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研究成果の刊行物・別冊

(2004.4～2005.3)

Biomaterials

Safety Evaluation of an Artificial O_2 Carrier as a Red Blood Cell Substitute by Blood Biochemical Tests and Histopathology Observations

YUBIN HUANG,* TERUYUKI KOMATSU,* HISASHI YAMAMOTO,† HIROHISA HORINOUCHI,‡ KOICHI KOBAYASHI,‡ AND EISHUN TSUCHIDA*

Recombinant human serum albumin (rHSA) incorporating synthetic heme with a covalently linked proximal base (albumin-heme [rHSA-heme]) is an artificial O_2 carrier that can transport O_2 like hemoglobin does in the blood stream. To evaluate the clinical safety of this compound, 20% and 40% exchange transfusions with rHSA-heme into anesthetized rats were followed by blood biochemical tests and histopathologic observations for 7 days. In the 20% rHSA-heme group, a total of 30 analytes by blood biochemical tests showed almost the same values as those observed in the reference 20% rHSA group. Although some abnormal values for liver parameters were found in the 40% rHSA-heme group, they returned to normal after 7 days. Histopathologic observations indicated that the administration of rHSA-heme in a volume of 20% total blood volume did not produce any negative side effects on the vital organs. *ASAIO Journal* 2004; 50:525–529.

Hemoglobin (Hb)-based O_2 carriers have been studied as red blood cell substitutes or as an O_2 therapeutic.[1–3] The advantages of these O_2 carriers are 1) the absence of a blood type antigen and infectious virus, 2) a small particle size for penetration through constricted vessels where red blood cells cannot penetrate, and 3) stability for long-term storage. The first generation products (Hemolink, Polyheme, and Hemopure),[4] which are currently in clinical testing, have been followed by second generation materials (Hemospan™ [MP4],[5] adenosine-GSH-Hb,[6] and SOD-catalase-Hb[7] under development. We have developed an entirely synthetic O_2 carrier without using Hb. Recombinant human serum albumin (rHSA) incorporates a synthetic heme (2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis {α,α,α,α-*o*-(1-methylcyclohexanamido)}phenylporphinatoiron(II) (**Figure 1**), providing an artificial hemoprotein (albumin-heme [rHSA-heme]), which has the potential to bind and release O_2 under physiologic conditions (pH 7.3, 37°C) in the same manner as Hb.[8–12] Because the rHSA-heme molecule is totally synthetic, there is absolutely no concern for infection with pathogens and virus. The *in vitro* experiments have indicated that the rHSA-heme solutions have a high compatibility with blood cell components.[12] Furthermore, we found that vasoconstriction was not observed after administration of rHSA-heme into the circulatory system because of its low permeability through the vascular endothelium; rHSA-heme does not deplete nitric oxide (endothelium-derived relaxing factor).[13] Our recent study of a 30% exchange transfusion with rHSA-heme after 70% hemodilution with 5% (by weight) rHSA in anesthetized rats showed that injection of this material improved the circulatory blood volume and resuscitated the animals from shock.[14] To evaluate the clinical safety of this material as a red blood cell substitute, 20% and 40% exchange transfusions in anesthetized rats were studied using blood biochemical testing and histopathologic observations for 7 days.

Materials and Methods

Preparation of rHSA-Heme

The rHSA (Albrec, 25% by weight) was provided by the NIPRO Corp. (Osaka, Japan). The rHSA-heme solution (rHSA: 4.9% rHSA by weight, pH 7.45; heme: 2.8 mmol/L heme, colloid osmotic pressure (COP) 18 torr, osmolarity 300 mOsm, viscosity 1.1 cP, and endotoxin <0.1 EU/ml) was prepared according to our previously reported procedure.[10,14,15] The other physicochemical properties of rHSA-heme (molecular weight 72.3 kDa, O_2 binding affinity [$p_{1/2}O_2$] 37 torr, and isoelectric point 4.8) have been reported elsewhere.[10] The half-life of the oxygenated rHSA-heme against the ferric state was 9 hours at 37°C *in vitro*.[10]

Exchange Transfusion with rHSA-Heme in Anesthetized Rats

The investigations were carried out in 60 male Wister rats (312 ± 3.0 gm). The details of the experimental setup (anesthesia and catheterization) were the same as our former protocol reported elswhere.[14,15] The total blood volume of a rat was estimated to be 64 ml/kg body weight. The 20% exchange transfusion was achieved by four cycles of repeated blood withdrawal *via* the common carotid artery (1 ml, 1 ml/min) and the rHSA-heme infusion into the femoral vein (1 ml, 1 ml/min; 20% rHSA-heme group, n = 12). The 40% exchange transfu-

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

This work was partially supported by a grant-in-aid for scientific research (No. 16350093) from JSPS, a grant-in-aid for exploratory research (No. 16655049) from MEXT Japan, and health science research grants (regulatory science) from MHLW Japan.

