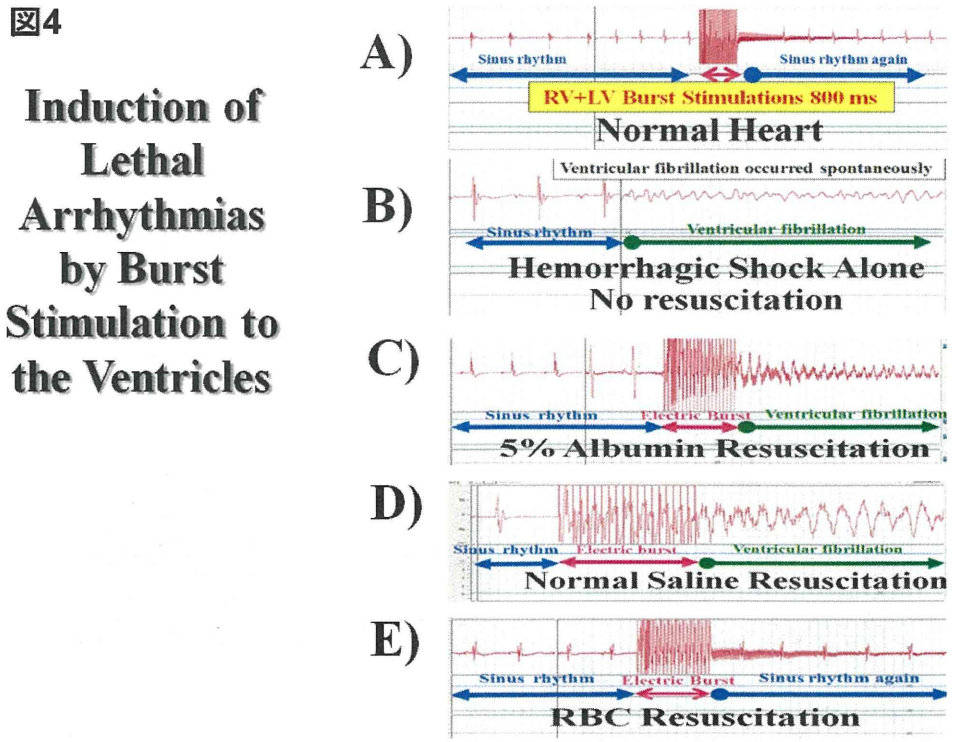
図4



さらに、催不整脈性を調べるために、右心室・左心室の3箇所、すなわち右心室心尖部、心臓基部、右室流出路を20回の連続刺激（burst pacing, 5, 50, 100 V; 40-ms interval, 20 trains） 各voltageにて3回づつ施行し、致死性不整脈の誘発の有無を検討した（**図４－A**）。

(2) Connexin43に関する免疫組織学的検討

摘出心臓を 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries Ltd.) にて48時間固定した。それぞれの標本は、70% ethanolにて脱水しparaffin固定した。組織学的検討では、心筋伝導蛋白であるconnexin43の心筋組織内発現の程度を定性的に評価するため、免疫組織染色を施行した。すなわち、Anti-connexin43 monoclonal antibody (1:2,000, Sigma-Aldrich, St. Louis, USA) を用いて、心筋組織内の心筋 gap-junction 蛋白 connexin 43の発現を検討した。

(3) 統計学的検討

各群において、興奮伝播時間及びAPD dispersionは平均±標準偏差で表した。興奮伝播様式及びconnexin43の発現様式は異常の有無を、異常有りまたは無しの定性的2分類でその頻度を検討し、致死性不整脈誘発頻度に関しても誘発の有り無しにつき各個体毎に検討し、その頻度を比較した。群間の比較にはANOVA法にて検定し、Bonferroni post hoc補正を実施した。頻度の検定にはカイ二乗検定を実施した。$P<0.05$を推計学的に有意とした。

Ｃ．結果

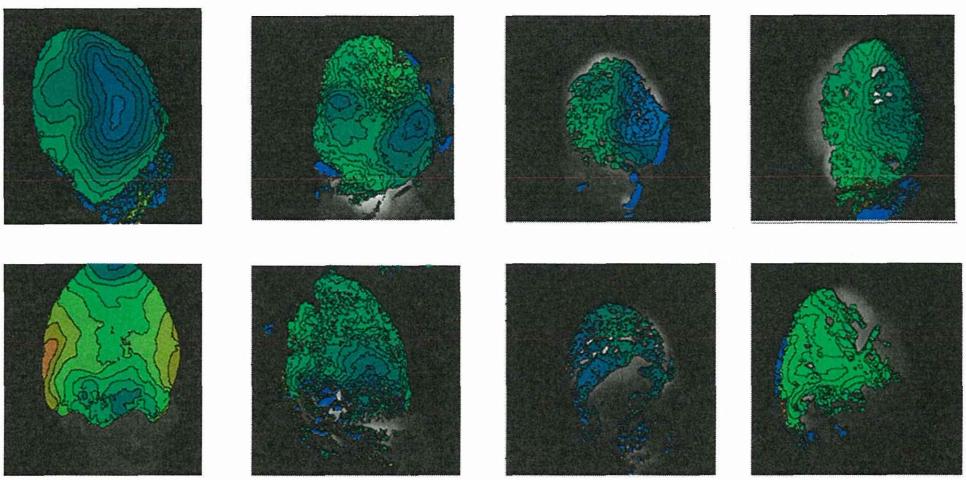
(1) Optical mapping analysis 法による興奮伝播時間・伝播様式及びAPD不均一性と不整脈誘発の結果

非蘇生群では全例Ratsは心室細動または徐脈性不整脈を惹起し、その後心停止を来たした（**図４－B**）。他の３群では、各蘇生液により全Rats血行動態はショック状態から蘇生された。これら３群のRatsから摘出された心臓の興奮伝播時間・伝播様式をOptical mapping systemにて検討した結果を図５に示した。

正常Ratsの洞調律における左心室の興奮伝播時間は24±1msであり、伝播様式は**図２**に示したpatternであった。一方、5%アルブミン群及び生理食塩水群では、ショック状態から蘇生されたにもかかわらず、興奮伝播時間はそれぞれ、35±3ms

