolar epithelial disorder in post-transplantation acute lung inflammation, we attempted detection of epithelial cadherin (E-cadherin)–soluble form in bronchoalveolar lavage fluid (BALF) of the injured lung.

METHODS

A more detailed description of the methods is available in the online supplement.

Animal Model

Specific pathogen-free inbred Lewis rats were used as both donor and recipient animals. All procedures described in this report were approved by the institutional review board for animal studies.

To perform orthotopic left lung transplantations in the rats, we have modified surgical techniques described by other investigators (19, 20). Donor animals were injected with heparin (1,000 U/kg) intravenously. A 14-gauge catheter was inserted into the main pulmonary artery through the right ventricle. Immediately after the inferior vena cava was divided and the left and right atrial appendages were amputated, the pulmonary artery was flushed with 100 ml/kg of one of the cold (4°C) preservation solutions described later here at a pressure of 18 cm H_2O. After the trachea was ligated and cut at an end-inspiratory phase during the ventilation, the donor heart and lungs were removed en bloc. Among five donor lung territories (right upper, middle and lower lobe, caudal lobe, and left lung), the right lower and caudal lobes

(Received in original form February 3, 2004; accepted in final form August 18, 2004)

Supported in part by a grant-in-aid for Fundamental Scientific Research from the Education Ministry of Japan 13,770,743 (T.G.).

Correspondence and requests for reprints should be addressed to Akitoshi Ishizaka, M.D., Department of Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160–8582, Japan. E-mail: ishizaka@cpnet.med.keio.ac.jp

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 170. pp 1239–1246, 2004
Originally Published in Press as DOI: 10.1164/rccm.200402-146OC on August 27, 2004
Internet address: www.atsjournals.org

immediately after excision were designated as "preischemic lung" and used for the evaluation of lung injury. Both the left lung and the right upper and middle lobes were wrapped in a bed of gauze soaked with 50 ml of preservation solution and stored at 4°C for a cold ischemic period. Then an orthotopic left lung transplantation was performed using a cuff technique for vessel and bronchial anastomoses, and blood flow and ventilation to the transplanted lung were reestablished after 6 hours of ischemia. The right upper and middle lobes of the donor were recovered from the preservation solution at the initiation of reperfusion and were designated as "postischemic lung" and used for the evaluation of lung injury. After the chest closure and awakening from the anesthesia, recipient animals were housed freely in room air. The rats were killed after 4 hours of reperfusion, and both the right and left lungs were evaluated for lung injury. Additional details are provided in the online supplement.

Experimental Protocol

Twenty-five Lewis rats were divided into three experimental groups (Figure 1). (*1*) In the transplantation group (n = 10), Euro-Collins solution was used as lung preservation solution, and physiologic saline was continuously infused via the tail vein at 0.75 ml/hour during 4 hours of reperfusion. (*2*) In the treatment group (n = 10), 1 mg/ml of TACE inhibitor (Y-41654) was added to the Euro-Collins solution. This modified Euro-Collins solution was used as lung preservation solution, and Y-41654 dissolved in physiologic saline was continuously infused via the tail vein at 3 mg/kg/hour (0.75 ml/hour) during 4 hours of reperfusion. (*3*) In the sham group (n = 5), after the same anesthesia, intubation, and artificial ventilation were given as in the previously mentioned two groups, only thoracotomy, dissection of the left hilum, and chest closure were performed. After dissection of the left hilum, physiologic saline was continuously infused via the tail vein at 0.75 ml/hour for 4 hours.

At 3 hours after transplantation, the recipient animals received an intravenous injection of 37-kBq ^{125}I-labeled bovine serum albumin into the tail vein as a permeability tracer. Next, 10 minutes before killing, a 10-kBq ^{51}Cr-labeled red blood cell was injected into the tail vein as a pulmonary blood tracer. Animals were killed by an intraperitoneal injection of 50 mg pentobarbital after 4 hours of reperfusion.

Blood samples were collected by cardiocentesis from each recipient at two points during the process: at the time of surgery (just before reperfusion) and at the time of killing. Bronchoalveolar lavage was performed in four specimens: (*1*) preischemic lung, (*2*) postischemic lung, (*3*) contralateral lung (recipient's right lung obtained at killing), and (*4*) graft lung (recipient's transplanted left lung obtained at killing). The BALF was centrifuged at 400 × *g* and 4°C for 10 minutes, and the supernatant was stored at −80°C until needed. Cell counts were done using a modified hemacytometer method. For differential counting of BALF cells, cell monolayers were prepared from BALF by cytocentrifugation. Differential counts were performed on 200 cells from smears stained with a modified Wright's stain. Additional details are provided in the online supplement.

Lung Water and ^{125}I-labeled Albumin Index

Pulmonary edema was assessed by using a wet-to-dry weight ratio (W/D ratio). The isotope-specific radioactivity of excised lungs, blood, and BALF samples was measured. Transvascular flux of ^{125}I-albumin was assessed by using the concentration ratio of lung tissue to plasma and that of BALF supernatant to plasma per unit weight, which were used as parameters of pulmonary endothelial and alveolar septal damage, respectively. Blood contamination in lung tissue and BALF was corrected using ^{51}Cr counts. Additional details are provided in the online supplement.

Cytokine Determination

The cytokine concentration of TNF-α, monocyte chemotactic protein-1 (MCP-1), and cytokine-induced neutrophil chemoattractant-1 (CINC-1) in each sample was determined using commercially available ELISA kits. Additional details are provided in the online supplement.

Measurement of Neutrophil Elastase Activity

Neutrophil elastase (NE) activity in BALF was determined with the synthetic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroaniline, which is highly specific for NE, by the method described by Yoshimura and colleagues (21).

Histopathologic Examination

A portion of each lung was fixed with 4% paraformaldehyde for histopathologic and immunohistochemical examinations. After incubation with anti-rat TNF-α antibody, sections 5 μm thick were treated sequentially with rabbit anti-goat IgG conjugated to peroxidase. In the same manner, dewaxed paraffin sections were stained by the immunoperoxidase method using anti-rat intercellular adhesion molecule-1 (ICAM-1) antibody and goat anti-mouse IgG conjugated to peroxidase. Finally, color was developed with diaminobenzidine, and the sections were counterstained with hematoxylin.

Hematoxylin and eosin–stained sections of the graft lung tissue (n = 4 in each experimental group) were examined under light microscopy for a histologic scoring of lung injury. A pulmonary pathologist, who was blinded to the animals' group assignments, scored the histologic level of lung injury according to the following scoring system: grade 0, no abnormal findings; grade 1, patchy alveolar edema with widened interstitium with occasional erythrocytes in air spaces; grade 2, patchy hemorrhage and diffuse alveolar edema with widened interstitium; and grade 3, diffuse alveolar hemorrhage, massive hemorrhage, and necrosis of parenchyma.

Next, a quantitative morphometric analysis was performed on the findings in 10 randomly selected fields per slide of graft lung tissue subjected to TNF-α immunostaining or ICAM-1 immunostaining. The staining intensity of TNF-α– and ICAM-1–immunostained images in the lung tissue was measured as previously published (22–25). The total staining intensity was calculated as the summation of optical density of the positive area and was shown using the arbitrary units. Fields

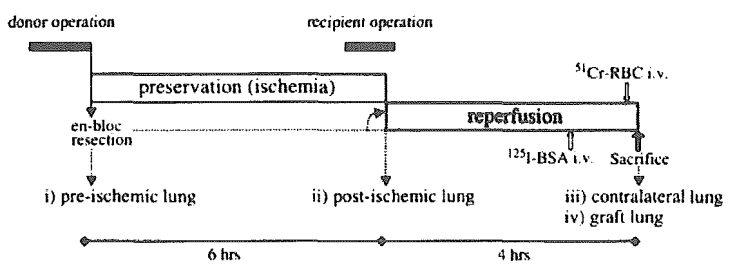


Figure 1. Schematized experimental protocol. First, the heart and lungs were excised en bloc from the donor, immersed in the preservation solution, and kept in a refrigerator for a cold ischemic period. The recipient operation was started at an appropriate time. The recipient's left lung was excised, and the preserved left lung was transplanted orthotopically. Subsequently, the recipient's chest was closed, and after recovery from anesthesia, the recipient was housed freely in room air. After 4 hours of reperfusion, the recipient was killed and evaluated for right and left lung injury. Thus, we established a model of ischemia–reperfusion lung injury resulting from 6 hours of ischemia and 4 hours of reperfusion. At 3 hours after the start of reperfusion, 37 kBq of ^{125}I-bovine serum albumin (BSA) were injected into the tail vein as a permeability tracer, and 10 kBq of ^{51}Cr-labeled erythrocytes were injected as a pulmonary blood tracer 10 minutes before killing. RBC = red blood cell.

containing large vessels or bronchi were excluded. The total staining intensity was normalized to alveoli per field to control for inflation of the lung. The results were averaged from four rats in each experimental group. An observer, blinded to the experimental group, examined complete digitized images of specimens with Canvas 9J and Photoshop, version 7.0. Additional details are provided in the online supplement.

