and GLP-based examinations on the safety of HbV are requisite before its clinical use.

In conclusion, HbV showed the effectiveness in two kinds of rat stroke models and was expected to provide a new therapeutic option for the treatment of stroke, especially acute ischemic stroke. To advance a clinical development of HbV as a new anti-stroke agent, further studies on a dose–response relationship, histopathological examination, and mechanism(s) of action including direct analysis of blood flow and oxygen delivery into the ischemic area would be conducted as next step.

Acknowledgements

The authors would like to thank Drs. Eishun Tsuchida, Shinji Takeoka, Makoto Suematsu and Hiromi Sakai for helpful comments on the present study, and Dr. Noam Ship for help of the manuscript preparation.

References

[1] Y. Aoki, M. Tamura, Y. Itoh, T. Seto, K. Nonaka, H. Mukai, Y. Ukai, Effective plasma concentration of a novel Na^+/Ca^{2+} channel blocker NS-7 for its cerebroprotective actions in rats with a transient middle cerebral artery occlusion, J. Pharmacol. Exp. Ther. 296 (2001) 306–311.

[2] D. Bochelen, M. Rudin, A. Sauter, Calcineurin inhibitors FK506 and SDZ ASM 981 alleviate the outcome of focal cerebral ischemic/reperfusion injury, J. Pharmacol. Exp. Ther. 288 (1999) 653–659.

[3] K. Burhop, D. Gordon, T. Estep, Review of hemoglobin-induced myocardial lesions, Artif. Cells Blood Substit. Immobil. Biotechnol. 32 (2004) 353–374.

[4] C.H. Chen, T.J. Toung, P.D. Hurn, R.C. Koehler, A. Bhardwaj, Ischemic neuroprotection with selective kappa-opioid receptor agonist is gender specific, Stroke 36 (2005) 1557–1561.

[5] D.J. Cole, J.C. Nary, J.C. Drummond, P.M. Patel, W.K. Jacobson, Alpha–alpha diaspirin crosslinked hemoglobin, nitric oxide, and cerebral ischemic injury in rats, Artif. Cells Blood Substit. Immobil. Biotechnol. 25 (1997) 141–152.

[6] M. Daffertshofer, Z. Huang, M. Fater, M. Popolo, H. Schroeck, W. Kuschinsky, M.A. Moskowitz, M.G. Hennerici, Efficacy of sonothrombolysis in a rat model of embolic ischemic stroke, Neurosci. Lett. 361 (2004) 115–119.

[7] L. Ding-Zhou, C. Marchand-Verrecchia, N. Croci, M. Plotkine, I. Margaill, L-NAME reduces infarction, neurological deficit and blood–brain barrier disruption following cerebral ischemia in mice, Eur. J. Pharmacol. 457 (2002) 137–146.

[8] D. Erni, R. Wettstein, S. Schramm, C. Contaldo, H. Sakai, S. Takeoka, E. Tsuchida, M. Leunig, A. Banic, Normovolemic hemodilution with Hb vesicle solution attenuates hypoxia in ischemic hamster flap tissue, Am. J. Physiol. Heart Circ. Physiol. 284 (2003) H1702–H1709.

[9] T.W. Furlow, N.H. Bass, Stroke in rats produced carotid injection of sodium arachidonate, Science 187 (1975) 658–660.

[10] I. George, G.-H. Yi, A.R. Schulman, B.T. Morrow, Y. Cheng, A. Gu, G. Zhang, M.C. Oz, D. Burkhoff, J. Wang, A polymerized bovine hemoglobin oxygen carrier preserves regional myocardial function and reduces infarct size after acute myocardial infarction, Am. J. Physiol. Heart Circ. Physiol. 291 (2006) H1126–H1137.

[11] H. Imai, H. Masayasu, D. Dewar, D.I. Graham, I.M. Macrae, Ebselen protects both gray and white matter in a rodent model of focal cerebral ischemia, Stroke 32 (2001) 2149–2154.

[12] Y. Izumi, H. Sakai, K. Hamada, S. Takeoka, T. Yamahata, R. Kato, H. Nishide, E. Tsuchida, K. Kobayashi, Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical tissue oxygen tension, Crit. Care Med. 24 (1996) 1869–1873.

[13] Y. Izumi, H. Sakai, T. Kose, K. Hamada, S. Takeoka, A. Yoshizu, H. Horinouchi, R. Kato, H. Nishide, E. Tsuchida, K. Kobayashi, Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model, ASAIO J. 43 (1997) 289–297.

[14] H. Kawai, H. Nakai, M. Suga, S. Yuki, T. Watanabe, K. Saito, Effects of a novel free radical scavenger, MCI-186, on ischemic brain damage in the rat distal middle cerebral artery occlusion model, J. Pharmacol. Exp. Ther. 281 (1997) 921–927.

[15] K. Kobayashi, H. Horinouchi, M. Watanabe, Y. Izumi, Y. Teramura, A. Nakagawa, Y. Huang, K. Sou, H. Sakai, T. Komatsu, S. Takeoka, E. Tsuchida, Safety and efficacy of hemoglobin-vesicles and albumin-hemes, in: K. Kobayashi, E. Tsuchida, H. Horinouchi (Eds.), Artificial Oxygen Carrier. Its Front Line, Vol. 12, Springer, Tokyo, 2005, pp. 1–21.

[16] C. Nito, T. Kamiya, S. Amemiya, K. Kato, Y. Katayama, The effects of a free radical scavenger, edaravone, combined with mild hypothermia on ischemic brain damage following transient middle cerebral artery occlusion in rats, Int. Congress Series 1252 (2003) 109–115.

[17] H. Sakai, P. Cabrales, A.G. Tsai, E. Tsuchida, M. Intaglietta, Oxygen release from low and normal P50 Hb vesicles in transiently occluded arterioles of the hamster window model, Am. J. Physiol. Heart Circ. Physiol. 288 (2005) H2897–H2903.