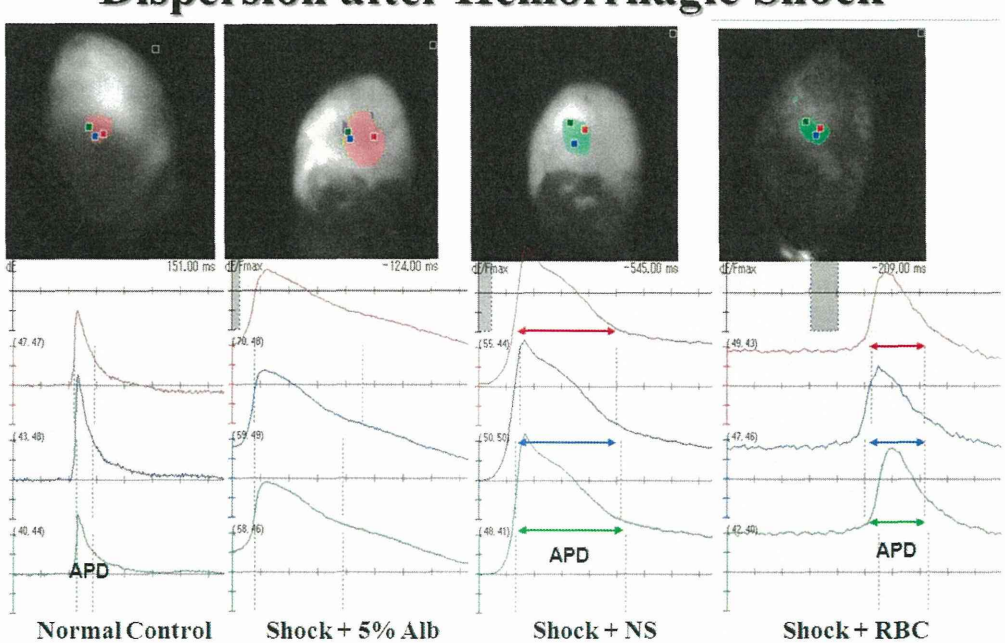
図5

Comparison of Activation Map



Normal Control　Shock + 5% Alb　Shock + NS　Shock + RBC

図6 **Comparison of Action Potential Duration Dispersion after Hemorrhagic Shock**



Normal Control　Shock + 5% Alb　Shock + NS　Shock + RBC

及び　39±3msとそれぞれ有意かつ著明に延長しており、伝播様式の明らかに正常patternと異なっていた（**図５**）。しかし、洗浄赤血球群で蘇生したRatsでは、全Rats正常興奮伝播時間（22±3ms）であり、かつ、正常伝播様式であることが認められた。さらにAPD dispersionは、洗浄赤血球群で全Rats、図３で示したnormal controlと差を認めず（normal control vs. 洗浄赤血球群; 14±2ms vs. 13±3ms, NS）なかったのに対し、5%アルブミン群及び生理食塩水群では、全Ratsで、図３で示したmoderateまたはsevere impairment patternを示し、APD dispersionはそれぞれ 34±27ms及び38±9msと有意（$P<0.05$）かつ著明に延長していた。また、活動電位持続時間そのものも正常Rats、洗浄赤血球群に比較し、5%

アルブミン群及び生理食塩水群では著明に延長していることが認められた（**図６**）。

致死性不整脈の誘発性の検討では、正常Ratsの摘出心臓では通常不整脈が誘発されない両心室へのburst pacing（**図４－A**）にて、5%アルブミン群及び生理食塩水群蘇生群Ratsでは全Ratsで致死性心室性不整脈（心室細動・心室頻拍）が容易に誘発された（**図４－C、D**）。しかし、洗浄赤血球群蘇生群Ratsでは、正常Ratsと同様に致死性不整脈は誘発されなかった（**図４－E**）。

(2) 心筋におけるconnexin43の発現（免疫組織学的検討）

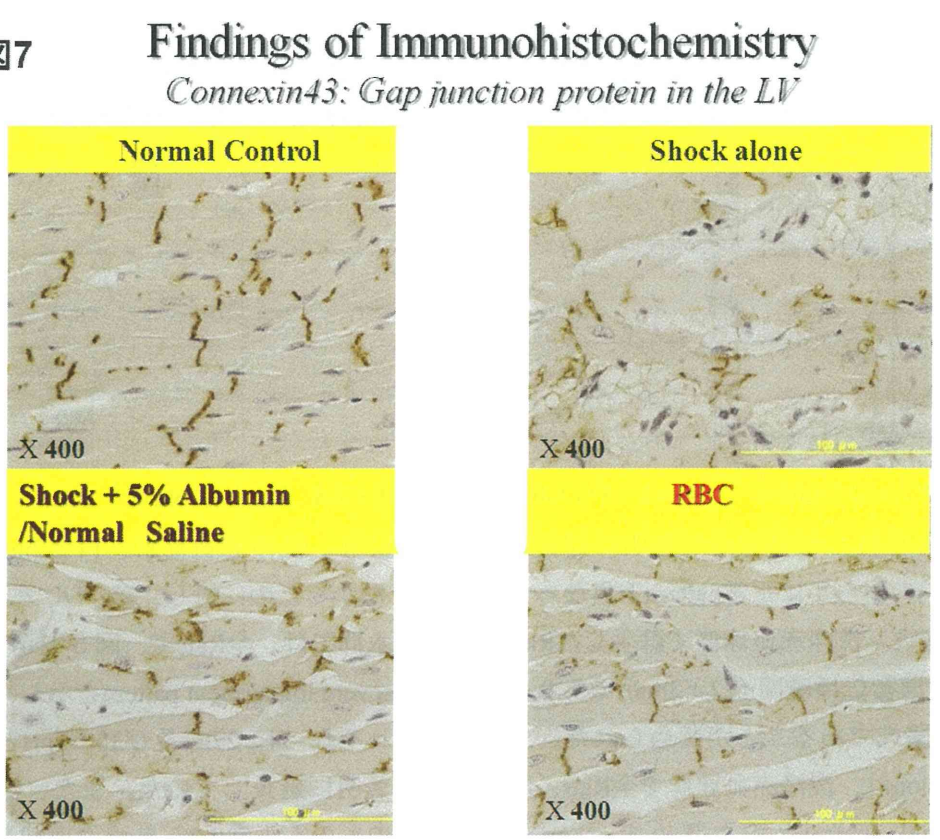
左心室におけるconnexin43の発現を免疫組織学的に検討した。**図７**に示した如く、視覚的定性的にconnexin43発現量・発現様式の異常の有無を検討した。その結果、正常Rats左心室心筋におけるconnexin43の正常発現量・発現様式に比較し、5%アルブミン群及び生理食塩水群では明らかにconnexin43の発現量・発現様式は異常であり、洗浄赤血球群では正常に保たれていた。

Ｄ．考案

今回の実験研究は、“出血性ショック心臓”において、洗浄赤血球蘇生が致死性不整脈誘発の抑制効果があり、その機序として心筋興奮伝播時間と伝播様式・活動電位持続時間の均一性・心筋Gap junctionの伝導蛋白connexin43等を正常に保つ作用が貢献している可能性があることが示唆された。

本研究では、非蘇生群が全Rats死亡する致死性再出血による血性ショックモデル（30%脱血）を用いた、いわゆる“出血性ショック心臓”において、通常臨床現場で用いられる5%アルブミン蘇生群、生理食塩水蘇生群及び洗浄赤血球蘇生群における致死性不整脈の誘発頻度やその機序を摘出心臓に対するOptical mapping systemとburst pacingによる致死性不整脈誘発法で検討した。その結果、5%アルブミン群及び生理食塩水群では血行動態は正常に復し、蘇生に成功したものの、摘出心臓におけるOptical mapping systemでは興奮伝播異常・再分極