Measurement of HMGB-1 Protein

HMGB-1 protein in BALF was quantified by ELISA with monoclonal antibodies, which do not cross-react with HMGB-2 by the method described by Yamada and colleagues (26).

Western Blot Analysis of Soluble E-cadherin Fragments

To detect the soluble fragments of E-cadherin in BALF, a Western blot analysis was performed using a rabbit polyclonal antiserum against the synthetic peptides for rat E-cadherin. Additional details are provided in the online supplement.

Statistical Analysis

All data were expressed as the mean ± SEM. One-way analysis of variance and a Tukey-Krammer multiple comparisons test were used to detect statistical significance between groups. Student's *t* test for paired data was used to detect significant changes in plasma TNF-α level within a group. A p value of less than 0.05 was used to determine significant differences between means.

RESULTS

TNF-α Level in Plasma and BALF

The plasma TNF-α level at the time of killing significantly increased as compared with at the time of surgery in the transplantation group, but not in the other two groups (Figure 2A). In BALF of the transplantation group, the TNF-α level increased in the bilateral lungs (graft and contralateral lungs) after the transplantation, particularly in the graft lung (Figure 2B). In the treatment group, however, the BALF TNF-α levels in the bilateral lungs after the surgery were not significantly different from those in the sham group and were significantly lower than those in the transplantation group.

Lung Water and ^{125}I-labeled Albumin Index

In the transplantation group, the W/D ratio significantly increased in the graft and contralateral lungs compared with those in the preischemic and postischemic lungs (Figure 2C). Furthermore, the W/D ratio was significantly higher in the graft lung than in the contralateral lung. In comparison of W/D ratios between the transplantation and treatment groups, the W/D ratio of the graft lung was significantly lower in the treatment group than in the transplantation group. However, no significant differences were observed in the W/D ratio of the preischemic, postischemic, or contralateral lungs between the transplantation and treatment groups.

Both lung tissue to plasma and BALF supernatant to plasma ratios in the graft lung were significantly lower in the treatment group than in the transplantation group (Figures 2D and 2E). In contrast, in the contralateral lung, there were no significant differences in the lung tissue to plasma or BALF supernatant to plasma ratio between the transplantation and treatment groups.

BALF Findings

In the transplantation group, no significant differences were observed in either the total or differential cell counts between the preischemic and postischemic lungs, and macrophages were predominant in the differential cell count in both lungs, which may have been the BALF feature of an almost normal lung (Table 1). The total cell count in BALF increased after reperfusion in the bilateral lungs, and the increase was marked in the

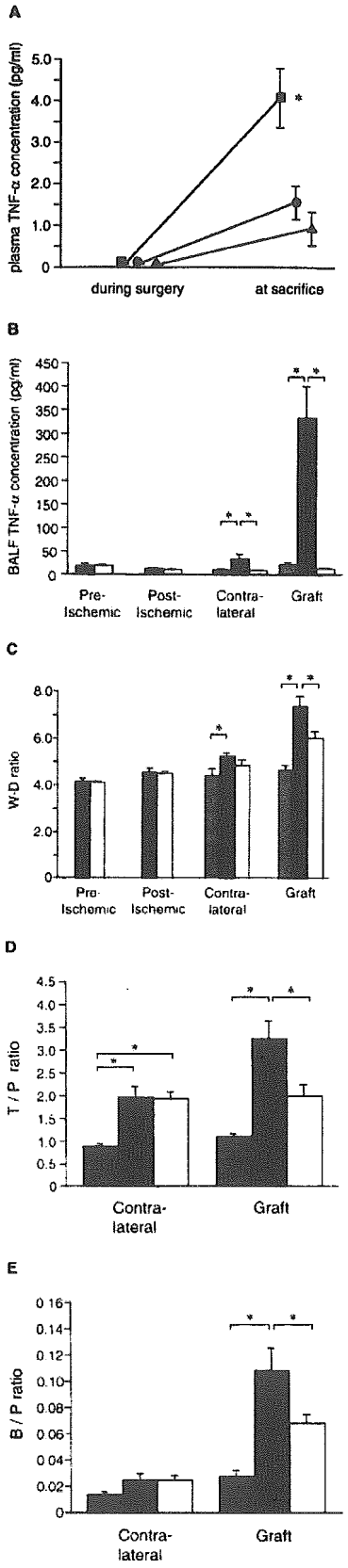


Figure 2. Tumor necrosis factor-α (TNF-α) level, lung water, and ^{125}I-labeled albumin index. (*A*) In the transplantation group, plasma TNF-α significantly increased with reperfusion ($p = 0.018$) (*closed triangle*, sham-operated group; *closed square*, transplantation group; *closed circle*, treatment group). *$p < 0.05$. (*B*) In the transplantation group, bronchoalveolar lavage fluid (BALF) TNF-α increased in the bilateral lungs (graft lung in particular) after reperfusion. In contrast, in the treatment group, BALF TNF-α in the bilateral lungs after reperfusion was significantly lower than that in the transplantation group. (*C*) In the transplantation group, the wet-to-dry weight ratio (W/D ratio) was significantly higher in the bilateral lungs (graft lung in particular) after reperfusion. In the graft lung, the W/D ratio was significantly lower in the treatment group than in the transplantation group. (*D* and *E*) Both lung tissue to plasma and BALF supernatant to plasma ratios in the graft lung were significantly lower in the treatment group than in the transplantation group. (*B–E*) *Gray bar*, sham group; *black bar*, transplantation group; *white bar*, treatment group. *$p < 0.05$.

TABLE 1. BRONCHOALVEOLAR LAVAGE FLUID CELL PERCENTAGE DIFFERENTIAL

	Total Cell (/μl)	Neutrophil (%)	Lymphocyte (%)	Macrophage (%)	Eosinophil (%)
Sham Group					
Right	245 ± 58	5.8 ± 0.9	2.4 ± 0.3	91.8 ± 0.6	0
Left	304 ± 19	29.5 ± 7.2	4.7 ± 1.0	65.8 ± 7.9	0
Transplantation Group					
Preischemic	291 ± 52	2.3 ± 0.8	3.3 ± 0.5	94.4 ± 1.1	0
Postischemic	268 ± 53	2.9 ± 0.6	5.4 ± 1.7	91.6 ± 1.8	0.1 ± 0.1
Contralateral	557 ± 79	11.4 ± 2.2	4.5 ± 1.2	84.0 ± 3.0	0.1 ± 0.1
Graft	927 ± 79	50.6 ± 5.7	16.7 ± 3.2	32.7 ± 6.8	0
Treatment group					
Preischemic	267 ± 63	0.9 ± 0.2	3.7 ± 1.6	95.4 ± 1.5	0
Postischemic	229 ± 43	2.2 ± 0.2	2.5 ± 0.6	95.3 ± 0.6	0
Contralateral	286 ± 64	25.6 ± 5.5	8.1 ± 5.3	66.3 ± 7.0	0
Graft	343 ± 58	36.3 ± 6.8	42.6 ± 9.6	21.1 ± 3.8	0

Values shown are mean ± SEM.

graft lung. As for the differential counts, neutrophils increased after reperfusion in the bilateral lungs, particularly in the graft lung. In the treatment group, the total and differential cell counts were similar to those in the transplantation group in both preischemic and postischemic lungs, and increases with reperfusion in the total cell and neutrophil counts in the graft lung BALF were less than those in the transplantation group.

In comparison of the BALF cell differential by the actual number of cells in the transplantation group graft lung and treatment group graft lung, the number of lymphocytes was similar, but the numbers of neutrophils and macrophages were significantly lower in the treatment group (Figure 3). As for differential cell count in BALF in the contralateral lung, the numbers of neutrophils and lymphocytes were similar in the transplantation and treatment groups, and only the number of macrophages was significantly lower in the treatment group.

Histopathologic Findings

In the transplantation group, graft lung, alveolar hemorrhages, and interstitial thickening were marked, and growth of type II alveolar epithelial cells was observed (Figures 4B and 4D). In contrast, in the treatment group graft lung, only faint alveolar hemorrhages were present, and no obvious interstitial thickening was noted (Figures 4C and 4D).

In TNF-α immunostaining, no staining was observed in the lung tissue or macrophages in the sham group left lung, suggesting that TNF-α synthesis was not induced (Figures 4E and 4H). In the transplantation group graft lung, pale staining of the macrophage cytoplasm was mainly observed (Figures 4F and 4H). In contrast, macrophages were entirely stained deeply in the treatment group graft lung (Figure 4G). MemTNF-α on macrophages may have increased because of inhibition of TACE. We found also deeply stained TNF-α in the alveolar epithelium and interstitium, which may have been memTNF-α originating from alveolar epithelial cells, fibroblasts, and vascular endothelial cells (Figure 4H).

Chemokine, Adhesion Molecules, and NE Activity

MCP-1 and CINC-1 in graft lung BALF were significantly lower in the treatment group than in the transplantation group (Figures 5A and 5B). In the contralateral lung, no significant differences were observed in the BALF MCP-1 or CINC-1 level between the transplantation and treatment groups.