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DOI: 10.1097/01.MAT.0000144361.60280.DA

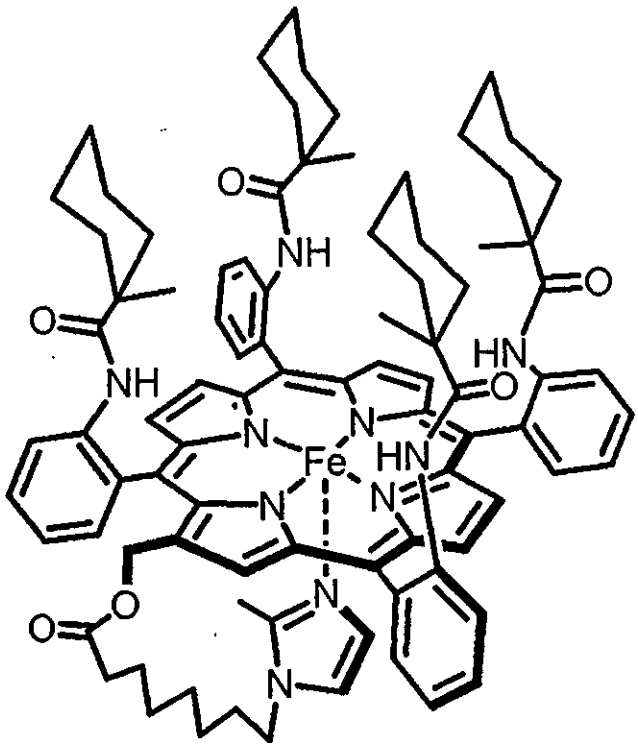

Figure 1. Chemical compound of entirely synthetic O_2 carrier without using Hb.

sion was performed by eight cycles of identical withdrawal/infusion (40% rHSA-heme group, n = 12). As a reference, a 5 gm/dl rHSA solution was given to other rats in the same ratios (20% rHSA group and 40% rHSA group, n = 12 each). Furthermore, 12 rats without blood exchange (anesthesia and surgery only) were also used as the control group. After the blood exchange transfusion, the animals were sutured and returned to their home cages. Their appearance and body weights were observed on days1, 3, and 7 after surgery.

After days 1 and 7, 4 ml of venous blood was taken from 6 rats in each group and centrifuged at 4°C (Beckman Coulter Co., Optima LE-80K for 3,500 rpm, 10 min). The plasma phase was frozen (−20°C) for the blood biochemical tests. The rats were sacrificed by venesection and the weights of the isolated liver, kidney, spleen, lung, and heart were measured. All animal handling and care were in accordance with NIH guidelines. The protocol details were approved by the Animal Care and Use Committee of Keio University.

Blood Biochemical Tests

A total of 30 analytes (total protein, albumin, albumin-globulin ratio, aspartate aminotransferase [AST], alanine aminotransferase [ALT], lactate dehydrogenase [LDH], alkaline phosphatase, γ-glutamyltransferase, leucine aminopeptidase [LAP], choline esterase, total bilirubin, direct bililubin, creatinine, blood urea nitrogen, uric acid, amylase, lipase, creatine phosphokinase, total cholesterol, free cholesterol, cholesterol ester [EChol], β-lipoprotein, high density lipoprotein [HDL] cholesterol, neutral fat [*i.e.*, triglyceride, TG], total lipid, free fatty acid, phospholipids [PhL], K^+, Ca^{2+}, and Fe^{3+}) were measured by the Kyoto Microorganism Institute (Kyoto, Japan).