図7



不均一性を示すAPD dispersion増加が認められ、かつ、不整脈の発生機序に関係する心筋Gap junctionの伝導蛋白connexin43の発現低下が認められるとともに、致死性不整脈の誘発の頻度が増加していた。これらの異常変化は、洗浄赤血球蘇生群では認められなかった。

これまでの報告では、急性血性ショックに伴い、侵襲の大きさに伴った心筋障害が惹起され、血性ショック早期の死亡率に関与しているとされている。これらの心筋障害には、血流の低下及び貧血による心筋虚血そのものの影響に加え、”出血性ショック心臓”に固有の2次的血流障害や代謝異常が関与する可能性を示唆する報告もある。従って、“出血性ショック心臓”では、単に5%アルブミンや生理食塩水による蘇生では、その回復は不十分と考えられる。洗浄赤血球治療群で、致死性不整脈やその病因となるOptical mapping system解析指標、心筋Gap junctionの伝導蛋白connexin43の発現が保たれた。このことは、血性ショック治療において、血行動態の改善のみならず、貧血を改善することにより“出血性ショック心臓”の心筋組織に充分は酸素供給を行うことが重要と考えられる。

本研究を、臨床現場における“出血性ショック心臓”の治療に直結させるには充分とはいえないものの、血性ショック後に遷延する血行動態の不安定性や心不全・致死性不整脈の発生予防に、充分な酸素運搬作用が治療上重要であることを示唆する結果と考えられた。

E．結論

出血性ショック心臓では、左心室伝導遅延とAPDd増大及びCx43発現異常を惹起し、電気的不安定性から致死性不整脈が誘発されると示唆された。RBC治療はこれら指標の保持と予防効果を有した。このモデルは、HbVの有効性を評価するのに適したものと考えられる。

F．健康危険情報

該当なし

G．研究業績

(原著論文)

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(学会発表)

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2. Takase B, Tanaka Y. Ishihara M. Pressure overload causes right ventricular lethal arrhythmias: analysis by optical mapping in pulmonary artery banding and pneumonectomy model. (American Heart Association Annual Scientific Session 2013, Dallas US A 2013/11/15-20)

3. Takase B, Tanaka Y, Higshimura Y, Hattori H, Ishihara M. Significant role of action potential duration dispersion and connexin 43 in lethal arrhythmogenesis in hemorrhagic shock heart. (第28回日本不整脈学会学術大会, Tokyo 2013/7/4-6)

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H. 知的財産権の出願。登録状況（予定を含む）

該当なし。

別添 5

表　研究成果の刊行に関する一覧表

刊行書籍又は雑誌名（雑誌のときは雑誌名、巻号数、論文名）	刊行年月日	刊行書店名	執筆者名
Primary and secondary immune responses to keyhole limpet hemocyanin in rats after the infusion of hemoglobin vesicle, an artificial oxygen carrier. *Artif. Organs* 38, 234-238 (2014)	2014 年 3 月	Wiley	M. Fujihara, D. Takahashi, H. Abe, H. Sakai, H. Horinouchi, K. Kobayashi, H. Ikeda, H. Azuma
Carbon monoxide-bound hemoglobin-vesicles as a potential therapeutic agent for the treatment of bleomycin-induced pulmonary fibrosis. *Biomaterials*	In press	Elsevier	S. Nagao, K. Taguchi, H. Sakai, R. Tanaka, H. Horinouchi, H. Watanabe, K. Kobayashi, M. Otagiri, T. Maruyama.
出血性ショック心臓における致死性不整脈の機序に関する実験的検討. *Therapeutic Research* 2013;34:1060- 1066.	2013 年 8 月	ライフサイエンス出版	高瀬凡平、東村悠子、木村一生、田中良弘、服部秀美、石原雅之
輸血代替としての人工赤血球(ヘモグロビン小胞体)製剤の安全性試験. 人工血液 21, 36-48 (2013)	2013 年 11 月	日本血液代替物学会	酒井宏水，堀之内宏久、東 寛、小田切優樹、小林紘一
Biocompatibility of a highly concentrated fluid of Hemoglobin-vesicles as a transfusion alternative. In: Selective Topics in Nanomedicine (T.M.S. Chang ed.) pp. 133-147, World Scientific, Singapore (2013)	2013 年	World Scientific, Singapore	H. Sakai
Cellular-type hemoglobin-based oxygen carriers to mimic the red blood cells structure. In: Hemoglobin-based oxygen carriers –principles, approaches and current status (H.W. Kim & A.G. Greenburg eds.) Springer-Verlag (Berlin/Heidelberg, Germany). Chapter 12, pp. 235-248 (2013)	2013 年 12 月	Springer-Verlag	H. Sakai
Biocompatibility of hemoglobin vesicles, a cellular-type artificial oxygen carrier, on blood cells and plasma proteins in vitro and in vivo. In: Hemoglobin-based oxygen carriers –principles, approaches and current status (H.W. Kim & A.G. Greenburg eds.) Springer-Verlag (Berlin/Heidelberg, Germany). Chapter 22, pp. 385-398 (2013)	2013 年 12 月	Springer-Verlag	H. Azuma, M. Fujihara, H. Sakai
Cellular-type hemoglobin-based oxygen carriers as a resuscitative fluid for hemorrhagic shock: acute and long-term safety evaluation using beagle dogs. In: Hemoglobin-based oxygen carriers –principles, approaches and current status (H.W. Kim & A.G. Greenburg eds.) Springer-Verlag (Berlin/Heidelberg, Germany). Chapter 28, pp. 501-526 (2013)	2013 年 12 月	Springer-Verlag	T. Ikeda, H. Horinouchi, Y. Izumi, H. Sakai, E. Tsuchida, K. Kobayashi

刊行書籍又は雑誌名（雑誌のときは雑誌名、巻号数、論文名）	刊行年月日	刊行書店名	執筆者名
International consortium for development of hemoglobin-based oxygen carriers, oxygen therapeutics and multifunctional resuscitation fluids–a white paper. In: Hemoglobin-based oxygen carriers –principles, approaches and current status (H.W. Kim & A.G. Greenburg eds.) Springer-Verlag (Berlin/Heidelberg, Germany). Chapter 39, pp. 737-746 (2013)	2013 年 12 月	Springer-Verlag	H.W. Kim, J.S. Jahr, A. Mozzarelli, H. Sakai
人工赤血球の開発状況と将来展望. *Anesthesia Network* 18, 37-41 (2014)	2014 年 1 月	下野印刷	酒井宏水
工赤血球による生体組織への酸素輸送. 「全人力・科学力・透析力に基づく透析医学」 平方秀樹 監修	印刷中	医薬ジャーナル社	酒井宏水

特許出願

PCT/JP2012/59233 (2012年4月4日出願): 小胞体の製造法.
(2013年度に国内段階移行完了: 米国, インド, 中国, EP, 日本)

報道

関西テレビ「スーパーニュースアンカー」 献血血液が将来的に不足することの解決策の一つとして、人工赤血球が紹介された（平成25年5月20日（月）16:48～19:00）。

その他

日本血液代替物学会 第20回年次大会
会　期:平成25年12月6日-7日
会　場:奈良県新公会堂
大会長:酒井 宏水（奈良県立医科大学医学部化学教室）

研究成果の刊行物・別冊

(2013. 4. － 2014. 3.)