On ICAM-1 immunostaining, almost no staining was observed in the lung tissue in the sham group left lung (Figures 5C and 5F). The vascular endothelium and alveolar epithelium were deeply stained in the transplantation group graft lung but were only weakly stained in the treatment group graft lung (Figures 5D–5F).

The NE activity in BALF increased in the transplantation group graft lung, whereas it remained significantly lower in the treatment group graft lung (Figure 5G). In the contralateral lung, the NE activity in BALF of transplantation and treatment groups was similar.

HMGB-1 and E-cadherin Soluble Form

HMGB-1 in BALF was significantly higher in the transplantation group graft lung than in the sham group left lung (Figure 6A). In the treatment group, the HMGB-1 level in graft lung BALF was significantly reduced compared with that in the transplantation group.

The soluble form of E-cadherin in BALF can be a parameter of lung epithelial damage. In this study, the BALF supernatants of the bilateral lungs after surgery in each experimental group were subjected to Western blotting described in the METHODS section (Figure 6B). A band corresponding to the molecular weight of E-cadherin soluble form, 85 kD, was detected in all samples. The variations in band intensity were concordant with the changes in BALF supernatant to plasma ratio, an isotopically derived index of alveolar septal damage, and the band intensity was macroscopically different between the transplantation group graft lung and treatment group graft lung.

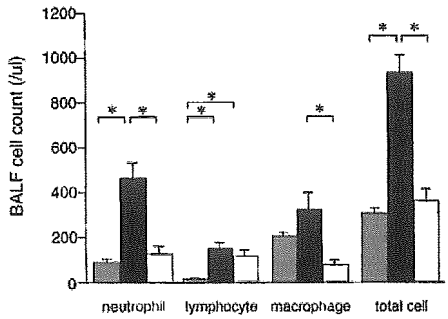


Figure 3. BALF cell count of the graft lung. In the treatment group, the number of lymphocytes was similar to that in the transplantation group, but the neutrophil and macrophage counts were significantly lower, resulting in significant decrease in the total cell count (*gray bar,* sham group; *black bar,* transplantation group; *white bar,* treatment group). *$p < 0.05$.

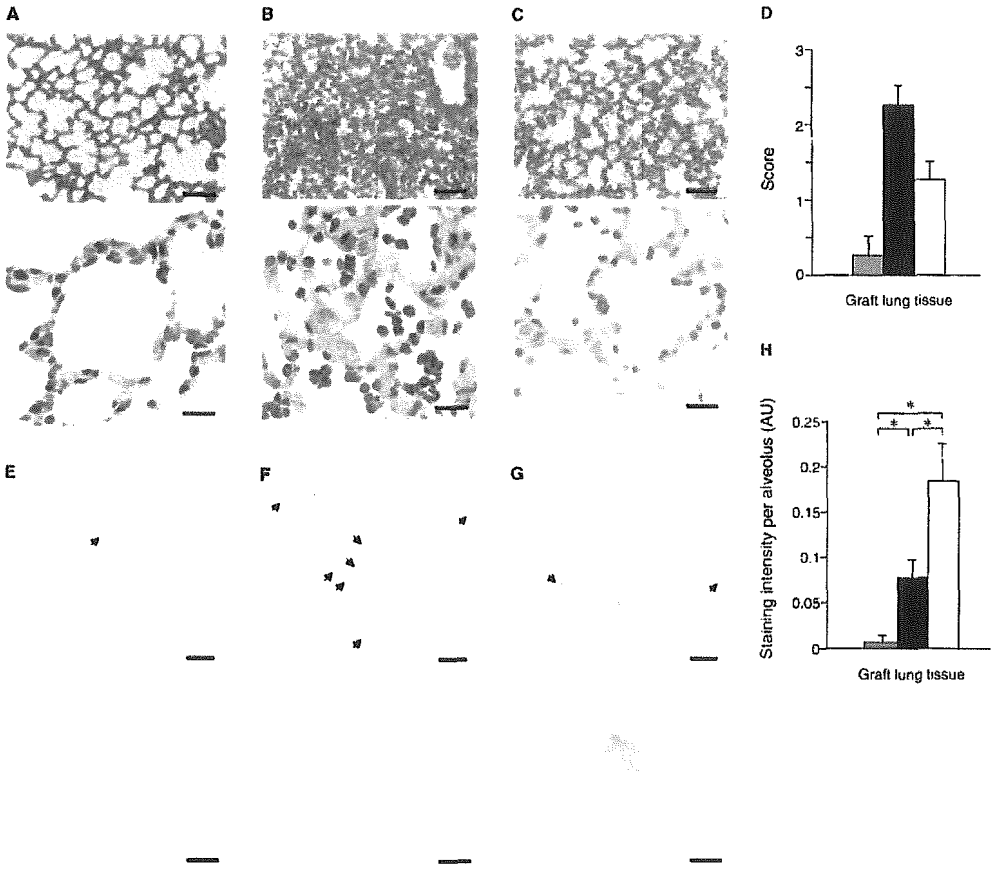


Figure 4. Histopathologic findings. (*A–D*) Hematoxylin and eosin staining of the graft lung tissue. (*A*) Sham group. (*B*) Transplantation group. (*C*) Treatment group. In the transplantation group, alveolar hemorrhages and interstitial thickening were marked, whereas only faint alveolar hemorrhages were observed in the treatment group. Scale bars, 150 μm (*top row*) or 25 μm (*bottom row*). (*D*) Histologic score for the graft lung injury. Data are shown as mean ± SEM (for grading, *see* the METHODS section). (*E–H*) TNF-α immunostaining of the graft lung tissue. (*E*) Sham group. (*F*) Transplantation group. (*G*) Treatment group. The *top row* presents immunostained lung tissues, and *arrows* indicate alveolar macrophages. The *bottom row* presents immunostained macrophages alone. In the sham group, lung tissue or macrophages were not immunostained. In the transplantation group, macrophage cytoplasm was mainly stained. In the treatment group, the entire macrophage, the alveolar epithelium, and the interstitium were prominently stained. Scale bars, 25 μm (*top row*) or 2 μm (*bottom row*). (*H*) Quantification of TNF-α immunostaining intensity in the graft lung tissue. In the graft lung, TNF-α staining was significantly increased in the treatment group. AU = arbitrary units. *$p < 0.05$. (*D* and *H*) *Gray bar,* sham group; *black bar,* transplantation group; *white bar,* treatment group.

DISCUSSION

In this experiment, TACE inhibitor was used to inhibit cleavage of memTNF-α and release of soluble TNF-α in a rat lung transplantation model. Regional and systemic treatment with TACE inhibitor attenuated the severity of reimplantation injury of the graft lung with decreased lung local concentration of soluble TNF-α, suggesting the involvement of soluble TNF-α in post-transplantation acute lung inflammation. The decrease in soluble TNF-α inhibited chemokine production and ICAM-1 expression in the lung local region, reducing accumulation of inflammatory cells and tissue-injuring activity.

In this study, lung injury was evaluated 4 hours after reperfusion, whereas previous reports described lung injury evaluated within a relatively short time after reperfusion, such as 15 minutes to 2 hours (19, 27, 28). In an experiment tracing the time course of lung ischemia–reperfusion disorder in rats, the development of the disorder was biphasic (29). Neutrophil-independent mild lung injury occurred approximately 30 minutes after reperfusion, and neutrophil-dependent severe lung injury occurred approximately 4 hours after reperfusion (29). Based on investigations of myeloperoxidase activity in lung homogenates and histology (29, 30), accumulation of neutrophils in the lung is considered to require 2 or more hours after reperfusion. In this experiment, therefore, lung injury was evaluated 4 hours after reperfusion because our main objective was to investigate the role of TACE in neutrophil-dependent lung injury.

We used alveolar–septal permeability calculated from the leakage of radiolabeled albumin for the evaluation of lung injury. In previous lung transplantation experiments, acute lung injury was evaluated mostly based on oxygenation capability (e.g., arterial O_2 tension), hemodynamics of the pulmonary circulation (pulmonary vascular resistance, pulmonary arterial flow), lung compliance, W/D ratio, or histologic examinations (19, 27, 28). We selected the isotopically derived indices because an accurate and quantitative evaluation of lung injury was anticipated (31, 32).