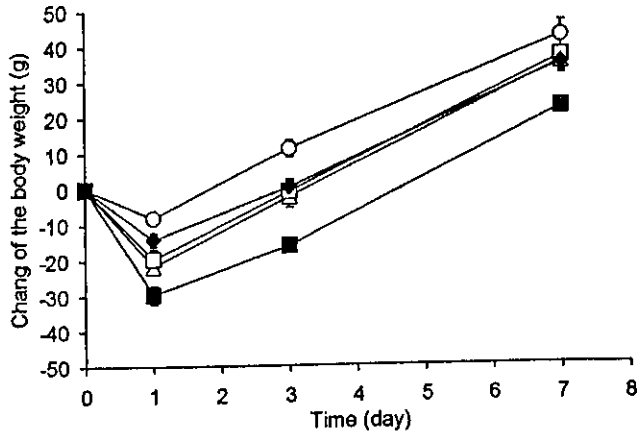

Figure 2. Body weight changes in male Wistar rats subjected to 20% and 40% blood exchange with recombinant human serum albumin (rHSA) and rHSA-heme solutions. Each value represents the mean±SEM. ○, Control group; ◆, 20% rHSA-heme group; △, 20% rHSA group; ■, 40% rHSA-heme group; and □, 40% rHSA group.

Histopathologic Observations

Paraffin sections were prepared from the 10% formalin fixed organs and stained with hematoxylin-eosin stain and Berlin blue. All histopathologic observations were carried out by Panapharm Laboratories Co., Ltd. (Kumamoto, Japan).

Data Analysis

The data for increased body weight, organ weight, and blood biochemical tests are expressed as mean ± SEM. A statistical analysis was performed using the Bartlett test followed by the Tukey-Kramer multiple comparison test. Values of $p < 0.05$ were considered significant. The statistical analytical software was StatView (SAS Institute Inc., Cary, NC).

Results

Appearance and Body Weight

In the control, 20% rHSA, and 20% and 40% rHSA-heme groups, all animals survived for 7 days without any change in their appearance and behavior. In contrast, one rat died after 3 days in the 40% rHSA group; the remaining five rats in this group did survive for 7 days.

In the control group, the change in body weight from the basal value decreased by 8.3 ± 1.5 gm after 1 day (**Figure 2**). However, it increased to 11.2 ± 2.4 gm after 3 days and to 42.1 ± 4.3 gm after 7 days.

In the 20% and 40% rHSA groups, body weights decreased by 21.8 ± 1.8 gm and 19.6 ± 2.8 gm, respectively, after 1 day, but they returned to basal levels after 3 days and increased to 34.9 ± 2.9 gm and 36.3 ± 4.0 gm, respectively, after 7 days.

In the 20% rHSA-heme group, body weight decreased by 14.3 ± 1.8 gm after 1 day. It recovered to the starting level after 3 days and increased to 34.9 ± 2.9 gm after 7 days. This change was almost the same as those observed in the 20% and 40% rHSA groups. In the 40% rHSA-heme group, the weight declined by 29.8 ± 2.5 gm after 1 day. Although it increased to 22.1 ± 2.1 gm after 7 days, the differences from the other groups were constant during the experimental period.

Weight of Vital Organs

We could not find any remarkable difference in the weights of the vital organs of the 20% rHSA and 20% rHSA-heme groups compared with the control group, except for spleen weight (**Figure 3**). Spleen weight was significantly increased in the 20% rHSA group after 1 and 7 days and in the 20% rHSA-heme group after 7 days.

In the 40% rHSA group, liver weight was significantly decreased after 1 day, and spleen weight increased after 7 days compared with that in the control group. In the 40% rHSA-heme group, spleen weight was significantly increased after 1 and 7 days *versus* the control group. There was no remarkable change in the weights of lungs, heart, liver, and kidney.

Blood Biochemical Tests

The 30 analytes from the blood biochemical tests of rat plasma are summarized in **Figure 4.** In the 20% and 40% rHSA groups, most of the parameters did not show any significant difference *versus* the control group, except for the iron decrease in the 20% rHSA group after 1 day and the choline esterase decrease after 7 days.

In the 20% rHSA-heme group, the decreases in LAP, TG, total lipid, PhL and iron after 1 day were significant compared with values in the control group. After 7 days, all analytes returned to the same levels as in the control group. With respect to the 20% rHSA group, the decrease in PhL after 1 day and the decrease in iron after 7 days were significant. In the 40% rHSA-heme group, the increase in total protein and AST and the decrease in total cholesterol, EChol, HDL cholesterol, and total lipid after 1 day were significant relative to the control group. Moreover, large increases in ALT and LDH were observed in three of the six samples. In comparison with the 40% rHSA group, the increase in LDH after 1 day, the decrease in EChol and HDL cholesterol after 1 day, the increase in HDL cholesterol after 7 days, and the decrease in TG after 7 days were significant.