[18] H. Sakai, H. Horinouchi, Y. Masada, S. Takeoka, E. Ikeda, M. Takaori, K. Kobayashi, E. Tsuchida, Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model, Biomaterials 25 (2004) 4317–4325.

[19] H. Sakai, H. Horinouchi, K. Tomiyama, E. Ikeda, S. Takeoka, K. Kobayashi, E. Tsuchida, Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system, Am. J. Pathol. 159 (2001) 1079–1088.

[20] H. Sakai, Y. Masada, H. Horinouchi, E. Ikeda, K. Sou, S. Takeoka, M. Suematsu, M. Takaori, K. Kobayashi, E. Tsuchida, Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days, J. Pharmacol. Exp. Ther. 311 (2004) 874–884.

[21] H. Sakai, Y. Masuda, H. Horinouchi, M. Yamamoto, E. Ikeda, S. Takeoka, K. Kobayashi, E. Tsuchida, Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats, Crit. Care Med. 32 (2004) 539–545.

[22] H. Sakai, Y. Suzuki, M. Kinoshita, S. Takeoka, N. Maeda, E. Tsuchida, O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permeable tube: comparison with RBCs and acellular Hbs, Am. J. Physiol. Heart. Circ. Physiol. 285 (2003) H2543–H2551.

[23] S. Shirakura, J. Sano, A. Karasawa, K. Kubo, Protective effects of benidipine on arachidonic acid-induced acute cerebral ischemia in rats, Jpn. J. Pharmacol. 59 (1992) 15–22.

[24] M. Suzuki, M. Suzuki, Y. Kitamura, S. Mori, K. Sato, S. Dohi, T. Sato, A. Matsuura, A. Hiraide, β-Hydroxybutyrate, a cerebral function improving agent, protects rat brain against ischemic damage caused by permanent and transient focal cerebral ischemia, Jpn. J. Pharmacol. 89 (2002) 36–43.

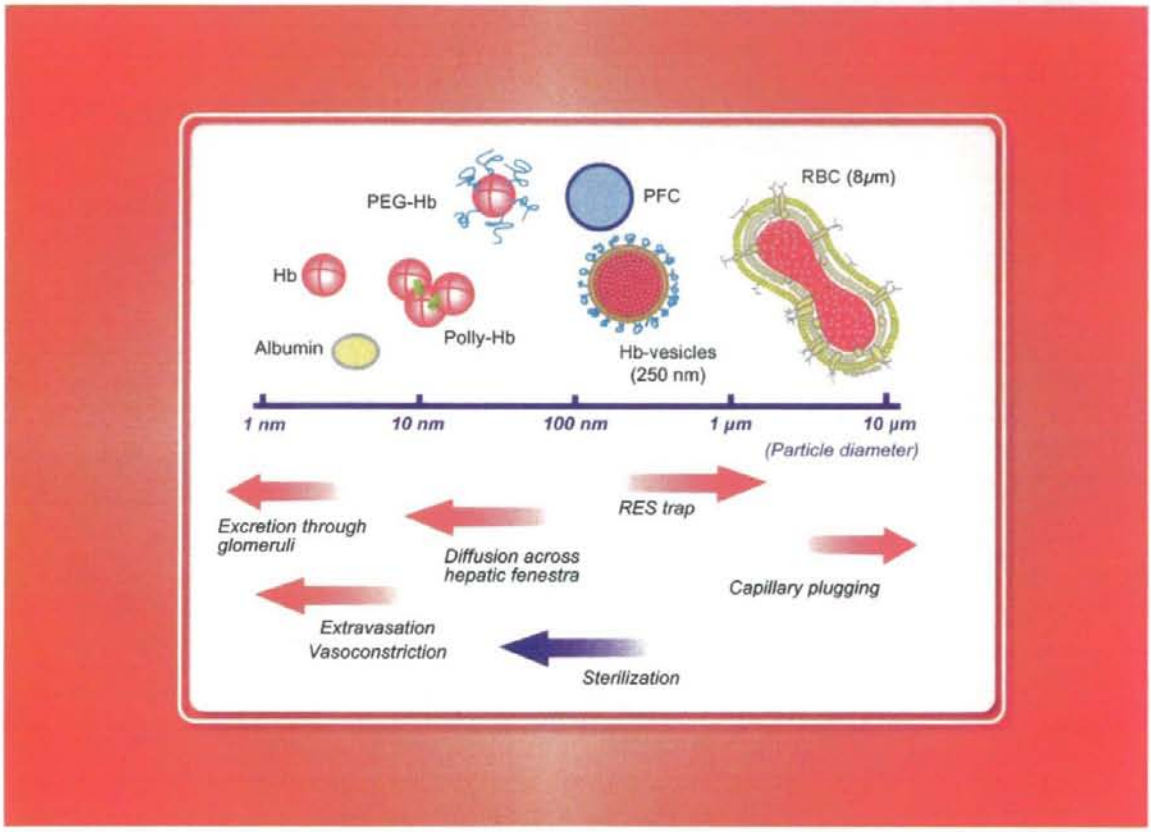
[25] T. Tanaka, M. Takei, Y. Fukuta, R. Higashino, Y. Fukuda, Y. Nomura, S. Ito, H. Tamaki, T. Kurimoto, Y. Suzuki, Arachidonic acid-induced hind limb gangrene: a new experimental rat model of peripheral vascular disease, Biol. Pharm. Bull. 22 (1999) 257–260.

[26] J. Yrjanheikki, T. Tikka, R. Keinanen, G. Goldsteins, P.H. Chan, J. Koistinaho, A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window, Proc. Natl. Acad. Sci. USA 96 (1999) 13496–13500.