Inhibition of the release of soluble TNF-α decreased neutrophils and macrophages in the local graft lung. Thus, we investigated whether TACE inhibition affects recruitment of inflammatory cells, including chemokine induction and expression of adhesion molecules (33). In the treatment group, the levels of CINC-1, a C-X-C chemokine, and MCP-1, a C-C chemokine, in BALF recovered from the graft lung were significantly lower in cooperation with a decrease in the local concentration of soluble TNF-α compared with those in the transplantation group. In a preliminary *in vitro* study, we confirmed that the TACE inhibitor itself had no inhibitory action on CINC-1 or MCP-1 (data not shown). We inferred that the impaired release of TNF-α could be responsible for the decreased production of CINC-1 and MCP-1, which we observed. It was also revealed that the expression of ICAM-1, an adhesion molecule on endothelial cells mediating firm adhesion of neutrophils and endothelial cells, in the graft lung tended

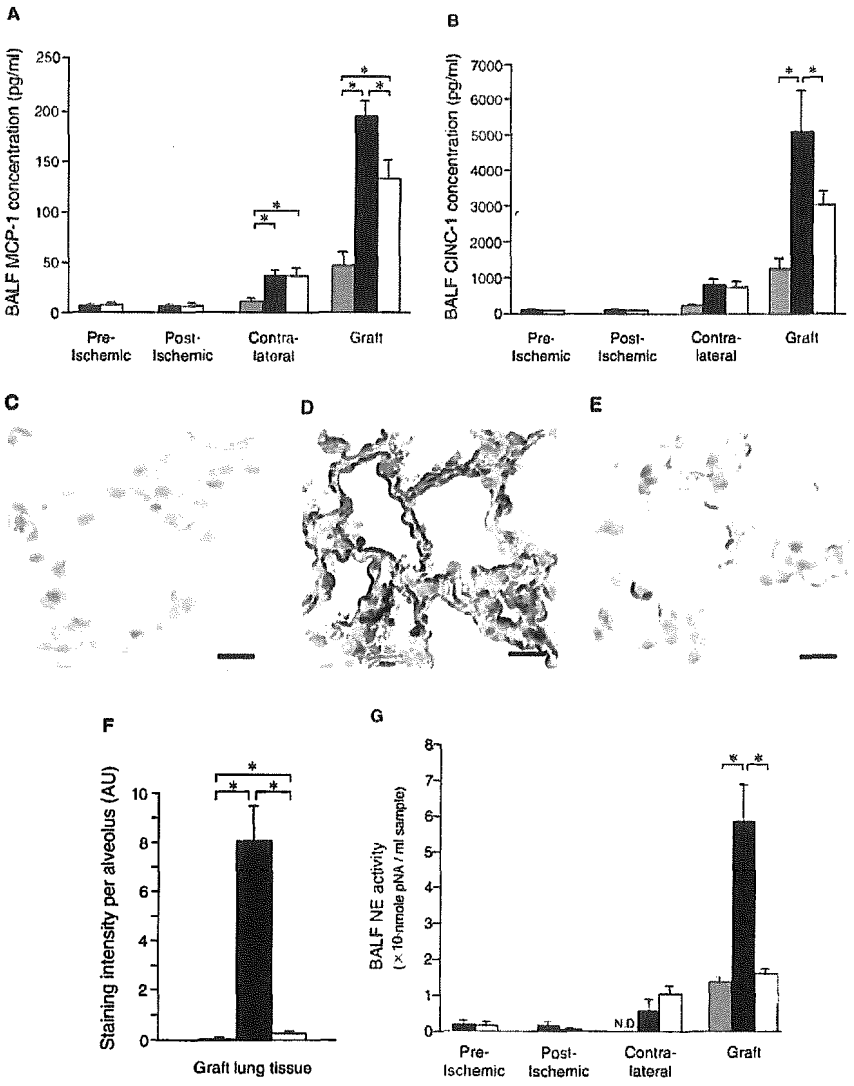


Figure 5. Chemokine, adhesion molecule, and neutrophil elastase (NE) activity. (*A* and *B*) Both monocyte chemotactic protein-1 (MCP-1) and cytokine-induced neutrophil chemoattractant-1 (CINC-1) in the graft lung BALF were significantly lower in the treatment group than in the transplantation group. (*C–F*) Intercellular adhesion molecule-1 (ICAM-1) immunostaining of the graft lung tissue. (*C*) Sham group. (*D*) Transplantation group. (*E*) Treatment group. In the transplantation group, vascular endothelium and alveolar epithelium were deeply stained, whereas these were only weakly stained in the treatment group. *Scale bars* = 25 μm. (*F*) Quantification of ICAM-1 immunostaining intensity in the graft lung tissue. In the graft lung, ICAM-1 staining was significantly increased in the transplantation group. AU = arbitrary units. (*G*) BALF NE activity increased in the transplantation group graft lung, but it was significantly decreased in the treatment group graft lung. N.D. = not detected; p-NA = p-nitroaniline. (*A, B, F,* and *G*) *Gray bar,* sham group; *black bar,* transplantation group; *white bar,* treatment group. *$p < 0.05$.

to be lower in the treatment group. Based on the findings mentioned previously here, we concluded that soluble TNF-α plays a critical role in the development of reimplantation lung injury by regulating accumulation of neutrophils and macrophages via induction of chemokines (CINC-1 and MCP-1) and upregulation of ICAM-1, which are consistent with previous reports (29, 30, 34, 35). Several investigators have shown that the administration of an antineutrophil antibody, a neutralizing monoclonal antibody against interleukin-8, or an anti–P-selectin antibody prevented neutrophil infiltration and tissue injury in the setting of lung transplantation (29, 30, 34). Also, the blocking antibody to MCP-1 was reported to be highly protective against lung reperfusion injury (35).

NE activity in the graft lung was decreased in the treatment group compared with the transplantation group. Because NE activity reflects the magnitude of neutrophil sequestration in the lung, decreased NE activity might be due to the decreased neutrophil recruitment by TACE inhibition, leading to attenuated lung injury.

In this study, we observed increased TNF-α levels in BALF after the transplantation, particularly in the graft lung, and in plasma 4 hours after transplantation, not during surgery, which suggests that the TNF-α production after reperfusion was mostly due to residential macrophages in the graft lung directly stimulated with ischemia–reperfusion and that plasma TNF-α may have been spilled over from the graft lung. It has been reported that during reperfusion oxidant stress activates macrophage nuclear factor-κB, leading to increased production of mRNA for TNF-α (36). Furthermore, soluble TNF-α in epithelial lining fluid, which is produced in the graft lung, may be more critical than TNF-α in plasma in the development of reimplantation injury. From this perspective, controlling the injurious potential of neutrophils and macrophages in the local lung may be effective as therapeutic strategy for reimplantation injury, and the addition of TACE inhibitor to the preservation solution may have significant clinical benefits. TACE inhibitor in the lung preservation solution could be distributed sufficiently in the lung tissue during 6 hours of preservation and may have formed the preparatory condition for inhibition of TNF-α release from residential macrophages after reperfusion (37). In addition, when the lung was stored in the preservation solution with TACE inhibitor, the total number and differential of BALF cells did not change after storage, which suggests exposure to the drug had no harmful effects.

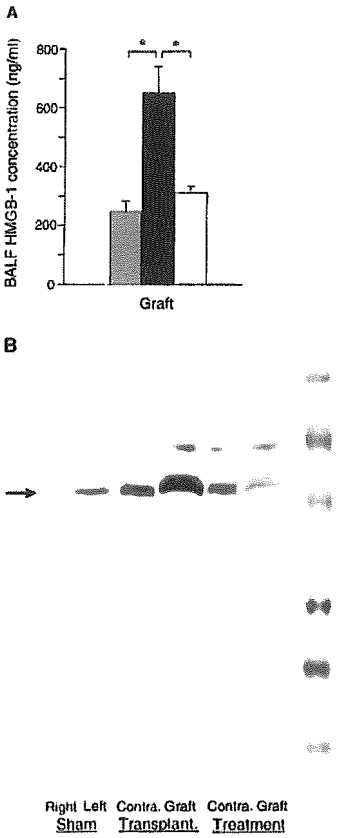


Figure 6. High mobility group box-1 (HMGB-1) protein and epithelial cadherin (E-cadherin)–soluble form. (*A*) The BALF HMGB-1 level was increased in the transplantation group graft lung, but it was significantly decreased in the treatment group graft lung (*gray bar*, sham group; *black bar*, transplantation group; *white bar*, treatment group). *$p < 0.05$. (*B*) Western blot analysis of E-cadherin–soluble form in the BALF from the bilateral lungs after the surgery. Molecular markers were applied in the extreme *right lane* (*blue*, 206 kD; *magenta*, 124 kD; *green*, 83 kD; *violet*, 42 kD; *orange*, 32 kD; *red*, 19 kD; and *blue*, 7 kD). A band consistent with the molecular weight of E-cadherin soluble form, approximately 85 kD, was detected in all samples. The BALF supernatant to plasma ratios were from the left, 0.0056, 0.0190, 0.0320, 0.1446, 0.0271, and 0.0683, and the band intensity changed in concordance with the BALF supernatant to plasma ratio. Contra = contralateral.

In this experiment, TACE inhibitor did not significantly attenuate a slight increase in lung transvascular permeability in the contralateral lung, which could be induced by inflammatory mediators in systemic circulation (35). In the transplantation group, the TNF-α level in the BALF recovered from the contralateral lung was significantly higher than in other two groups. In contrast, no significant differences were observed in the BALF MCP-1 level, CINC-1 level, NE activity, or number of BALF neutrophils between the transplantation and treatment groups in the contralateral lung. Lung injury of the contralateral lung caused by indirect stimulation may not be mediated only by TNF-α but by several other mediators.