Artificial Organs

Primary and Secondary Immune Responses to Keyhole Limpet Hemocyanin in Rats After Infusion of Hemoglobin Vesicle, an Artificial Oxygen Carrier

*Mitsuhiro Fujihara, *Daisuke Takahashi, *Hideki Abe, §Hiromi Sakai, †Hirohisa Horinouchi, †Koichi Kobayashi, *Hisami Ikeda, and ‡Hiroshi Azuma

*Japanese Red Cross, Hokkaido Block Blood Center, Sapporo; †Department of Surgery, School of Medicine, Keio University, Tokyo; ‡Department of Pediatrics, Asahikawa Medical College, Asahikawa, Japan; and §Waseda Bioscience Research Institute in Singapore, Singapore, Singapore

Abstract: Hemoglobin vesicles (HbVs), artificial oxygen carriers encapsulating concentrated Hb solution on phospholipid vesicles (liposomes), are promising candidates for clinically useful transfusion. Although HbV infusion transiently suppressed the proliferative response of rat splenic T-cells to concanavalin A or keyhole limpet hemocyanin (KLH), a T-cell-dependent antigen, in ex vivo culture conditions, HbV infusion did not affect the primary IgG antibody response. We extended our assessment of the effects of HbV infusion on the systemic immune response using primary and secondary responses to KLH in rats. We observed that the generation of primary anti-KLH IgM antibody in HbV-infused rats was not suppressed but was instead higher than those in saline-infused rats. Furthermore, HbV infusion did not suppress the increase of IgG subclass of KLH antibody in secondary response. The T cell response to KLH of bulk spleen cells, as derived from 2–3 months after secondary KLH immunization, was unaffected by infusion of HbV, suggesting that HbV loading has no suppressive effect on homeostatic survival of memory T-cells against KLH. These results indicate that HbV is highly biocompatible in systemic immune responses in rats. **Key Words:** Liposome-encapsulated hemoglobin—Hemoglobin-vesicles—Biocompatibility—Antibody response—Keyhole limpet hemocyanin.

Hemoglobin vesicles (HbVs), which are artificial oxygen carriers encapsulating concentrated Hb solution on phospholipid vesicles (liposomes), are promising candidates for clinically useful transfusion alternatives because several lines of evidence have demonstrated that HbVs have excellent oxygen transporting efficacy in animal models without noteworthy adverse reactions (1). In addition, several lines of data show that HbVs have great biocompatibility to human blood cells and plasma proteins in vitro (2).

Because of particulate features, liposomes accumulate in the mononuclear phagocytic systems present in the liver, spleen, bone marrow, and other tissues when injected intravenously into experimental animals (3). In fact, HbVs reportedly accumulate in the liver, spleen, and bone marrow (4). The amount of HbVs to be infused as a blood substitute is expected to be large. Therefore, it is assumed that the accumulation of considerable amounts of HbVs and empty liposomes in the mononuclear phagocytic system can affect immunologic response. In this regard, we recently reported that rat splenic immature monocytes phagocytosed HbVs as did empty liposomes, and that they became transiently and highly immunosuppressive in ex vivo culture conditions (5). In contrast, our preliminary data showed that the systemic immune response was apparently unaffected because IgG production in primary response to keyhole limpet hemocyanin (KLH), which depends on T-cells, occurred irrespective of HbV infusion (5).

To clarify the effects of HbVs on the systemic immune response, we assessed the effects of HbV infusion on IgM production in primary response to KLH and secondary responses to KLH in rats. Here, we show that HbVs did not inhibit the generation of primary IgM production to KLH or secondary immune response in rats, further extending our initial results showing that HbV infusion has no adverse effect on systemic immune response in rats.

MATERIALS AND METHODS

Preparation of HbV suspension

The HbVs were prepared as described in a previous report (6,7). In brief, a hemoglobin solution prepared

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Address correspondence and reprint requests to Professor Hiroshi Azuma, Department of Pediatrics, Asahikawa Medical University, Higashi 2-1-1-1, Midorigaoka, Asahikawa 078-8510, Japan. E-mail: azuma5p@asahikawa-med.ac.jp

from outdated red blood cells was heated under a CO gas atmosphere to inactivate any contaminating virus. After centrifugation and filtration, the hemoglobin solution was mixed with lipids and then extruded through membrane filters with a 0.22-μm pore size to produce liposomes. The lipid composition (molar ratio) was as follows: 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC)/cholesterol (CHOL)/ 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG)/ polyethyleneglycol-conjugated 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (PEG5000-DSPE) = 5:5:1:0.033. The mean particle size was 250 nm. All lipids were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan) except for PEG5000-DSPE (NOF Corp., Tokyo, Japan). The HbVs were suspended in normal saline; the suspension contained 10 g of hemoglobin/dL, 5.7 g of lipid/dL, and <0.1 endotoxin unit of lipopolysaccharide/mL.

Animals

Male Wistar–King Aptekman Hokkaido (WKAH) rats, 8–12 weeks old and weighing approximately 220–300 g, were purchased from Japan SLC, Inc. (Shizuoka, Japan). This study was approved by the Ethics Committee for Animal Experiments at the Hokkaido Red Cross Blood Center.

Evaluation of the effects of HbVs on primary antibody response

Rats were infused with HbV or saline 6 h before the intravenous injection of 100 μg of KLH (Calbiochem, La Jolla, CA, USA) on day 0. HbV-induced suppression of splenic T-cell proliferation in ex vivo culture systems was observed from 6 h to 3 days after HbV infusion (5). Therefore, we chose 6 h for this experiment. HbV or saline were infused intravenously into the tail as a top load under ether anesthesia. The injection volume was 20% of whole blood volume according to a previous report (5), as estimated as follows such that the whole blood volume is 56 mL/kg of body weight in rats. This dosage is consistent with approximately 1 L of whole blood in a 70-kg male human. Peripheral blood was taken from the tail vein on days 0, 5, 7, 10, and 14. Subsequently, serum was separated and stored at −80°C until anti-KLH antibody was measured. The serum concentration of anti-KLH IgM was measured via enzyme immunoassay. Ninety-six-well microtiter plates (Nalgene Nunc International, Rochester, NY, USA) were coated with 0.5 μg of KLH in 100 μL phosphate-buffered saline (PBS) per well and incubated overnight at 4°C. Plates were washed with PBS once and blocked with 5% dry skim milk in PBS. After incubation for 2 h at room temperature, plates were washed three times with PBS-0.1% Tween 20. Rat sera were added at a concentration of 1:1000 40%FCS/0.05%Tween 20/PBS. Appropriately diluted standard rat anti-KLH IgM (Biolegend, San Diego, CA, USA) in 40%FCS/0.05%Tween 20/PBS was also added to the appropriate plates. Plates were incubated for 60 min and washed three times with 0.1% Tween 20/PBS. Then, 100 μL of horseradish peroxidase-conjugated goat anti-rat IgM antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) was added at a concentration of 1:10 000 in 0.05% skim milk/0.1% Tween 20/PBS to the appropriate plates and incubated for 60 min. Plates were washed three times with 0.1% Tween 20/PBS, and 100 μL of 3, 3′, 5, 5′,-tetramethylbenzidine (TMB) One-Step Substrate System (Dako Japan, Inc., Tokyo, Japan) was added to all wells. Plates were incubated for 15 min and read at an optical density of 450 nm.