Histopathologic Observations

A mild or modulate extramedullary hematopoiesis in the spleen was often found in all groups after the surgical operation at 7 days. In the 20% rHSA group, a slight erythrophagocytosis in the Kupffer cells of the liver (three samples) was observed after 7 days. In the 40% rHSA group, slight mineralization in the tubule of the kidney (three samples) after 1 day and a mild hyaline droplet in the tubule epithelial cells of the

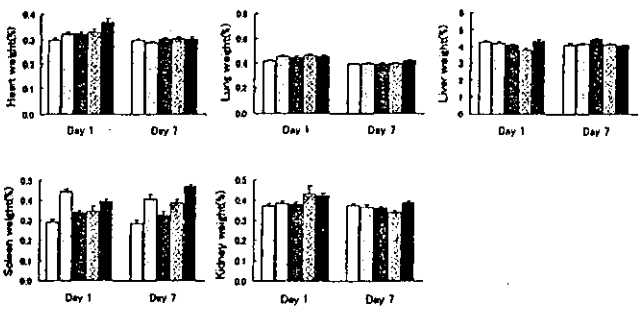

Figure 3. Relative organ weights (as a percentage of body weight) in male Wistar rats subjected to 20% and 40% blood exchange with recombinant human serum albumin (rHSA) and rHSA-heme solutions. Each value represents the mean±SEM. □, Control group; □, 20% rHSA group; □, 20% rHSA-heme group; ▨, 40% rHSA group; and ■, 40% rHSA-heme group.

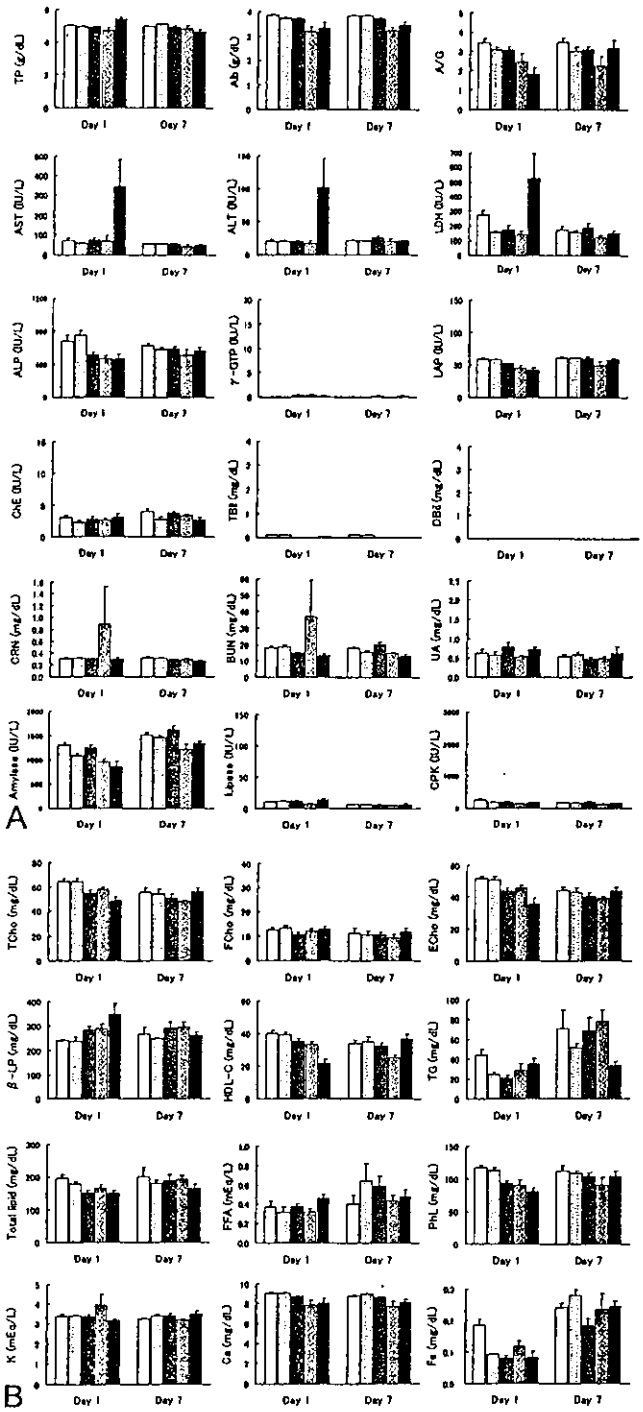

Figure 4. Blood biochemical tests of rat plasma after 20% and 40% blood exchange with recombinant human serum albumin (rHSA) and rHSA-heme solutions after 1 and 7 days. Each value represents the mean±SEM. □, Control group; □, 20% rHSA group; □, 20% rHSA-heme group; ▨, 40% rHSA group; and ■, 40% rHSA-heme group.