In addition to its role as a transcriptional regulatory factor, HMGB-1 protein has recently been identified as a late mediator of endotoxin lethality (38). Macrophages release HMGB-1 when exposed to early, acute cytokines, indicating that it is also positioned as a mediator of inflammatory conditions. Previous studies have demonstrated that TNF-α functions as an upstream regulator of HMGB-1 release (39). Our observations are consistent with the role of HMGB-1 as a distal inflammatory mediator and with its release induced primarily by soluble TNF-α. The role of HMGB-1 in the pathogenesis of acute lung injury seems distinct from any effects of earlier acting proinflammatory cytokines. It was reported that intratracheal administration of HMGB-1 causes acute lung injury, and antibodies against HMGB-1 attenuate lipopolysaccharide-induced pulmonary edema (40). In that study, anti–HMGB-1 antibody did not significantly reduce the concentrations of proinflammatory cytokines in lipopolysaccharide-induced lung injury, suggesting that HMGB-1 occupies a more distal position in the proinflammatory cascade (40). In our model as well, the delayed release of HMGB-1 may have participated in the downstream development of post-transplantation lung injury.

Soluble E-cadherin in BALF was measured as a direct index of alveolar epithelial injury. E-cadherin is a 120-kD transmembrane glycoprotein, predominantly localized to the lateral cell border and associated with the contractile cytoskeleton (41). Most epithelial cells express E-cadherin, and soluble E-cadherin may reflect loosened intercellular adhesion among epithelial cells. In this study, Western blot analysis showed soluble E-cadherin released into BALF, which might suggest the involvement of alveolar epithelial disorder in reimplantation lung injury.

In summary, TACE inhibition markedly attenuated reimplantation injury. A modest reduction in lung graft failure and early mortality rates caused by reimplantation injury would exert a significant effect on overall long-term survivals. The lung specimens were preserved for 6 hours in this study, but it is possible that organ preservation solution containing TACE inhibitor reduces reperfusion injury even though it was kept in ischemic condition for a prolonged period, and clinically, the ability to preserve donor lungs effectively for longer periods of time would increase the pool of potential donors.

Some studies have used anti–TNF-α antibodies for TNF-α inhibition in lung ischemia–reperfusion injury (42, 43) and noted improvement in lung injury. On the other hand, studies have reported that the complete blocking of the physiologic functions of TNF-α tends to result in pulmonary infections such as tuberculosis and fungal infection (44, 45). The administration of anti–TNF-α antibody in the perioperative period of lung transplantation might be risky and impractical. In general, the half-life of antibodies in the blood is relatively long up to 14 days, and anti–TNF-α antibody binds to soluble and memTNF-α in a specific and high-affinity manner, blocking all biological activities of TNF-α. Therefore, we speculate that anti–TNF-α antibodies might have disadvantages, such as potential for making the host vulnerable to infections. In contrast, TACE inhibitor inhibits only soluble TNF-α, which is distributed through the systemic circulation, and does not completely block the biological activities of TNF-α. Because TACE inhibitor with high water solubility used in the experiment has a very short half-life of approximately 20 minutes, we presume that the drug might have some advantage compared with anti–TNF-α antibodies because its blood concentration could be easily controlled, making it safer and more convenient compared with long-acting anti–TNF-α antibody.

In the graft lung after transplantation, TNF-α synthesis begins in alveolar macrophages in response to reperfusion stimulation after ischemia, but memTNF-α expressed on the cell surface does not cause pulmonary disorder. The lung injury cascade via soluble TNF-α does not start until memTNF-α is cleaved by TACE. TACE may be very important as a trigger of TNF-α–induced lung injury.

Conflict of Interest Statement: T.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; F.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; I.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Mitsubishi Pharma Corporation (Osaka, Japan) for supplying the TACE inhibitor and pertinent information regarding the drug.

References

1. Zaas D, Palmer SM. Respiratory failure early after lung transplantation. *Chest* 2003;123:14–16.
2. Chatila WM, Furukawa S, Gauchan JP, Criner GJ. Respiratory failure after lung transplantation. *Chest* 2003;123:165–173.
3. Thabut G, Vinatier I, Stern JB, Leseche G, Liorat P, Fournier M, Mal H. Primary graft failure following lung transplantation. *Chest* 2002;121: 1876–1882.
4. Fiser SM, Kron IL, Long SM, Kaza AK, Kern JA, Cassada DC, Jones DR, Robbins MC, Tribble CG. Influence of graft ischemic time on outcomes following lung transplantation. *J Heart Lung Transplant* 2001;20:1291–1296.
5. Serrick C, Giaid A, Reis A, Shennib H. Prolonged ischemia is associated with more pronounced rejection in the lung allograft. *Ann Thorac Surg* 1997;63:202–208.
6. Fiser SM, Tribble CG, Long SM, Kaza AK, Kern JA, Jones DR, Robbins MK, Kron IL. Ischemia-reperfusion injury after ling transplantation increases risk of late bronchiolitis obliterans syndrome. *Ann Thorac Surg* 2002;73:1041–1048.
7. Strieter RM, Kunkel SL, Bone RC. Role of tumor necrosis factor-α in disease states and inflammation. *Crit Care Med* 1993;21:S447–S463.
8. Ermert M, Pantazis C, Duncker HR, Grimminger F, Seeger W, Ermart L. In situ localization of TNFalpha/beta, TACE and TNF receptors TNF-R1 and TNF-R2 in control and LPS-treated lung tissue. *Cytokine* 2003;22:89–100.
9. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castnar BJ, Stocking KL, Reddy P, Srinivasan S, *et al.* A metalloproteinase disintegrin that releases tumor-necrosis factor-α from cells. *Nature* 1997;385:729–733.
10. Moss ML, Catherine JSL, Milia ME, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, *et al.* Cloning of a disintegrin metalloproteinase that processes precursor tumor-necrosis factor-α. *Nature* 1997;385:733–736.
11. Shurety W, Pagan JK, Prins JB, Stow JL. Endocytosis of uncleaved tumor necrosis factor-α in macrophages. *Lab Invest* 2001;81:107–117.
12. Sherry B, Cerami A. Cachectin/tumor necrosis factor exerts endocrine, paracrine, and autocrine control of inflammatory responses. *J Cell Biol* 1988;107:1269–1277.
13. Karp SE, Hwu P, Farber A, Restifo NP, Kriegler M, Mule JJ, Rosenberg SA. In vivo activity of tumor necrosis factor (TNF) mutants: secretary but not membrane-bound TNF mediates the regression of retrovirally transduced murine tumor. *J Immunol* 1992;149:2076–2081.
14. Grell M, Douni E, Wajant H, Lohden M, Clauss M, Maxeiner B, Georgopoulos S, Lesslauer W, Kollias G, Pfizenmaier K, *et al.* The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 1995;83:793.
15. Mohler KM, Sleath PR, Fitzner JN, Cerretti DP, Alderson M, Kerwar SS, Torrance DS, Otten-Evans C, Grrenstreet T, Weerawarna K, *et al.* Protection against a lethal dose of endotoxin by an inhibitor of tumor necrosis factor processing. *Nature* 1994;370:218–220.
16. Aversa G, Punnonen J, de Vries JE. The 26-kD transmembrane form of tumor necrosis factor α on activated CD4$^+$ T cell clones provides a costimulatory signal for human B cell activation. *J Exp Med* 1993; 177:1575–1585.
17. Solomon KA, Covington MB, DeCicco CP, Newton RC. The fate of pro-TNF-α following inhibition of metalloprotease-dependent processing to soluble TNF-α in human monocytes. *J Immunol* 1997;159: 4524–4531.
18. Olleros ML, Guler R, Corazza N, Vesin D, Eugster HP, Marchal G, Chavarot P, Mueller C, Garcia I. Transmembrane TNF induces an efficient cell-mediated immunity and resistance to Mycobacterium bovis bacillus Calmette-Guerin infection in the absence of secreted TNF and lymphotoxin-α. *J Immunol* 2002;168:3394–3401.
19. Aeba R, Keenan RJ. Hardesty RL, Yousem SA, Hamamoto I, Griffith BP. University of Wisconsin solution for pulmonary preservation in a rat transplant model. *Ann Thorac Surg* 1992;53:240–246.
20. Mizobuchi T, Sekine Y, Yasufuku K, Fujisawa T, Wilkes DS. Comparison of surgical procedures for vascular and airway anastomoses that utilize a "modified" non-suture external cuff technique for experimental lung transplantation in rats. *J Heart Lung Transplant* 2004;23:889–893.
21. Yoshimura K, Nakagawa S, Koyama S, Kobayashi T, Honma T. Roles of neutrophil elastase and superoxide anion in leukotriene B4-induced lung injury in rabbit. *J Appl Physiol* 1994;76:91–96.
22. Junaid A, Amara FM. Osteopontin: correlation with interstitial fibrosis in human diabetic kidney and PI3-kinase-mediated enhancement of expression by glucose in human proximal tubular epithelial cells. *Histopathology* 2004;44:136–146.
23. Lehr HA, Mankoff DA, Corwin D, Santeusanio G, Gown AM. Application of Photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. *J Histochem Cytochem* 1997;45: 1559–1565.
24. Esteban JM, Kandalaft PL, Mehta P, Odom-Maryon TL, Bacus S, Battifora H. Improvement of the quantification of estrogen and progesterone receptors in paraffin-embedded tumors by image analysis. *Am J Clin Pathol* 1993;99:32–38.
25. Bolender RP, Hyde DM, Dehoff RT. Lung morphometry: a new generation of tools and experiments for organ, tissue, cell, and molecular biology. *Am J Physiol* 1993;265:L521–L548.
26. Yamada S, Inoue K, Yakabe K, Imaizumi H, Maruyama I. High mobility group protein 1 (HMGB1) quantified by ELISA with a monoclonal antibody that does not cross-react with HMGB2. *Clin Chem* 2003; 49:1535–1537.
27. Fisher S, Hopkinson D, Liu M, Keshavjee S. Raffinose improves the function of rat pulmonary grafts stored for twenty-four hours in low-potassium dextran solution. *J Thorac Cardiovasc Surg* 2000;119:488–492.
28. Hausen B, Ramsamooj R, Hewitt CW, Schroder F, Beuke M, Poets CF, Schafers HJ. The importance of static lung inflation during organ storage. *Transplantation* 1996;62:1720–1725.
29. Eppinger MJ, Jones ML, Deeb GM, Bolling SF, Ward PA. Pattern of injury and the role of neutrophils in reperfusion injury of rat lung. *J Surg Res* 1995;58:713–718.
30. Sekido N, Mukaida N, Harada A, Nakanishi I, Watanabe Y, Matsushima K. Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8. *Nature* 1993;365:654–657.
31. Naidu BV, Woolley SM, Farivar AS, Thomas R, Fraga C, Mulligan MS. Simvastatin ameliorates injury in an experimental model of lung ischemia-reperfusion. *J Thorac Cardiovasc Surg* 2003;126:482–489.
32. Dodd-o JM, Hristopoulos ML, Faraday N, Pearse DB. Effect of ischemia and reperfusion without airway occlusion on vascular barrier function in the in vivo mouse lung. *J Appl Physiol* 2003;95:1971–1978.
33. Colletti LM, Cortis A, Lukacs N, Kunkel SL, Green M, Strieter RM. Tumor necrosis factor up-regulates intercellular adhesion molecule 1, which is important in the neutrophil-dependent lung and liver injury associated with hepatic ischemia and reperfusion in the rat. *Shock* 1998;10:182–191.
34. Naka Y, Toda K, Kayano K, Oz MC, Pinsky DJ. Failure to express the P-selectin gene or P-selectin blockade confers early pulmonary protection after lung ischemia or transplantation. *Proc Natl Acad Sci USA* 1997;94:757–761.
35. Eppinger MJ, Deeb GM, Bolling SF, Ward PA. Mediators of ischemia-reperfusion injury of rat lung. *Am J Pathol* 1997;150:1773–1784.
36. Baeuerle PA, Baltimore D. NF-κB: ten years later. *Cell* 1996;87:13–20.
37. Chiang CH, Hsu K, Yan HC, Harn HJ, Chang DM. PGE_1, dexamethasone, U-74389G, or Bt_2-cAMP as an additive to promote protection by UW solution in I/R injury. *J Appl Physiol* 1997;83:583–590.
38. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, *et al.* HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999;285:248–251.
39. Wang H, Vishnubhakat JM, Bloom O, Zhang M, Ombrellino M, Sama A, Tracey KJ. Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. *Surgery* 1999;126:389–392.
40. Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ. Cutting edge: HMG-1 as a mediator of acute lung inflammation. *J Immunol* 2000; 165:2950–2954.
41. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 1991;251:1451–1455.
42. Krishnadasan B, Naidu BV, Byrne K, Fraga C, Verrier ED, Mulligan MS. The role of proinflammatory cytokines in lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 2003;125:261–272.
43. Khimenko PL, Bagby GJ, Fuseler J, Taylor AE. Tumor necrosis factor-alpha in ischemia and reperfusion injury in rat lungs. *J Appl Physiol* 1998;85:2005–2011.
44. Gomez-Reino JJ, Carmona L, Valverde VR, Mola EM, Montero MD. Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk. *Arthritis Rheum* 2003;48:2122–2127.
45. Hage CA, Wood KL, Winer-Muram HT, Wilson SJ, Sarosi G, Knox KS. Pulmonary cryptococcosis after initiation of anti-tumor necrosis factor-α therapy. *Chest* 2003;124:2395–2397.