Evaluation of the effect of HbV injection on secondary antibody response

Rats were immunized using KLH as follows. KLH solution (1 mg/mL of KLH in saline) was mixed with the same volume of incomplete Freund's adjuvant (Sigma-Aldrich Corp., St. Louis, MO, USA). Then, 0.2 mL of the mixture (100 μg of KLH) was injected subcutaneously. Fourteen days after first injection of KLH, HbVs (20% of whole blood volume) were infused intravenously into the tail at the top load under ether anesthesia. Saline was infused as a control. Secondary KLH immunization was conducted 16 h after HbV or saline injection. KLH solution with complete Freund's adjuvant (100 μg of KLH in 0.2 mL) or saline with complete Freund's adjuvant was injected subcutaneously. Peripheral blood was taken from the tail vein immediately after secondary immunization, and at 1 day, 3 days, 5 days, and 7 days. Subsequently, serum was separated and stored at −80°C until anti-KLH antibody was measured. Serum concentrations of anti-KLH IgG and IgM were measured using an ELISA kit (Shibayagi Co. Ltd, Gunma, Japan).

Assay of the proliferation of splenic T-cells in response to Con A or KLH

The spleens from HbV-treated or saline-treated rats that received secondary KLH immunization were excised at 2–3 months after secondary immunization aseptically under ether anesthesia. Then, a single-cell suspension was prepared. Erythrocytes were depleted using red blood cell lysing buffer (IBL International GmbH, Hamburg, Germany). Spleen

cells were washed in RPMI-1640 medium containing 10% fetal calf serum (FCS). Single spleen cell suspensions in RPMI-1640 medium supplemented with 10% FCS and mercaptoethanol (50 μM) were plated in 96-well plates in a volume of 0.2 mL/well. The cells were cultured in triplicate for 72 h in the presence of 0.3 and 3 μg/mL of Con A (Sigma-Aldrich Corp.) or KLH (1, 3, 10, 30 μg/mL) and pulsed with [^{3}H]-thymidine (18.5 kBq) for the final 18 h of incubation. Subsequently, the cells were harvested onto glass fiber paper. Radioactivity was measured using a liquid scintillation counter (LS5000 TD; Beckman Coulter, Inc., Fullerton, CA, USA).

Statistical analyses

Experimental differences from the controls were assessed using one-way nonrepeated ANOVA with Dunnet's post hoc test and two-tail unpaired Student's *t* tests. Software was used for statistical analyses (ystat2004; Igaku Tosho Press, Co. Ltd, Tokyo, Japan). Values of $P < 0.05$ were inferred as statistically significant.

RESULTS AND DISCUSSION

Effects of HbV infusion on the primary responses to KLH in rats

Our previous study showed that KLH infusion caused the elevation of IgG contents of anti-KLH antibody with no apparent difference between HbV and saline-treated rats (5). In primary response, however, the generation of IgM subclass of antibody occurs earlier and stronger than that of IgG type of antibody. Therefore, we evaluated the effects of HbV infusion on the primary IgM responses to KLH in rats. The IgM contents of anti-KLH antibody in saline-treated rats increased on Day 5 after immunization with a gradual decrease until Day 14 (Fig. 1A). In HbV-treated rats, the IgM contents of anti-KLH antibody were generally higher than those in the saline-treated rats at all time points after KLH immunization. Results show that HbV infusion had no suppressive effect on the generation of IgM as well as IgG antibody to primary KLH immunization.

The KLH antibody response requires the integrated activity of various components of the immune system, namely antigen-presenting cells (MHC class II$^+$ cells), T-cells, and B-cells. Therefore, the quantities of these cells and the ratio of CD4$^+$ and CD8$^+$ cells are regarded as important biomarkers for the evaluation of immunotoxicity testing for better prediction of unintended immunosuppressive potential of new pharmaceuticals (8). In this regard, we observed no differences in these parameters between HbV-injected rats and control rats after primary KLH immunization (data not shown), supporting the observation that HbV infusion had no suppressive effect on the generation of antibody response to primary KLH immunization.

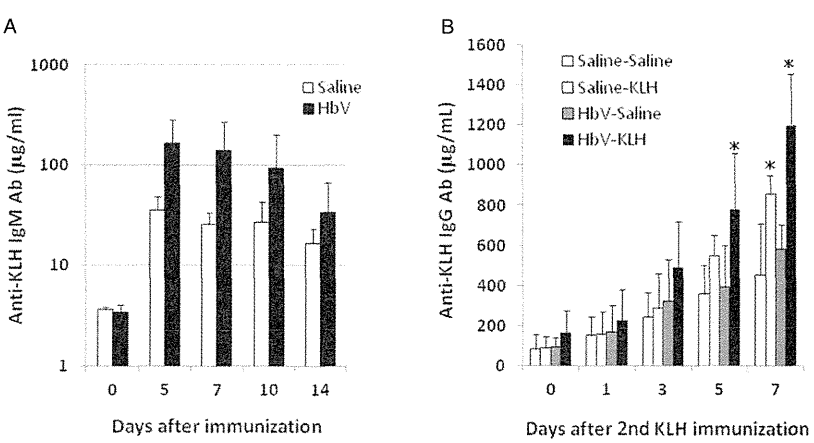

FIG. 1. (A) Effects of HbV infusion on primary IgM antibody response to KLH in rats. Male rats were treated either with HbV (closed column) or saline (open column). At 6 h later, rats were administered KLH by i.v. injection. Serum anti-KLH IgM antibodies were measured before immunization (Day 0) and at the indicated time-points after immunization using quantitative ELISA: $n = 3$, mean ± standard deviation (SD). (B) Effects of HbV infusion on secondary IgG antibody response to KLH in rats. All rats were immunized with KLH by s.c. injection once. At 14 days after the first immunization of KLH, rats were administered either HbV or saline by i.v. injection. After 16 h, those rats were immunized with or without KLH by s.v. injection. Serum anti-KLH IgG and IgM antibodies were measured before immunization (Day 0) and at the indicated time-points after immunization using quantitative ELISA: $^*P < 0.05$ compared with control (saline–saline group); $n = 5$, mean ± SD.