[27] Y.M. Yu, J.B. Kim, K.W. Lee, S.Y. Kim, P.L. Han, J.K. Lee, Inhibition of the cerebral ischemic injury by ethyl pyruvate with a wide therapeutic window, Stroke 36 (2005) 238–2243.

ISSN 0954-6820 Vol. 263 No. 1 January 2008
www.blackwellpublishing.com/jim
www.jim.se

Journal of INTERNAL MEDICINE



Blackwell Publishing

Review | Journal of INTERNAL MEDICINE

doi: 10.1111/j.1365-2796.2007.01893.x

Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions

■ H. Sakai[1], K. Sou[1], H. Horinouchi[2], K. Kobayashi[2] & E. Tsuchida[1]

From the [1]Oxygen Infusion Project, Advanced Research Institute for Science and Engineering, Waseda University; and [2]Department of General Thoracic Surgery, School of Medicine, Keio University; Tokyo, Japan

Abstract. Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E (Research Institute for Science and Engineering, Waseda University; and School of Medicine, Keio University; Tokyo, Japan). Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions (Review). *J Intern Med* 2008; **263**: 4–15.

During the long history of development of haemoglobin (Hb)-based O_2 carriers (HBOCs), many side effects of Hb molecules have become apparent. They imply the physiological importance of the cellular structure of red blood cells. Hb-vesicles (HbV) are artificial O_2 carriers that encapsulate concentrated Hb solution with a thin lipid membrane. We have overcome the intrinsic issues of the suspension of HbV as a molecular assembly, such as stability for storage and in blood circulation, blood compatibility and prompt degradation in the reticuloendothelial system. Animal tests clarified the efficacy of HbV as a transfusion alternative and the possibility for other clinical applications. The results of ongoing HbV research make us confident in advancing further development of HbV, with the expectation of its eventual realization.

Keywords: artificial oxygen carrier, biocompatibility, liposome, nanotechnology, polyethylene glycol.

Introduction

Since the discovery of blood type antigen by Landsteinter in 1900, allogeneic blood transfusion has developed into a routine clinical practice; it has contributed to human health and welfare. Infectious diseases such as hepatitis and HIV have become widespread social problems, but a strict virus test by nucleic acid amplification test (NAT) is extremely effective to detect trace presences of a virus to minimize infection (although it is available mainly in a few developed countries). Even so, NAT poses problems such as detection limits during a window period and limited species of viruses for testing. Emergence of new viruses (such as West Nile virus, avian influenza and Ebola) and a new type of pathogen, prions, also threaten humans throughout the world. The preservation period of donated red blood cells (RBCs) is limited to 3 weeks in Japan. Immunological responses (such as anaphylaxis and graft-versus-host disease), and contingencies of blood type incompatibility further limit the utility of blood products. To obviate or minimize homologous transfusion, the transfusion trigger has been reconsidered, and roughly reduced from 10 to 6–8 g dL^{-1}. Bloodless surgery and preoperational enhancement of erythropoiesis for storing autologous blood have become common. However, these epoch-making treatments are not always practical for all patients. Some developed countries with ageing populations are confronting a decreasing number of young donors and an increasing number of aged recipients. Prohibition of blood donation from people who have travelled certain countries during a specific period also exacerbates the blood shortage in Japan. On the other hand, in some developing countries, establishment of a safe blood donation system is difficult. Under such circumstances, research of blood substitutes has gathered great attention and has been developed worldwide [1–4]. In Japan, for example, the government has given strong support to development of blood

substitutes in the wake of two tragedies: infection, by AIDS, of haemophiliac patients who had received nonpasteurized plasma products and the Great Hanshin Earthquake disaster. The requisites for artificial oxygen carriers that we develop should be not only effectiveness for tissue oxygenation, but also the following:

1 No blood type antigen and no infection (no pathogens);
2 Stability for long-term storage (e.g. over 2 years) at room temperature for stockpiling for any emergency;
3 Low toxicity and prompt metabolism, even after massive infusion;
4 Physichochemical properties that are adjustable to resemble those of human blood and
5 Reasonable production expense and cost performance.
Realization of such an artificial oxygen carrier will revolutionize transfusion medicine.

Physiological significance of cellular structure

Physicochemical measurements of O_2-releasing behaviours have revealed that the cellular structure of RBCs might not be effective for facilitating O_2 releasing in comparison with a homogeneous haemoglobin (Hb) solution [5–7]. However, nature has selected this cellular structure through evolution. The reasons for Hb encapsulation in RBCs are: (i) a decrease in the high colloidal osmotic pressure of Hb; (ii) prevention of the removal of Hb from blood circulation and (iii) preservation of the chemical environment in cells, such as the concentration of phosphates (2,3-diphosphoglyceric acid (DPG), ATP, etc.) and other electrolytes. Moreover, during the long history of the development of Hb-based O_2 carriers (HBOCs), many side effects of Hb molecules have become apparent, such as the dissociation of tetrameric Hb subunits into two dimers ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$) that might induce renal toxicity, and entrapment of gaseous messenger molecules (NO and CO) inducing vasoconstriction, hypertension, reduced blood flow and tissue oxygenation at microcirculatory levels [8, 9], neurological disturbances, and the malfunctioning of oesophageal motor function [10]. These side effects of Hb molecules imply the importance of the cellular structure (Fig. 1).

Pioneering work of Hb encapsulation to mimic the cellular structure of RBCs was performed by Chang in 1957 [1], who prepared microcapsules (5 μm) made of nylon, collodion, etc. Toyoda in 1965 [11] and the Kambara-Kimoto group [12] also covered Hb solutions with gelatine, gum Arabic, silicone, etc. Nevertheless, it was shown to be extremely difficult to regulate the particle size that was appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne reported in 1964 that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior [13], it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller in 1977 prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acids, etc. [14]. In the US, Naval Research Laboratories showed remarkable progress of LEH [15].