日外会誌　106（1）：31～37，2005

特　集

外科領域における輸血と血液製剤の現状と展望

7. 人工血液

慶應義塾大学外科

小林　紘一

キーワード　人工血液，人工酸素運搬体（人工赤血球），ヘモグロビン小胞体（Hemoglobin-vesicles，HbV），アルブミンヘム，人工血小板

I. 内容要旨

赤血球輸血はK. Landsteinerによる血液型の発見によりその有効性と安全性が確立され，臨床医学は大きく進歩した．しかし，緊急輸血時の血液型判定の煩雑さ，長期保存ができない，輸血を介するウィルス感染やgraft versus host disease（GVHD）の副作用の問題などがある．これらに対処するために血液型を考慮せずに使用でき，しかも棚置きできる人工酸素運搬体が開発されつつある．また，血小板輸血はがん，造血器腫瘍などに対する抗腫瘍療法の際の血小板減少や，外科手術の際には病態によっては欠くことのできない治療法となる．しかし，血小板需要の増加と血小板の短い保存期間のために起こる供給の不足や，血小板輸血後のウィルス感染症の副作用などの問題がありこれに対処するために，人工血小板の開発も始まっている．

II. はじめに

輸血は現代医学にとって欠かせない治療法の1つである．血液は血球成分と血漿成分から構成されるが，輸血に際しては全血輸血ではなく成分輸血が，また赤血球輸血では同種血輸血に変わり自己輸血が推奨されるようになった．これは血液成分の有効利用になるばかりでなく，輸血に伴う副作用の予防という面でも重要である．医工学の進歩は緊急事態に対応でき，同種血輸血を回避できるような人工血液の開発を可能にしつつある．本稿では血球成分の代替物である人工酸素運搬体（人工赤血球）と人工血小板について述べる．

III. 人工酸素運搬体

現在のところ人工酸素運搬体には①フッソの化合物で酸素溶解能の高い液体であるperfluorocarbon（PFC），②ヒトやウシのヘモグロビンを利用するもの，③酸素運搬体としてヘム自体も合成し，このヘムの担体としてアルブミンを用いたもの（アルブミンヘム）などがある（図1）[1][2]．PFCは酸素運搬機能が低いことや使用時に酸素吸入を必要とすること，粒子が網内系に蓄積するなどの問題が指摘されている．

ヒト由来の分子状のヘモグロビンをそのまま投与した時には図2に挙げたような問題が存在するのでこれを改善するための方法が必要である[2]．その方法には2種類あり，1つはcell freeまたはacellular型と呼ばれるもので，ヘモグロビンを分子内あるいは分子間架橋させて分子量を増加させ，腎からの排出を防止し，血中滞留時間を調整しようとするものであり，修飾ヘモグロビンとも呼ばれている（図1のpoly-HbやPEG-Hb）[2]．これらは，膠質浸透圧が高くなるためにヘモグロビン濃度が5～8g/dlに制限されるため酸素運搬能が十分でないといわれている．これらの製品は欧米で研究されており，前臨床や，臨床治験に入ったものがあり，その状況を表1に示す[1]．しかしこれらは分子が小さいので血管内皮に接近するため血管内皮により産生されるnitric oxide（NO）を吸着し，血圧上昇をきたすのでこの欠点をどのように修正するかが問題となって

ARTIFICIAL BLOOD
Koichi Kobayashi
Department of Surgery, School of Medicine, Keio University, Tokyo, Japan

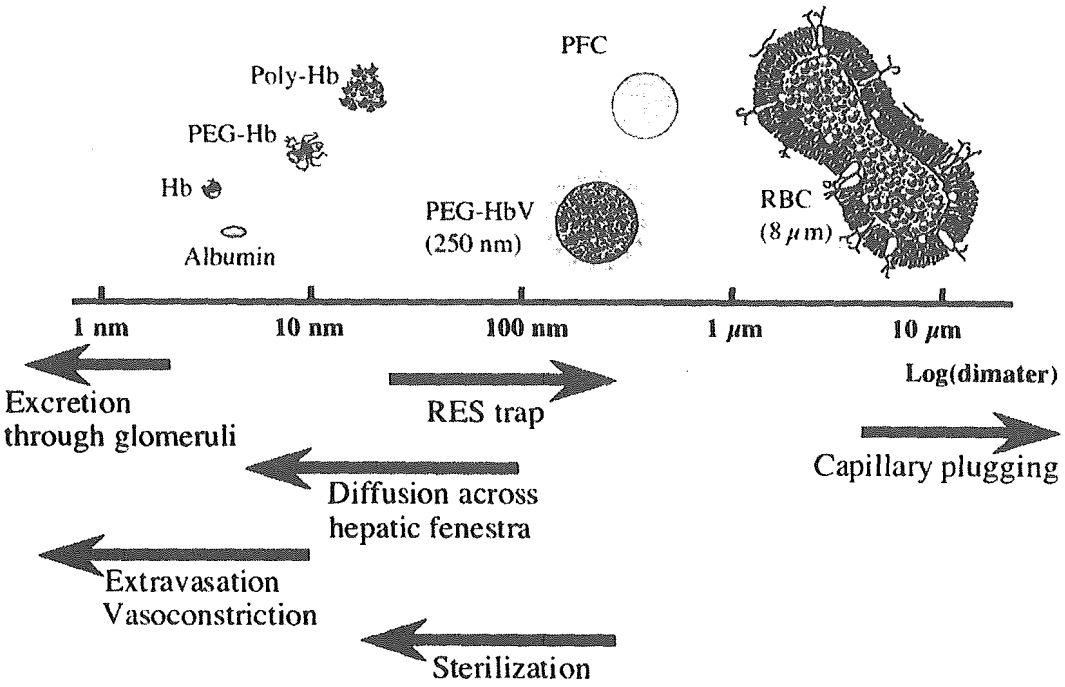


図1　What is the optimal dimension of O_2 carriers?