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

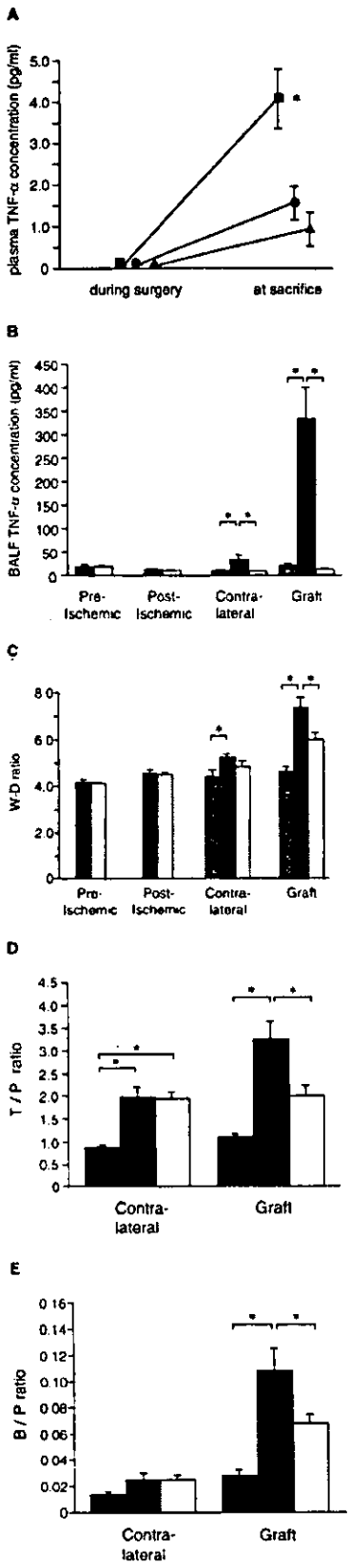

Figure 2. Tumor necrosis factor-α (TNF-α) level, lung water, and ^{125}I-labeled albumin index. (*A*) In the transplantation group, plasma TNF-α significantly increased with reperfusion ($p = 0.018$) (*closed triangle*, sham-operated group; *closed square*, transplantation group; *closed circle*, treatment 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.

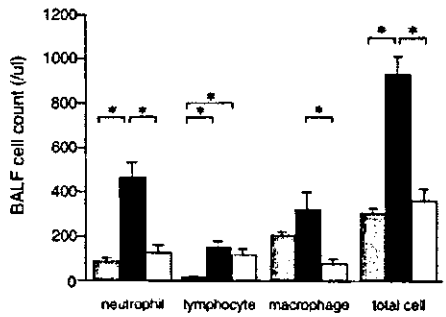

Figure 3. BALF cell count of the graft lung. In the treatment group, the number of lymphocytes was similar to that in the transplantation group, but the neutrophil and macrophage counts were significantly lower, resulting in significant decrease in the total cell count (*gray bar*, sham group; *black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