However, some intrinsic issues of encapsulated Hbs remained, mainly related to molecular assembly and particle dispersion. What we call Hb-vesicles (HbV) with high-efficiency production processes and their improved properties, were established by Tsuchida's group [16–18] based on technologies of molecular assembly and precise analysis of pharmacological and physiological aspects (Fig. 2). The salient characteristics of HbV are the following:

1 Human Hb is purified completely via pasteurization at 60 °C and ultrafiltration; no viruses exist [19–21];
2 A concentrated Hb solution, nearly 35 g dL^{-1}, is encapsulated with a thin bilayer membrane [16–18];
3 A new synthetic lipid is used to prevent platelet (PLT) activation [22, 23];
4 PEG-modification guarantees long-term storage over 2 years at room temperature, blood compatibility and extended circulation half-life [24–30];

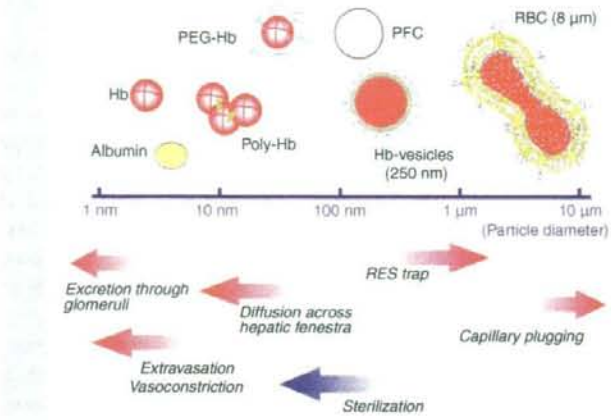


Fig. 1 What is the optimal dimension of artificial oxygen carriers? There is an upper limitation, below the capillary diameter, to prevent capillary plugging, and for the sterilization by membrane filters. On the other hand, the much smaller ones show higher rates of renal excretion and vascular wall permeabilities with side effects such as hypertension and neurological disturbances. Hb-vesicles show very low level of vascular wall permeabilities. Therefore, the Hb-vesicles seems appropriate from the viewpoint of hemodynamics. However, we have to clarify the influence of Hb-vesicles on the reticuloendothelial system (RES) because the fate of Hb-vesicles is RES trapping (see Fig. 3).

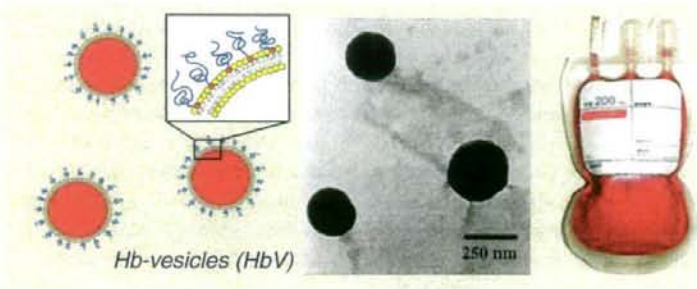


Fig. 2 (Left) Schematic representation of Hb-vesicle (HbV). One particle contains about 30 000 Hb molecules. The surface of one HbV is modified using polyethylene glycol chains that ensure the dispersion stability of HbV during storage and during circulation in the bloodstream. (Middle) The transmission electron micrograph depicts the well-regulated particle size (250 nm) and high Hb content within the vesicles. (Right) The packed HbV suspension looks turbid, like a mixture of milk and red wine, because of light-scattering of the particle suspension.

5 The cellular structure, which resembles that of RBCs, shields all side effects of Hb molecules, such as scavenging NO and CO [8, 9, 27];

6 The particle size (250 nm) is appropriate for sterilization, circulation persistence and biodistribution [18, 28] and

7 Hb-vesicles do not show colloid osmotic pressure. Addition of a plasma substitute solution such as recombinant albumin is effective to regulate colloid osmotic pressure [31–33].

Stabilized HbV for a long-term storage

Because Hb autoxidizes to form metHb and loses its O_2-binding ability during storage as well as during blood circulation, prevention of metHb formation is necessary. Some groups have reported a method to preserve deoxygenated Hbs in the liquid state [34] using well-known intrinsic characteristics of Hb: the Hb oxidation rate in a solution is dependent on the O_2 partial pressure; also, deoxyHb is not autoxidized at ambient temperatures [35]. For HbV, not only the inside Hb, but also the cellular structure (liposome) must be physically stabilized to prevent intervesicular aggregation, fusion and leakage of the encapsulated Hb.

Liposomes, as molecular assemblies, have been generally inferred to be structurally unstable. Many researchers have sought to develop stabilization methods that use polymer chains [36]. Polymerization of phospholipids that contain dienoyl groups was studied extensively in our group. For example, gamma-ray irradiation induces radiolysis of water molecules and generates OH radicals that initiate intermolecular polymerization of dienoyl groups in phospholipids. This method produces enormously stable liposomes, like rubber balls, which are resistant to freeze-thawing, freeze-drying and rehydration [37–39]. However, the polymerized liposomes were so stable that they were not degraded easily in the macrophages, even 30 days after injection [40]. It was concluded that polymerized lipids would not be appropriate for intravenous injection. Subsequently, it was clarified that selection of appropriate lipids (phospholipid/cholesterol/negatively charged lipid/PEG-lipid) and their composition are important to enhance the stability of liposomes without polymerization. Surface modification of liposomes with PEG chains is sufficient for dispersion stability [24–30].