表1　Hemoglobin based oxygen carriers in development as red blood cell substitutes

Product	Hb source	Technology	Developer	Status
DCL-Hb (HemAssist)	Human red cells	α-α crosslinked Hb	Baxter Healthcare (Deerfield, IL, U.S.A.)	Phase III (trauma) (suspended)
RHb1.1/1.2 (Optro)	*E. coli*	Recombinant human αα fused Hb	Baxter Healthcare (Somatogen < 1998)	Phase II (discontinued)
RHb2.0	*E. coli*	Recombinant Hb	Baxter Healthcare	Preclinical
HBOC-201 (Hemopure)	Bovine red blood cells	Gultaraldehyde polymerization	Biopure (Cambridge, MA, U.S.A.)	BLA filed (elective surgery). Approved for clinical use in S. Africa
Human POE-Hb (PHP)	Human red cells	PEG conjugation	Curacyte (Apex) (Munich, Germany)	Phase III (septic shock)
Hb-raffimer (Hemolink)	Human red cells	Oligermerization with o-raffinose	Hemosol (Toronto, Canada)	Phase III (cardiac surgery) (suspended)
Pyridoxal polyHb (PolyHeme)	Human red cells	PLP-Hb polymerized Hb with glutaraldehyde	Northfield Laboratories (Evanston, IL, U.S.A.)	Phase III (trauma) Filed BLA
Hemospan	Human red cells	Conjugated with maleiimide PEG	Sangart (San Diego, CA, U.S.A.)	Phase II (elective surgery)
HemoZyme	Human red cells	Polynitroxylated Hb	SynZyme (Irvine, CA, U.S.A.)	Preclinical
PolyHb-SOD-CAT	Bovine red cells	Hb modified with SOD and catalase	McGill University (Montreal, Canada)	Preclinical
PEG-Hb	Bovine red cellls	PEG conjugated Hb	Enzon (Piscataway, NJ, U.S.A.)	Phase Ia (discontinued)
OxyVita	Human/bovine red cells	Stabilized Hb with sebacoly diaspirin	IPBL Pharm. (Goshen, NJ)	Preclinical
HemoTech	Bovine red cells	Modified Hb with o-ATP, o-adenosine, and glutathione	HemoBioTech (Amarillo, TX, U.S.A.)	Preclinical

いる[1)2)].

一方，土田らにより開発されたヘモグロビンをリポソームの人工膜でカプセル化した細胞型（cellular 型）の酸素運搬体は，膠質浸透圧や粘度の調整が容易である．本物質はヘモグロビン小胞体(hemoglobin vesicle-HbV)と呼ばれておりこの HbV はリポソームの内側に35% 以上の濃度でヘモグロビン粒子が詰め込まれており，十分な酸素運搬能力を持っている[2),3)~5)]．リポソー

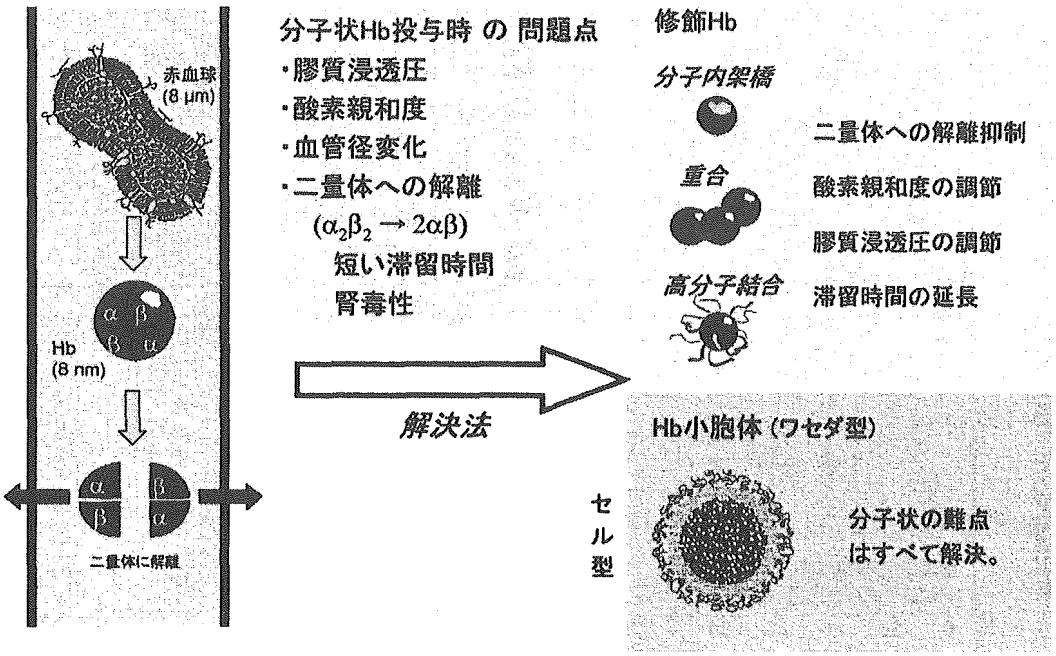


図2　分子状 Hb の問題点と解決法

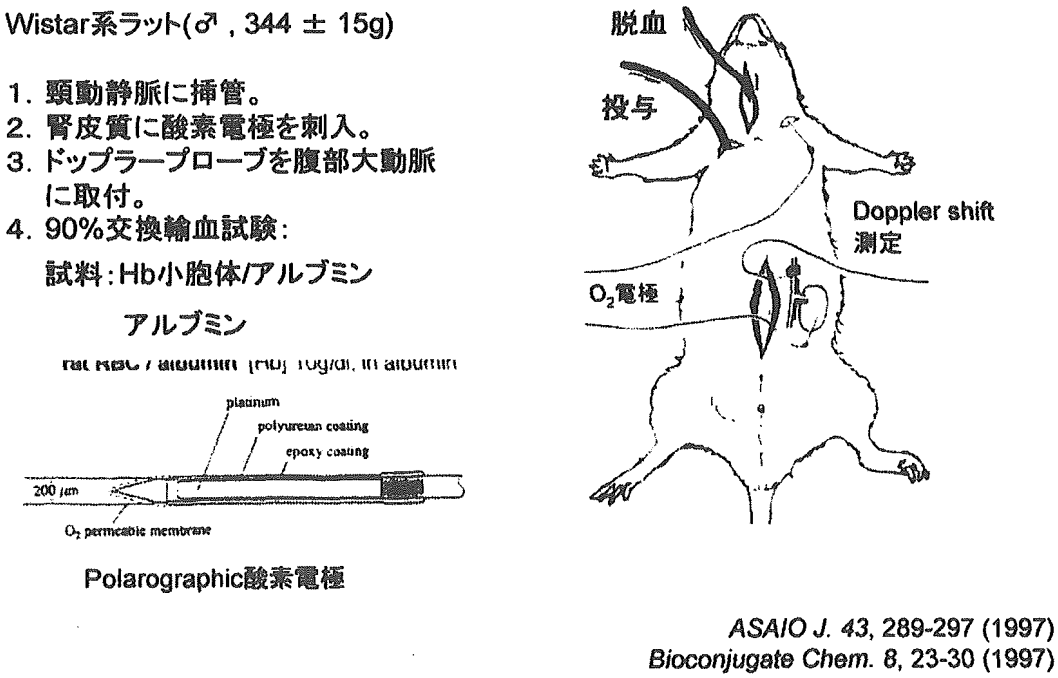
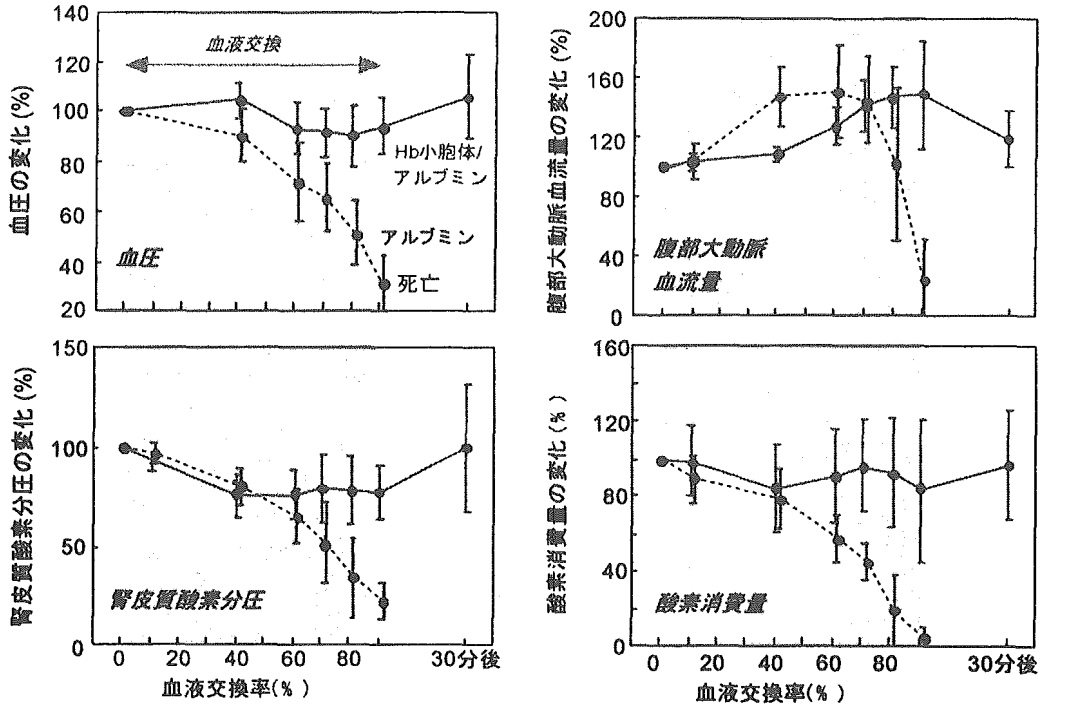