Effects of HbV infusion on secondary responses to KLH in rats

As both primary and secondary responses to KLH were applied for the testing of immunotoxicity of chemicals (9,10), we then evaluated the effects of HbV infusion on the secondary responses to KLH in rats. As presented in Fig. 1B, all groups showed similar IgG concentrations of anti-KLH antibody at 14 days after primary immunization. In saline-injected rats, the IgG contents of anti-KLH antibody in the secondarily KLH-immunized group were increased gradually at 5 and 7 day as compared with the group without secondary immunization. In this case, significance was found at 7 days after secondary immunization. In HbV-injected rats, secondary immunization of KLH also increased IgG contents of anti-KLH antibody, with significance found at 5 and 7 days after secondary immunization. The IgG antibody response to secondary KLH injection in the HbV-injected rats appeared to be earlier and stronger than that in the saline-injected rats.

In contrast to IgG subclass, no significant increase was found in IgM contents of anti-KLH antibody with KLH secondary immunization irrespective of HbV infusion (data not shown). It is generally accepted that the production of IgG antibody becomes dominant in the secondary antibody responses. Therefore, the present results suggest that infusion of HbVs had no suppressive effect on the secondary antibody response to KLH.

Effects of HbV infusion on the survival of KLH-responsive memory T-cells in spleen

The memory T-cells play a crucial role in long-term immunity to infection and cancer because they have the dual ability to persist within the host for long periods and to respond rapidly to rechallenge of the antigen. The survival and recall of memory T-cells are tightly regulated by complex homeostatic mechanisms (11,12). In this sense, it is important to investigate whether HbV infusion might affect the survival of T-cell memory cells or not. The dose-dependent proliferation in response not only to ConA but also KLH was observed in both HbV-loaded and control rats, with no apparent difference between them (Fig. 2). Consequently, HbV infusion into rats did not affect the long-term survival of memory T-cells against KLH.

In the current study as well as in our previous studies, the effects of HbV infusion on immune response were investigated using a 20% top load protocol. The question of whether the transient suppression of T-cell proliferation by HbV infusion might also occur in a hemorrhagic shock model is an important aspect related to the clinical setting. Further study is necessary to verify this issue.

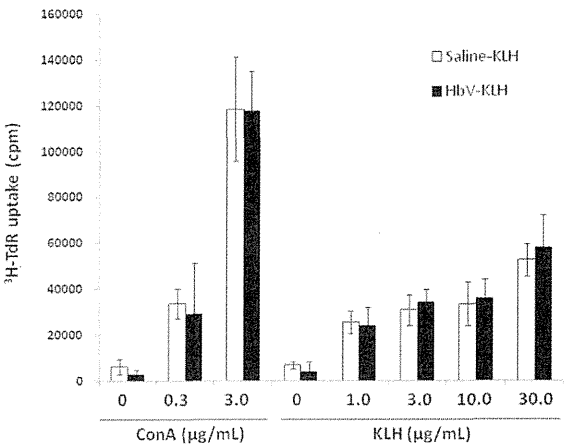

FIG. 2. Effects of HbV infusion on proliferation of memory T-cells against KLH in an ex vivo condition. Immunization of KLH and HbV infusion were conducted in the same manner as depicted in Fig. 1B. Splenocytes were excised from the secondarily KLH-immunized rats at 2–3 months after immunization. The bulk splenocytes were stimulated with ConA or KLH: $n = 4$, mean ± SD.

CONCLUSION

Results of the present study demonstrated that HbV does not interfere with primary and secondary antibody responses to keyhole limpet hemocyanin in rats.

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Carbon monoxide-bound hemoglobin-vesicles for the treatment of bleomycin-induced pulmonary fibrosis

Saori Nagao[a,1], Kazuaki Taguchi[b,1], Hiromi Sakai[c], Ryota Tanaka[a], Hirohisa Horinouchi[d], Hiroshi Watanabe[a,c], Koichi Kobayashi[d], Masaki Otagiri[a,b,f**], Toru Maruyama[a,e*]

[a]Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan,

[b]Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto 860-0082, Japan,

[c]Department of Chemistry, Nara Medical University, Kashihara 634-8521, Japan,

[d]Department of Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan,

[e]Center for Clinical Pharmaceutical Sciences, School of Pharmacy, Kumamoto University, Kumamoto 862-0973, Japan,

[f]DDS Research Institute, Sojo University, Kumamoto 860-0082, Japan.

[1]These authors contributed equally to this work.

*Corresponding author:

Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan,

Tel: +81-96-371-4150; fax: +81-96-362-7690;

email: tomaru@gpo.kumamoto-u.ac.jp (T. Maruyama)

**Corresponding author:

Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto 860-0082, Japan,

Tel: +81-96-326-3887; fax: +81-96-326-5048;

email: otagirim@ph.sojo-u.ac.jp (M. Otagiri)

Abstract

Carbon monoxide (CO) has potent anti-inflammatory and anti-oxidant effects. We report herein on the preparation of a nanotechnology-based CO donor, CO-bound hemoglobin-vesicles (CO-HbV). We hypothesized that CO-HbV could have a therapeutic effect on idiopathic pulmonary fibrosis (IPF), an incurable lung fibrosis, that is thought to involve inflammation and the production of reactive oxygen species (ROS). Pulmonary fibril formation and respiratory function were quantitatively evaluated by measuring hydroxyproline levels and forced vital capacity, respectively, using a bleomycin-induced pulmonary fibrosis mice model. CO-HbV suppressed the progression of pulmonary fibril formation and improved respiratory function compared to saline and HbV. The suppressive effect of CO-HbV on pulmonary fibrosis can be attributed to a decrease in ROS generation by inflammatory cells, NADPH oxidase 4 and the production of inflammatory cells, cytokines and transforming growth factor-β in the lung. This is the first demonstration of the inhibitory effect of CO-HbV on the progression of pulmonary fibrosis via the anti-oxidative and anti-inflammatory effects of CO in the bleomycin-induced pulmonary fibrosis mice model. CO-HbV has the potential for use in the treatment of , not only IPF, but also a variety of other ROS and inflammation-related disorders.

Keywords: Antioxidant, Fibrosis, Inflammation, Liposome, Lung

Abbreviation

CO: carbon monoxide, **HbV**: Hemoglobin-vesicles, **CO-HbV**: CO-bound hemoglobin-vesicles, **IPF**: idiopathic pulmonary fibrosis, **FVC**: forced vital capacity, **ROS**: reactive oxygen species, **TGF-β**: transforming growth factor-β, **CO-RM**: CO-releasing molecules, **HbCO**: carboxyhemoglobin, **RBC**: red blood cell, **PEG**: polyethylene glycol, **BLM**: bleomycin, **8-OH-dG**: 8-hydroxy-2'-deoxygenase, **NO_2-Tyr**: nitrotyrosine, **TNF-α**, tumour necrosis factor-α, **IL-6**: interleukin-6, **IL-1β**: interleukin-1β, **Nox4**: nicotinamide adenine dinucleotide phosphate oxidase 4, **Poldip2**: polymerase delta interacting protein 2, **EMT**: epithelial-mesenchymal transition

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive type of fibrous interstitial pneumonia with an unknown cure, except for lung transplantation. Patients with IPF have an estimated median survival of 2 to 5 years [1-3]. Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is currently the only orally administered drug approved for clinical use in the treatment of IPF in both the EU and Japan. Recently, the CAPACITY (Clinical Studies Assessing Pirfenidone in idiopathic pulmonary fibrosis: Research of Efficacy and Safety Outcomes) program showed that pirfenidone has a favorable benefit-risk profile, and, as a result, represents an appropriate treatment option for patients with IPF [4]. On the other hand, this multinational, double-blind, placebo-controlled study (CAPACITY006) also showed that pirfenidone treatment does not completely improve the clinically meaningful effects on forced vital capacity (FVC) and survival benefit [4]. In addition, it is well-known there are some significant side effects associated with the use of pirfenidone, which include photosensitivity (more than 50% of patients) [5]. Therefore, the development of drugs designed to suppress the progression of this disease or to improve respiratory function is of great importance.