We investigated the possibility of long-term preservation of HbV through a combination of two techniques, e.g. deoxygenation and PEG modification during storage for 2 years [24]. The PEG chains on the vesicular surface stabilize the dispersion state and prevent aggregation and fusion for 2 years because of their steric hindrance. The original metHb content (approximately 3%) before preservation decreased gradually to <1% in all samples after 1 month because of the presence of a reductant, such as homocysteine, inside the vesicles that consumed the residual O_2 and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the O_2 partial pressure: no increase in the metHb formation was observed because of the intrinsic stability of the deoxygenated Hb. In fact, the metHb content did not increase for 2 years. These results clearly indicate the possibility that the HbV suspension can be stored at room temperature for at least 2 years, which would enable stockpiling of HbV for any emergency.

Blood compatibility of Hb-vesicles

Liposome is not a solute but a particle in a suspension. Once injected, the surface is sometimes recognized by, or interacted with blood components. The so-called 'injection reaction', or pseudo-allergy is caused by complement activation with liposomal products [41] and a perfluorocarbon emulsion. Therefore, examination of blood compatibility of liposomal particles is important for clinical use. Transient thrombocytopenia in relation to complement activation is an extremely important haematological effect observed in rodent models after infusion of LEH (containing DPPG: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl glycerol), developed by the Naval Research Laboratory [42, 43]. In our group, exchange transfusion with the old-type HbV (containing DPPG, no PEG modification) in anesthetized rats resulted in thrombocytopenia [31]. Similar effects were also observed for administration of negatively charged liposomes [44, 45]. The transient reduction in PLT counts caused by liposomes was also associated with sequestration of PLTs in the lung and liver. Such non-physiological PLT activation would engender initiation and modulation of inflammatory responses because PLTs contain an array of potent proinflammatory

substance. However, the present HbV apparently does not induce thrombocytopenia in animal experiments, probably because the present HbV contains PEG-modification and a different type of negatively charged lipid (DHSG: 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate), not DPPG or a fatty acid [22, 23].

Detailed blood compatibility of HbV in relation to negatively charged lipid was examined by Dr H. Ikeda at Hokkaido Red Cross Blood Center (Sapporo) and his colleagues [22, 23, 25, 46]. The present PEG-modified HbV containing DHSG did not affect the extrinsic or intrinsic coagulation activities of human plasma, whereas HbV containing DPPG and no PEG modification tended to shorten the intrinsic coagulation time. The kallikrein-kinin cascade of the plasma was activated slightly by DPPG-HbV, but not by the present PEG-DHSG-HbV. Moreover, the complement consumption of the plasma was observed by incubation with DPPG-HbV, but not with the present PEG-DHSG-HbV. These results indicate that the present PEG-DHSG-HbV has a higher biocompatibility with human plasma. Moreover, the exposure of human PLTs to high concentrations of the present HbV (up to 40%) *in vitro* did not cause PLT activation and did not adversely affect the formation and secretion of prothrombotic substances or proinflammatory substances that are triggered by PLT agonists. These results imply that HbV, at concentrations of up to 40%, has no aberrant interactions with either unstimulated or agonist-induced PLTs.

Biodistribution and fate of Hb-vesicles in reticuloendothelial system

The dose rate of blood substitutes would be considerably larger than those of other drugs, and their circulation time would be considerably shorter than RBC. Therefore, their biodistribution, metabolism, excretion and side effects must be characterized in detail especially about the reticuloendothelial system (RES).

Normally, free Hb released from RBC is bound rapidly to haptoglobin and is consequently removed from circulation by hepatocytes. However, when the Hb concentration is greater than the haptoglobin-binding capacity, unbound Hb is filtered through the kidney, where it is actively absorbed. Haemoglobinuria and eventual renal failure occur when the reabsorption capacity of the kidney is exceeded. The encapsulation of Hb in vesicles completely suppresses renal excretion. However, HbV in the bloodstream is ultimately captured by phagocytes in the RES (or mononuclear phagocytic system) in much the same manner as senescent RBC are, as confirmed by radioisotope ^{99m}Tc-labelled HbV injection [15, 28]. Gamma camera images of ^{99m}Tc-HbV showed that HbV remains in the bloodstream immediately after infusion so that the heart and liver that contain much blood showed strong intensity (Fig. 3a). However, HbV are finally distributed mainly in the liver, spleen and bone marrow. The circulation half-life is dose dependent; when the dose rate was 14 mL kg^{-1}, the circulation half-life was 32 h. The circulation time in the case of the human body can be estimated as twice or three times longer; or about 2 or 3 days at the same dose rate.

The time course of liver uptake was monitored using a confocal fluorescence microscope after fluorescence-labelled HbV was infused intravenously in an anesthetized hamster. Even though the individual particles of HbV were indistinguishable, they are recognizable with strong fluorescence when HbV are accumulated in phagosomes of Kupffer cells (Fig. 3b). Transmission electron microscopy (TEM) of the spleen 1 day after infusion of HbV clearly demonstrated the presence of HbV particles in macrophages, where HbV particles that appear as black dots are captured by the phagosomes [47] (Fig. 3c). However, after 7 days, the HbV structure cannot be observed. We confirmed transient splenomegaly with no irreversible damage to the organs and complete metabolism within a week. Immunochemical staining with a polyclonal anti-human Hb antibody was used as the marker of Hb in the HbV, and clarified that HbV almost disappeared after 7 days in both the spleen and liver (Fig. 3d) [47].