図3　Hb 小胞体による 90% 交換輸血試験

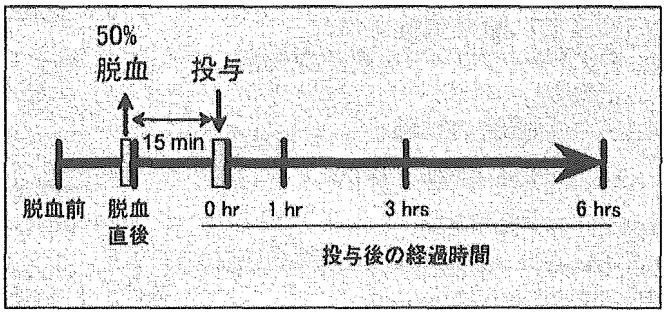
ムの表面は親水性高分子(ポリエチレングリコール)により被覆されておりこの親水性高分子により，HbV同士の融合や血液との接触による凝集を防止することができる[2)5)]．この製品の直径は約 250nm である(図1，図2)．この製品は室温溶液状態で2年間，また乾燥状態ではさらに長期の保存が可能である．粉末は適量の溶液に溶かすだけで使用できるという特徴がある[2)]．HbV を用いたラットにおける 90% 交換輸血実験（図3）のデーターを示す（図4)[6)]．5% アルブミンに HbV を溶解した液で交換輸血を行った群ではそれぞれのコ



ASAIO J. **43**, 289-297 (1997)
Bioconjugate Chem. **8**, 23-30 (1997)

図4　Hb小胞体による90%交換輸血試験

1. 雄性Wistar rat (280 ± 27 g)
2. 吸入麻酔(1.5% sevoflurene)
3. 右頸静脈に挿管
4. 測定項目:
   血圧, 心拍数, ヘマトクリット,
   PaO_2, $PaCO_2$, pH, 乳酸値
   組織病理



蘇生液:

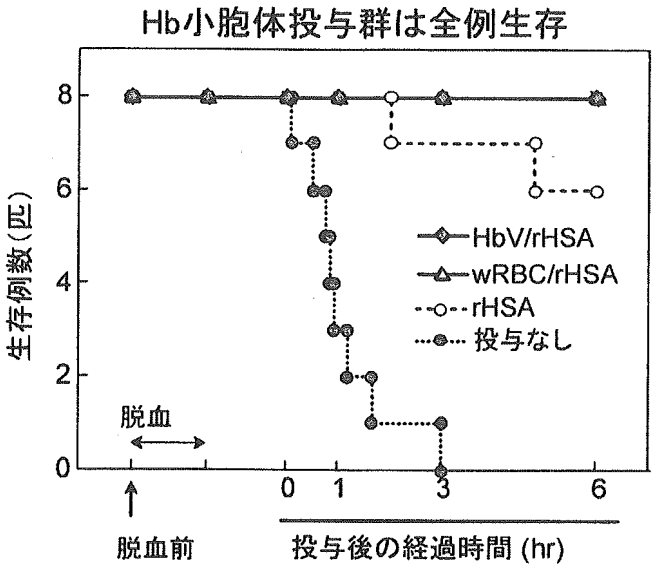
HbV/rHSA	HbV suspended in rHSA, [Hb] = 8.6 g/dL
wRBC/rHSA	Washed RBC suspended in rHSA, [Hb] = 8.6 g/dL
rHSA	recombinant human serum albumin, 5 g/dL

Crit. Care Med. **32**, 539-545 (2004)

図5　Hb小胞体の投与による出血ショックからの蘇生

ントロール値を維持したが，5%アルブミンのみで交換輸血を行った群では全例死亡した．

HbVのショックからの蘇生実験(図5)では，5%のrecombinantアルブミンにHbVを溶解した液を投与したHbV/rHSA群では，recombinantアルブミンに自己洗浄赤血球を浮遊させた液を投与した群と同様に8例全例が6時間後も生存していた（図6)[7]．このようにHbVが酸素運搬体として機能していることが証明され



Crit. Care Med. **32**, 539-545 (2004)

図6　出血ショックと蘇生

た．現在われわれは臨床応用を踏まえた研究を継続中である[8]．

前述したアルブミンヘムは，アルブミンの立体構造の中にヘムを包接させた酸素運搬体であるが，アルブミンによる膠質浸透圧をもったresuscitation fluidとしての可能性を持ち，現在動物実験が進行中である[9][10]．

人工酸素運搬体には出血性ショックに対する治療薬としてばかりでなく，体外循環の補填液としての利用，心筋梗塞，脳梗塞などへの虚血部位[10]への酸素運搬，固形腫瘍を酸素化することによる抗癌剤や放射線治療に対する増感作用[10]なども期待されている．

IV. 人工血小板

血小板製剤は保存可能な時間が短い(72時間)，週末などに入手困難，ウィルス感染や，発熱などの反応を伴うことがあること，生体内での寿命が短いので頻回投与が必要なことが多い，濃厚血小板の使用量が増加の傾向にあるなどの理由で止血を目的とした人工血小板の開発が行われている[11][12][13]．

血小板の最も重要な機能は傷害された血管内皮に接触し，粘着，凝集反応により止血を行うことであるが，人工血小板に求められている機能には出血部位で内皮下組織が露呈された部位にのみ作用し，効率よく血栓を形成し，出血をコントロールすることであり，組織の修復後には血栓は溶解し，吸収されなければならない．人工血小板開発の基本は膜受容体蛋白とそれらを運ぶ担体にこれを固相化することで，膜受容体蛋白としてはフォンビルブランド因子（vWF）の受容体であるGP1b/1Xや凝集に必要なGP1b/IIIaなどが，また膜受容体蛋白の担体としてはリポソーム，ヒアトルブミンや赤血球などが考えられている．現在開発されつつある人工血小板としては，以下のようなものがある[1]．

Fibrinogen-RBC, Erythorocytes with covalently bound fibrinogen：正常ヒト赤血球を担体とし，これにフィブリノーゲンを共有結合させたもので，血小板減少ラットに輸注するとラットの出血時間が短縮するという[14]．

Thromboerythrocyte：血小板の絶対値を補う目的で開発されたものでフィブリノーゲンのアミノ酸配列でGP1b/IIIaとの結合に関係するAraGly-Asp（RGD）配列を持った合成ペプチドAcCGGRGDF-NH2を共有結合で赤血球膜に固定したもので，この赤血球は活性化血小板（GP1b/IIIa）と特異的に反応する．自己の赤血球を担体として使用することも可能である[15]．

Plateletsome：可溶化した血小板膜に固定したもの．GP1b，GpIIIb/IIIa，GpIVなど15種類以上の蛋白質が含まれている[16]．

Infusible platlet membrane(IPM)：期限切れヒト血小板を反復凍結融解により破壊し，粉末にして乾燥させたもので4℃で36カ月保存可能であるという．主成