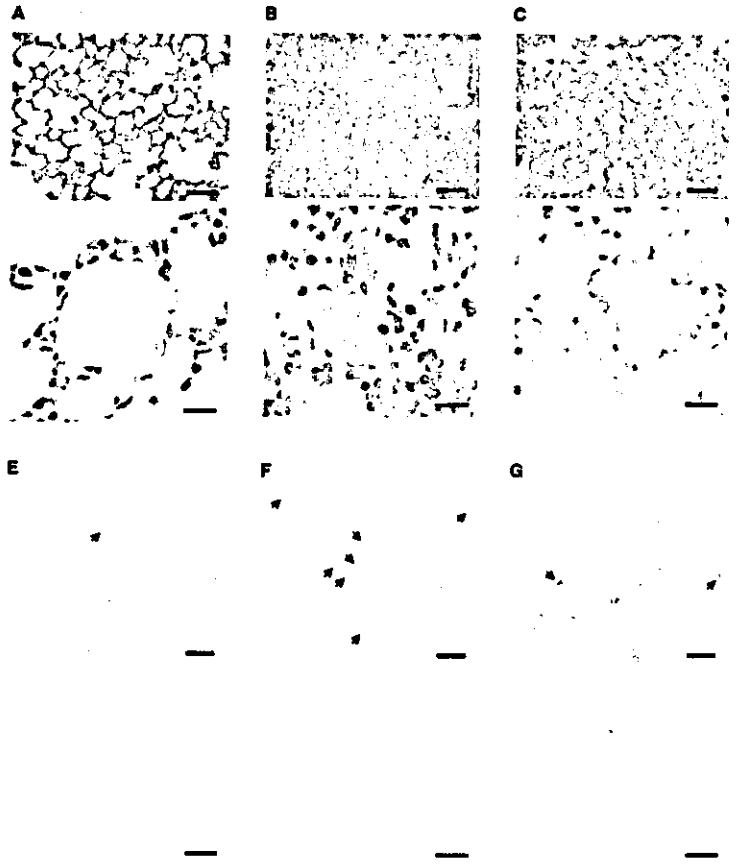

Figure 4. Histopathologic findings. (*A–D*) Hematoxylin and eosin staining of the graft lung tissue. (*A*) Sham group. (*B*) Transplantation group. (*C*) Treatment group. In the transplantation group, alveolar hemorrhages and interstitial thickening were marked, whereas only faint alveolar hemorrhages 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

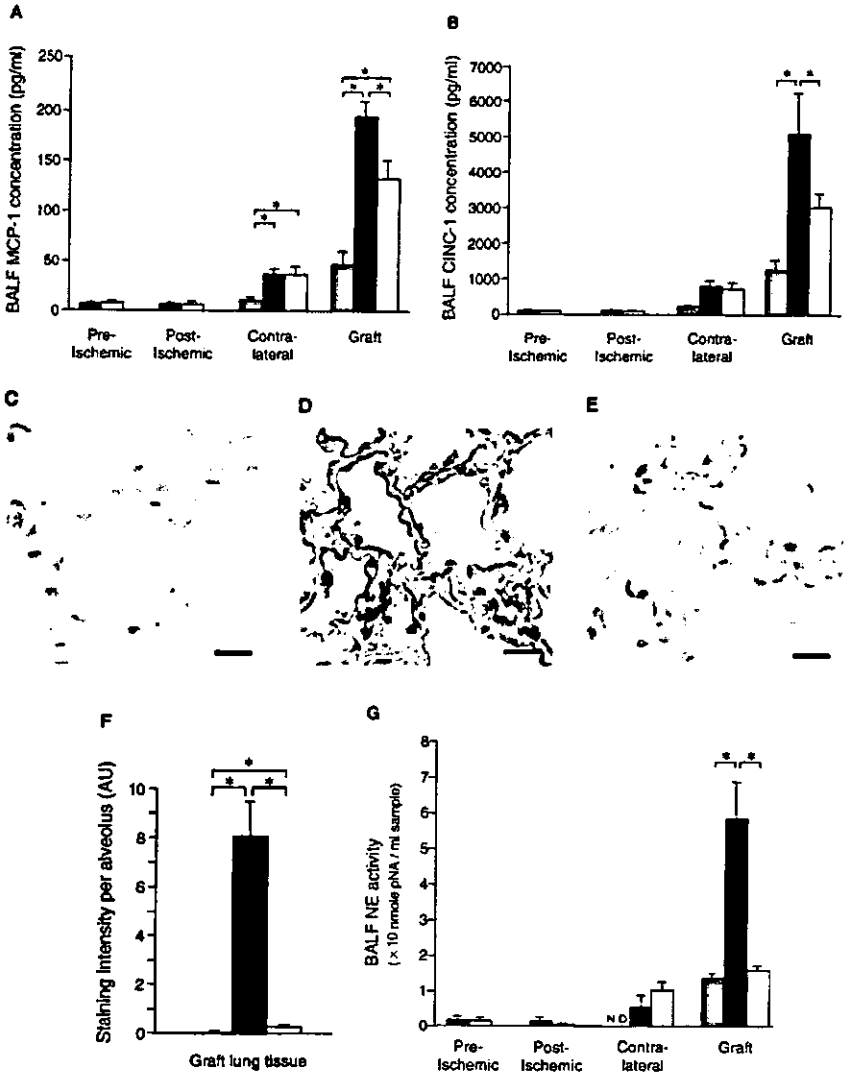

Figure 5. Chemokine, adhesion molecule, and neutrophil elastase (NE) activity. (*A* and *B*) Both monocyte chemotactic protein-1 (MCP-1) and cytokine-induced neutrophil chemoattractant-1 (CINC-1) in the graft lung BALF were significantly lower in the treatment group than in the transplantation group. (*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.