During metabolism of Hb, bilirubin and iron would be released. However, in our animal experiments of topload infusion, daily repeated infusions, and 40% blood exchange, neither of those products increased

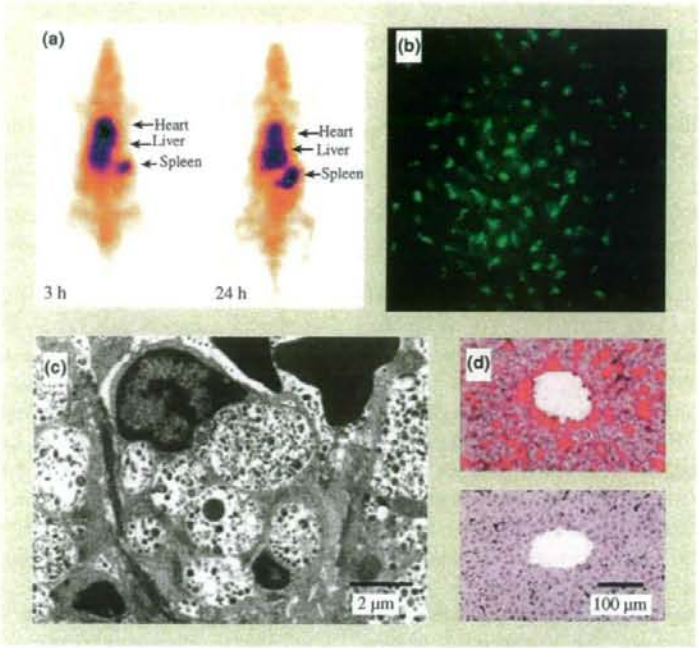


Fig. 3 Biodistribution and fate of Hb-vesicle (HbV). (a) Gamma-camera images of the distribution of ^{99m}Tc-labelled HbV in rats. At 3 h after injection, the heart and the liver showed a strong intensity because of the large blood volume. However, 24 h later, the intensity increased in the liver and spleen, the so-called reticuloendothelial system. (b) The liver surface of an anesthetized golden hamster 40 min after injection of fluorescence-labelled HbV observed using laser confocal scanning microscopy. The individual HbV particles flowing in the sinusoid are not detected, but the strong fluorescence is observed only in the Kupffer cells when they phagocyte HbV. (c) Transmission electron micrograph of rat spleen 1 day after intravenous injection of HbV. The small black dots are HbV near red blood cell in the capillaries and in the phagosomes of spleen macrophages. They disappear completely within 1 week. (d) Staining with anti-human Hb antibody revealed the presence of HbV in the liver Kupffer cells and sinusoids 1 day after infusion. However, they disappear within 1 week.

in the plasma within 14 days [33, 48, 49]. The released haeme from Hb in HbV might be metabolized by the inducible form of haeme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages. Bilirubin would normally be excreted in the bile as a normal pathway, and no obstruction or stasis of the bile should occur in the biliary tree. Berlin blue staining revealed considerable deposition of haemosiderin in the liver and spleen, even after 14 days. Normally, iron from a haeme is stored in the ferritin molecule. Both ferritin and haemosiderin release iron. They are anticipated to induce hydroxyl radical production followed by lipid peroxidation. The iron release rate from haemosiderin, however, is substantially less than that from ferritin. Consequently, the excess amount of iron would then normally be stored in an insoluble and less toxic form as haemosiderin. Hemosiderosis often occurs in patients who have received repeated blood transfusions because of the shorter half-life of the stored RBCs. Moderate splenomegaly and haemosiderin deposition were also confirmed in the spleen after injection of stored RBCs, partly because of the accumulation and degradation of stored RBCs with the lowered membrane deformability and shortened circulation half-life [33, 50].

As for the membrane components of HbVs, it was reported that the infused lipid components of

liposomes are entrapped in the Kupffer cells, and that phospholipid is metabolized and reused as a component of the cell membrane, or excreted in bile, especially as fatty acids and CO_2 in exhaled air. It was recently clarified using a ^{3}H-cholesterol that cholesterol of HbV is released from macrophages to blood, and is ultimately excreted in faeces. The PEG chain is widely used for surface modification of liposomal products. The chemical crosslinker of PEG-lipid is susceptible to hydrolysis to release PEG chains during metabolism. The released PEG chains, which are known as inert macromolecules, should be excreted in urine through the kidneys [51].

More precise data are necessary. However, these results imply that the metabolism of HbV and the excretion are within the physiological capacity that has been well characterized for the metabolism of senescent RBCs and conventional liposomal products.

Rheological properties and efficacy of an Hb-vesicle suspension as a transfusion alternative

A single HbV particle (approximately 250 nm diameter) contains about 30 000 Hb molecules. The HbV is much smaller than RBC, PLT or white blood cell (WBC) particles (Fig. 4a). Nevertheless, HbV acts as a particle in the blood and not as a solute; the colloid osmotic pressure of the HbV suspension is nearly zero. Addition of a plasma expander is necessary for a large substitution of blood to maintain the blood volume. The plasma expander candidates are human serum albumin (HSA), hydroxyethyl starch, dextran or gelatine, depending on the clinical setting, cost, country and clinician. Recombinant human serum albumin (rHSA) is an alternative [32, 33]. The impossibility of transmission of any infectious disease from humans is the greatest advantage of rHSA, which will soon be approved for clinical use in Japan [52].