Since new pathogenic pathways and mediators of IPF are discovered, the progression of IPF appears to result from a complex combination a number of factors, including inflammation, reactive oxygen species (ROS) and transforming growth factor (TGF)-β. Although recent studies have suggested that the repeated administration of drugs with either anti-oxidative or anti-inflammatory properties would be expected to be useful in the treatment of IPF [6, 7], the clinical use of these agents for IPF have not been approved worldwide. This is likely because these agents targeted only one of the many pathogenesis pathways, and, because mechanism responsible for the development of IPF

is complex, little alleviation occurs. Therefore, a shift in the effective treatment strategy for IPF from agents that block a single functional action to an agent that can address multiple functions is clearly needed.

Carbon monoxide (CO) possesses anti-inflammatory, anti-oxidant and anti-proliferative effects, and has attracted interest as a possible clinically viable medicinal agent [8, 9]. Similar to medical gasses that are routinely used in clinical situations, such as nitric oxide and oxygen, clinical applications of CO take the form of inhaled gaseous therapy and the use of CO-releasing molecules (CO-RM) [10, 11]. In fact, several studies have demonstrated the efficacy of inhaled CO and CO-RM in preclinical animal models such as disorders related to inflammation and redox [12-14]. In addition, it was reported that inhaled CO and CO-RM also exerts protective effects in the case of several types of lung diseases, including pulmonary hypertension, asthma and ischemia reperfusion [15-17]. Taking these findings into consideration, CO holds enormous potential for use in the treatment of pulmonary disorders, including IPF. However, CO-RM rapidly liberate CO, with a half-life of 1-21 min, which is extremely short in terms of producing a significant therapeutic impact [18]. To achieve a sustainable therapeutic effect of CO, the continuous or repeated administration of CO-RM would be required. In addition, although high serum carboxyhemoglobin (HbCO) levels can cause several toxicity [19], it is difficult to control the serum HbCO levels as the result of inhaled CO and avoid CO intoxication. Therefore, it should be noted that an alternative pathway for the therapeutic delivery of CO to the lungs is essential in the successful clinical application of CO.

Recent developments in nanotechnology-based carriers, namely, Hemoglobin-vesicles (HbV), would offer great potential for effective CO delivery, and

could lead to strategies in the development of new CO donors. To date, several preclinical trials have evaluated the histology, biochemical analysis and pharmacokinetic properties after the single or repeated administration of a putative dose of HbV in rodent, pig and monkey [20-24]. The results show that HbV possesses good biological compatibility (low complement activation) and is promptly metabolized (no accumulation in the body) even after a massive single or repeated infusion. Furthermore, the size of HbV is controlled at ca. 250 nm, because it can prevent capillary plugging, renal excretion and vascular wall permeability. Fortunately, CO easily and stably binds to hemoglobin (Hb) in the form of HbV as well as red blood cell (RBC), because the cellular structure of HbV most closely mimics the characteristics of a natural RBC, in which a highly concentrated Hb is encapsulated within a liposome with polyethylene glycol (PEG). In addition, in a previous study, we reported that CO was exhaled within 6 hour after administering CO-bound HbV (CO-HbV) to hemorrhagic-shocked rats [25]. These findings led us to the hypothesis that HbV has the potential for use a carrier of CO to the lungs. Given the known therapeutic effects of CO-HbV on IPF, we decided to first evaluate whether CO-HbV could protect against IPF using an IPF animal model of bleomycin (BLM)-induced pulmonary fibrosis. In subsequent experiments, we investigated the reason why CO-HbV functions to suppress the progression of IPF.

Materials and Methods

Preparation of HbV and CO-HbV solution

HbVs and CO-HbV were prepared under sterile conditions, as previously reported [26]. In short, the Hb solution was purified from outdated donated RBC, which was provided by the Japanese Red Cross Society (Tokyo, Japan), and the oxyhemoglobin converted into HbCO by bubbling with CO gas. The lipid bilayer was a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan) at a molar ratio of 5/5/1, and 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-*N*-PEG (NOF Corp., Tokyo, Japan) (0.3 mol%). The CO-HbV particles were prepared by the extrusion method, and suspended in a physiological salt solution, filter-sterilized (Dismic, Toyo-Roshi, Tokyo, Japan; pore size, 450 nm). By illumination with visible light under an oxygen atmosphere, CO-HbV was converted to HbV. The HbV particles suspended in physiological salt solution bubbled with nitrogen for storage. The average diameters of the HbV and CO-HbV were maintained at approximately 250 nm *via* stepwise extrusion through cellulose acetate membrane filters with a final pore size of 0.2 μm (Fig. 1). The HbV and CO-HbV suspended in physiological salt solution were at [Hb] = 10 g/dL amd [lipid] = 9.0 g/dL. The HbCO rate in CO-HbV was nearly 100%, while that in HbV was less than 5%.

Production of BLM-induced pulmonary fibrosis mice model

All animal experiments were conducted in accordance with the guidelines of Kumamoto University for the care and use of laboratory animals. To create BLM-induced

pulmonary fibrosis model mice, Sea-ICR mice (6 weeks, male; Kyudo Co.,Ltd, Saga, Japan) were intratrachealy treated with BLM (5 mg/kg; Nippon Kayaku, Tokyo, Japan) in PBS (1 ml/kg) under anesthesia with chloral hydrate (500 mg/kg) as previous report [27]. Saline, HbV, or CO-HbV was administered *via* the tail vein at 30 min before BLM treatment and 24 hour after BLM treatment.

Plasma biochemical parameters

At 7 and 14 days after the HbV injection, BLM-induced pulmonary fibrosis model mice were anesthetized with ether and collected blood. Blood samples were immediately centrifuged (3000 g, 10 min) to produce plasma. The plasma samples were then ultracentrifuged to remove HbV (50000 g, 30 min), because HbV interferes with some of the laboratory tests [28]. All plasma samples were stored at -80 °C until used. All plasma samples were analyzed by Clinical Chemistry Analyzer (JEOL, JCA-BM6050, Tokyo, Japan).