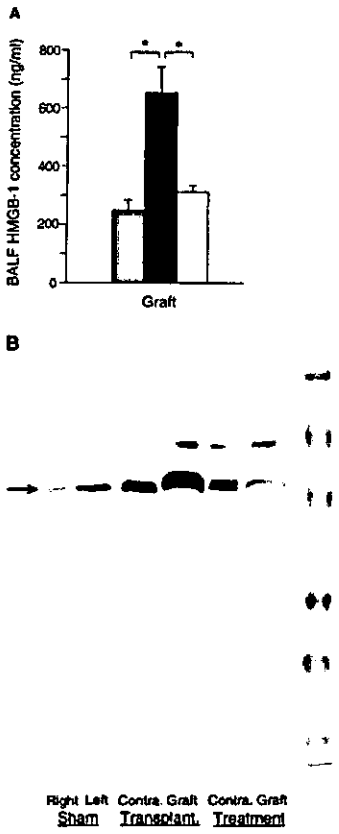

Figure 6. High mobility group box-1 (HMGB-1) protein and epithelial cadherin (E-cadherin)–soluble form. (*A*) The BALF HMGB-1 level was increased in the transplantation group graft lung, but it was significantly decreased in the treatment group graft lung (*gray bar*, sham group; *black bar*, transplantation 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.

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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
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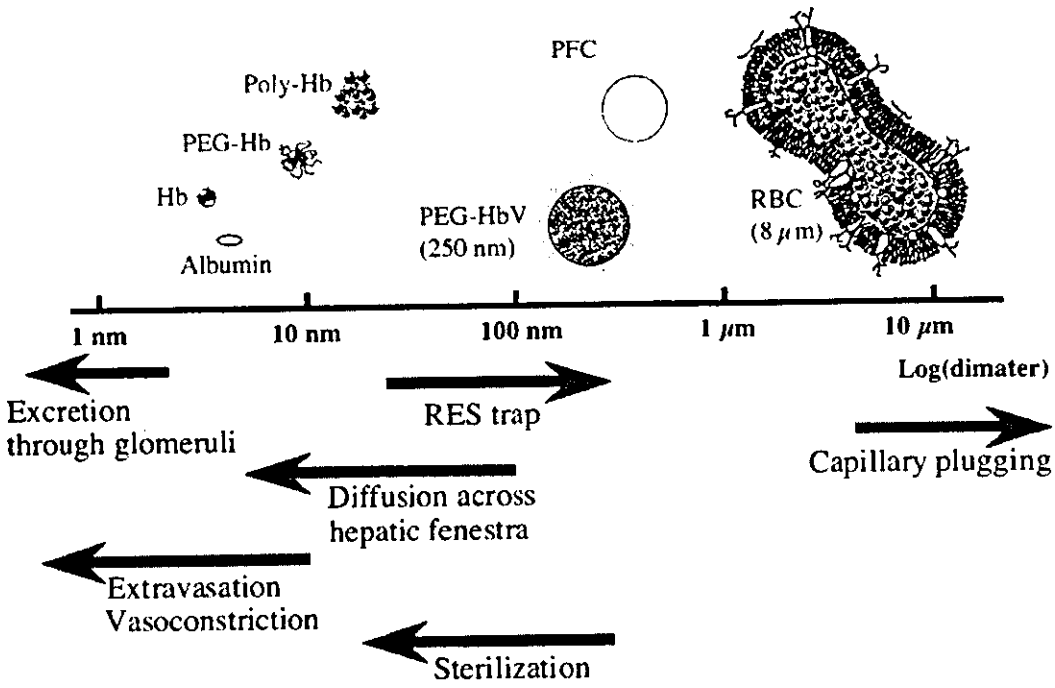

図1　What is the optimal dimension of O_2 carriers?

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