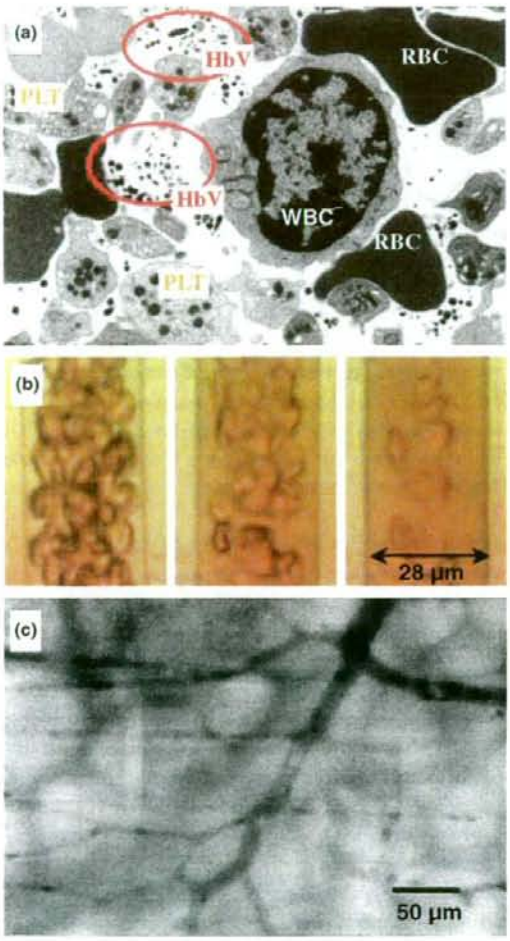


Fig. 4 How small is Hb-vesicle (HbV)? (a) The transmittance electron micrograph of rat blood 1 day after infusion of HbV. The buffy coat, obtained by a centrifugation of blood, was fixed using a 2.5% glutaraldehyde solution. Many HbV particles are visible in the red circles. They are much smaller than red blood cell (RBC), WBCs or PLT. (b) Flow patterns of the mixture of HbV and RBC suspended in recombinant human serum albumin in a narrow tube (centerline flow velocity: 1 mm s^{-1}). From left to right, the mixing ratios, RBC/HbV by volume are 100/0, 50/50 and 10/90 at a constant (Hb) = 10 g dL^{-1}. The RBCs tend to flow in the centerline, whereas HbV particles are dispersed homogeneously in a suspension medium. (c) Micrograph of a hamster skin microvasculature after 80% exchange transfusion with HbV suspended in 5% HSA solution, with an illumination with a wavelength of about 420 nm, being absorbed at the *Soret* band of Hb in HbV and RBC. The capillaries are blackened because of the homogeneous dispersion of HbV in the plasma phase. This homogeneous distribution believed to be effective for tissue oxygenation.

The rheological property of an artificial oxygen carrier is important because the infusion amount should be considerably large, which might affect the blood viscosity and hemodynamics. The viscosity of HbV suspended in 5%-rHSA was similar with that of blood, and the mixtures with RBC at various mixing ratios showed viscosities of 3–4 cP [53]. The main component to determine blood viscosity is RBC; the results indicate no great interaction between HbV and RBC. To observe the flow pattern of the mixture of HbV and RBC, they were mixed in various volume ratios. Then the suspension was perfused through an O_2-permeable narrow tube (28 μm inner diameter) and exposed to a deoxygenated environment [6]. Because HbV was dispersed homogeneously in the rHSA solution, increasing the volume of the HbV suspension thickened the marginal RBC-free layer and the plasma phase became semitransparent (Fig. 4b). The measurement of the O_2-release rate showed that HbV releases O_2 similarly to RBCs. On the other hand, an acellular Hb solution, in a comparative study, showed the facilitated O_2-release attributable to the effect of diffusion of small HbO_2. The slow O_2-release rate of HbV, which resembles that of RBC, is important to prevent autoregulatory vasoconstriction. Microvascular observation after 80% exchange transfusion with HbV suspended in HSA in conscious hamsters with a dorsal skin-fold window model of Prof. Intaglietta (UCSD) also showed that HbV was distributed homogeneously in the plasma phase; the capillary shape was visualized (Fig. 4c). This homogeneous distribution is inferred to be effective for improved blood flow and homogeneous tissue oxygenation.

Extensive *in vivo* studies of such HbV suspended in plasma-derived HSA or rHSA revealed sufficient O_2 transporting efficiency that is apparently comparable to RBCs in extreme blood exchange experiments [29–31, 33, 54–56] and fluid resuscitation from hemorrhagic shock [32, 57–60]. It was confirmed in rat models that haematopoietic activity was preserved and the decreased haematocrit returned to the original level within 1 or 2 weeks, whilst HbV captured in RES disappeared completely [33]. A recent experiment of HbV suspended in rHSA as a priming solution for cardiopulmonary bypass (CPB) in a rat model showed that HbV protects neurocognitive function by transporting O_2 to brain tissue even when the haematocrit is reduced markedly [61]. Homologous blood use is considered to be the gold standard for CPB priming in infants despite exposure of patients to potential cellular and humoral antigens. However, the results indicate that the use of HbV for CPB priming might prevent neurocognitive decline in infants because of considerable hemodilution. Other studies investigating HbV suspension as a possible perfusate for organ transplantation are also underway for the heart, liver, intestine, etc.

New concepts to design HbV

Development of artificial O_2 carriers was initiated originally with a simple idea and an expectation that the materials that bind or dissolve O_2 can behave similarly to RBCs in the bloodstream. Unfortunately, it was not so simple. During its long history of development, unexpected side effects were clarified such as capillary plugging, renal toxicity, vasoconstriction, vascular injury and accumulation. Decades-long R&D of artificial O_2 carriers has yielded no commercially available material for clinical use in Europe, Japan or the US. Recent advanced biotechnology enables *ex vivo* RBC production from haematopoietic stem cells [62]. However, problems remain of large-scale production and long-term storage for stockpiling. On the other hand, no doubts persist about the strong demand and expectation of a blood substitute.

The importance of the sophisticated function of RBCs in concert with vascular physiology has been clarified. New concepts are proposed in terms of the physicochemical properties of Hb-based artificial O_2 carriers. Historically, it has been regarded that the O_2 affinity is regulated similarly to RBCs (25–30 torr). Theoretically, this enables sufficient O_2 unloading during blood microcirculation, as can be evaluated according to the arterio-venous difference in O_2 saturation in accordance with an O_2 equilibrium curve. It has been expected that decreasing O_2 affinity (increasing P_{50}) increases O_2 unloading. However, this concept is controversial in light of recent findings because an excess O_2 supply would cause autoregulatory vasoconstriction

and microcirculatory disorders. A new conceptualization is that HBOCs with a high O_2 affinity (low P_{50}) retain O_2 in the upstream artery or arteriole and release O_2 in the capillaries of the targeted tissue. This hypothesis has been supported recently by results of PEG-modified Hbs and HbV by microcirculatory observations [55, 56, 63, 64]. The P_{50} of HbV is easily regulated by manipulating the content of an allosteric effector, pyridoxal 5′-phosphate (PLP), inside the HbV [55, 65]. For example, equimolar PLP to Hb (PLP/Hb = 1/1 by mol) was coencapsulated, and P_{50} was regulated to 18 torr. When the molar ratio PLP/Hb was 3/1, P_{50} was regulated to 32 torr. The present HbV contains PLP at PLP/Hb = 2.5 by mol; the resulting P_{50} is about 25–28 torr, which shows sufficient O_2 transporting capacity as a transfusion alternative for extreme hemodilution, resuscitative fluid for hemorrhagic shock and prime solution for extracorporeal circulation. The P_{50} of HbV without PLP and Cl^- is 8–9 torr.