Histological and immunohistochemical analyses

The whole lungs were removed and fixed with 10% phosphate buffered formalin. The tissue was then dehydrated at room temperature through a graded ethanol series and embedded in paraffin. The prepared tissues were cut into 4-μm-thick sections for histological and immunohistochemical evaluation. Hematoxylin and Eosin (HE) stain and Masson's trichrome stain were performed as previously described [27]. The immunostaining for 8-hydroxy-2'-deoxygenase (8-OH-dG) and nitrotyrosine (NO_2-Tyr) were performed as described in a previous report with minor modifications [29]. In short, the primary antibody reaction was conducted below 4°C overnight, and the secondary

antibody reaction at room temperature for 90 min. In addition, the primary antibody containing NO_2-Tyr (Millipore, Tokyo, Japan, cat#: AB5411) and 8-OH-dG [15A3] (Santa Cruz, California, USA, cat#: sc-66036) was diluted 50 fold prior to use. The secondary antibodies for 8-OH-dG and NO_2-Tyr were Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen, Eugene, USA, cat#: AB11008) and Alexa Fluor 546 goat anti-rabbit IgG (H+L) (Invitrogen, Eugene, USA, cat#: AB11010), respectively. In each case the secondary antibody was diluted 200 times before use. After the reaction, the slide was observed using Microscope (Keyence, BZ-8000, Osaka, Japan).

Determination of hydroxyproline level in lung tissues

On day 14 after BLM administration, the left lung was removed and hydroxyproline content was determined as described previously [30]. The absorbance was measured at 550 nm to determine the amount of hydroxyproline.

Measurement of lung mechanics and FVC

Measurement of lung mechanics and FVC were performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ), as described previously [31]. Mice were mechanically ventilated at a rate of 150 breaths/min, using a tidal volume of 8.7 mL/kg and a positive end-expiratory pressure of 2 to 3 cm H_2O. Total respiratory system elastance and tissue elastance were measured by the snap shot and forced oscillation techniques, respectively.

Counting of cells in bronchoalveolar lavage fluid (BALF)

At days 3 after the BLM administration, BALF was collected as described

previously [27]. Total cell number was counted using a hemocytometer. Cells were stained with Diff-Quick reagents (Kokusai Shiyaku, Kobe, Japan), and the ratios of alveolar macrophages, neutrophils, and lymphocytes to total cells were determined. More than 200 cells were counted for each sample.

Quantification of tumour necrosis factor (TNF)-α, interleukin-6 (IL-6), interleukin-1β (IL-1β) and activated TGF-β1 in lung tissue

At days 7 and 14 after the BLM administration, whole lungs were removed and homogenized in 0.5 ml of buffer (PBS, 1% protease inhibitor cocktail, 10 mM EDTA, 0.05% Tween-20). After centrifugation at 21,000 g for 10 min at 4°C (twice), the supernatants were recovered. The amount of TNF-α, IL-6, IL-1β on Day 7 and activated TGF-β1 on Day 14 in the supernatant was measured by ELISA kit (TGF-β1 ELISA kit; R&D Systems Inc., Minneapolis, USA, IL-6, TNF-α and IL-1β ELISA kit; Biolegend, San Diego, USA).

Western blotting analysis

At day 7 after the BLM administration, whole lungs were removed and homogenized in a homogenization buffer composed of 70 mmol/l sucrose, 10 mmol/l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 210 mmol/l mannitol, 1 mmol/l EDTA, 1 mmol/l EGTA (ethylene glycol tetraacetic acid), pH 7.5, 200 mmol/l dithiothreitol, and 1% protease inhibitor cocktail. The homogenate was centrifuged at 720 g for 5 min at 4 °C, and the supernatants were recovered. The supernatants were centrifuged at 10000 g for 5 min at 4 °C and further centrifuged at 100000 g for 1 hour. The resultant pellet is referred to as the crude membrane fraction. After measurement of

the protein content, each sample was mixed in a loading buffer (2% sodium dodecyl sulfate, 62.5 mmol/l Tris-HCl and 1% 2-mercaptoethanol). These samples (40 mg) were run on 12.5% sodium dodecyl sulfate polyacrylamide gels, followed by electrophoretic transfer to nitrocellulose membranes. The membranes were blocked by treatment with 5% skimmed milk in PBS for 1hour at room temperature and then incubated with rabbit polyclonal anti-human nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) [H-300] (Santa Cruz, California, USA, cat#: sc-30141, 1:500) antibodies, goat polyclonal anti-human $p22^{phox}$ [C-17] (Santa Cruz, California, USA, cat#: sc-11712, 1:600) antibodies, rabbit polyclonal anti-human polymerase delta interacting protein 2 (Poldip2) (Abgent, California, USA, cat#: AP7626b, 1:200) antibodies, or mouse monoclonal anti-human β-actin antibody (1:5000) overnight at 4 °C. The membranes were washed with 0.05% Tween-20 (T-PBS), and a horseradish peroxidase–conjugated anti-goat IgG antibody (Santa Cruz, California, USA, cat#: sc-2768, 1:5000), an anti-rabbit IgG antibody (Santa Cruz, California, USA, cat#: sc-2004, 1:5000), and an anti-mouse IgG antibody (Santa Cruz, California, USA, cat#: sc-2005, 1:10000) were then used for the detection of the target proteins. SuperSignal Western blotting detection reagents (Thermo Scientific, Rockford, IL) were used for immunodetection.

Detection of superoxide

Dihydroethidium was used to evaluate lung superoxide concentrations *in situ*, as described in detail elsewhere [32]. After the reaction, the slide was observed under a microscope (Keyence, BZ-8000, Osaka, Japan).

Statistics

Statistical analyses were performed using the analysis of variance. A probability value of $p<0.05$ was considered significant.

Results

Evaluation of the optimal dosing of CO-HbV in BLM-induced pulmonary fibrosis.

Figure 2A shows a schematic summary of the experimental protocols used in the study. Pulmonary fibrosis was induced in the mice by a single intratracheal administration of BLM (at day 0) and confirmed 14 days later. In order to determine the optimal dosing of CO-HbV in BLM-induced pulmonary fibrosis, histopathological analysis (HE stain and Masson's trichrome stain) and hydroxyproline levels were evaluated after the administration of CO-HbV at 30 min prior to the BLM treatment and day 1 after the BLM treatment at doses of 250, 500, 1000 and 1400 mg Hb/kg. As shown in Figure 2B and 2C, pulmonary fibrosis was suppressed as the result of the administration of CO-HbV and suppression was dose-dependent. The maximum ameliorative effect was achieved at a concentration of 1000 mg Hb/kg.

We also evaluated the toxic effects of CO-HbV administration in BLM-induced pulmonary fibrosis mice. No evidence of the development of signs of hypoxia or abnormal behavior was found when CO-HbV as administered at a dose of 1000 mg Hb/kg. Furthermore, no changes in of serum laboratory parameters reflecting hepatic, renal and pancreatic function were found at 7 and 14 days after CO-HbV administration, except for an elevation in cholesterol levels, compared to the saline treatment in BLM-induced pulmonary fibrosis mice (Table 1). Based on these results, we concluded that the optimal dose of CO-HbV was 1000 mg Hb/kg.

Effect of CO-HbV on BLM-induced pulmonary fibrosis

To assess the effect of CO-HbV on the development of BLM-induced pulmonary fibrosis, BLM-induced pulmonary fibrosis mice were treated with saline, HbV (1000 mg