Because infusion of an artificial O_2 carrier necessitates the substitution of a large volume of blood, its impact on hemorheology is remarkable. It has been regarded that lower blood viscosity after hemodilution is effective for tissue perfusion. However, microcirculatory observation shows that, in some cases, lower viscosity decreases shear stress on the vascular wall, engendering vasoconstriction and reduced functional capillary density [66]. Therefore, an appropriate viscosity might exist, which maintains the normal tissue perfusion level. A large molecular dimension such as HbV can provide viscous fluids. In relation to this, our recent studies clarified that HbV suspended a series of plasma substitutes can provide non-Newtonian viscous fluid without capillary plugging [67]. A large molecular dimension is also effective to reduce vascular permeability and to minimize the reaction with NO and CO as vasorelaxation factors. These new concepts suggest reconsideration of the design of artificial O_2 carriers [68]. Actually, new products are appearing, although they are in the preclinical stage, not only HbV, but also zero-link polymerized Hb [69] and others with larger molecular dimensions and higher O_2 affinities. Erni *et al.* clarified that HbV with a high O_2 affinity (low P_{50}, such as 9–15 torr) and high viscosity (such as 11 cP) suspended in a high-molecular-weight HES solution was effective for oxygenation of an ischaemic skin flap [63, 70–72]. That study showed that HbV would retain O_2 in the upper arterioles, then perfuse through collateral arteries and deliver O_2 to the targeted ischaemic tissues. The results imply the further application of HbV for other ischaemic diseases such as myocardial and brain infarction and stroke.

Concluding remarks

Advantages of artificial O_2 carriers including HbV are the absence of blood-type antigens and infectious viruses, along with stability for a long-term storage for any emergency that might overwhelm the RBC transfusion capacity. The shorter half-lives of the HbV in the bloodstream (2–3 days) limit their use, but they are applicable as a transfusion alternative for shorter periods of use. Easy manipulation of physicochemical properties of HbV supports the possible tailor-made O_2 carriers that suit various clinical indications. The achievements of ongoing HbV research described above make us confident in advancing further development of HbV, with the expectation of its eventual realization.

Conflicts of interest statement

Among the authors, ET, HS, KS and KK are consultants of Oxygenix Inc. (Tokyo, Japan).

Acknowledgements

This work was partly supported by Health Sciences Research Grants (Research on Regulatory Science), the Ministry of Health, Labour and Welfare, Japan. The authors gratefully acknowledge Prof. S. Takeoka (Waseda University); Prof. Yozu, Prof. Suematsu, Dr M. Watanabe, Dr Y. Izumi, Dr M. Yamamoto, Dr T. Ikeda, Dr E. Ikeda (Keio University); Dr H. Ikeda, Dr H. Azuma, Dr M. Fujiwara, Dr H. Abe (Hokkaido Red Cross Blood Center); Dr M. Takaori (Higashitakarazuka Satoh Hospital); Prof. M. Otagiri, Dr M. Anraku (Kumamoto University); Prof. N. Maeda, Dr Y. Suzuki (Ehime University); Prof. M. Intaglietta,

Dr A.G. Tsai, Dr P. Cabrales (University of California, San Diego); Prof. W.T. Phillips (University of Texas, San Antonio); Prof. D. Erni (Inselspital Hospital, University of Berne) and their active colleagues for meaningful discussions and contributions to this research.

References

1 Chang TMS. *Blood Substitutes: Principles, Methods, Products, and Clinical Trials*. Basel: Karger, 1997.

2 Tsuchida E, ed. *Blood Substitutes: Present and Future Perspectives*. Amsterdam: Elsevier, 1998.

3 Kobayashi K, Tsuchida E, Horinouchi H, eds. *Artificial Oxygen Carrier: its Front Line, Keio University International Symposia for Life Sciences and Medicine*, Vol. 12. Tokyo: Springer-Verlag, 2005.

4 Winslow R, ed. *Blood Substitutes*, Amsterdam: Elsevier, 2006.

5 Page TC, Light WR, McKay CB, Hellums JD. Oxygen transport by erythrocyte/hemoglobin solution mixtures in an in vitro capillary as a model of hemoglobin-based oxygen carrier performance. *Microvasc Res* 1998; 55: 54–66.

6 Sakai H, Suzuki Y, Kinoshita M, Takeoka S, Maeda N, Tsuchdia E. O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permaeble tube: comparison with RBCs and acellular Hbs. *Am J Physiol Heart Circ Physiol* 2003; 285: H2543–55.

7 Vandegriff KD, Olson JS. The kinetics of O_2 release by human red blood cells in the presence of external sodium dithionite. *J Biol Chem* 1984; 259: 12609–18.

8 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* 1998; 101: 604–12.

9 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* 2000; 279: H908–15.

10 Murray JA, Ledlow A, Launspach J, Evans D, Loveday M, Conklin JL. The effects of recombinant human hemoglobin on esophageal motor function in humans. *Gastroenterology* 1995; 109: 1241–8.

11 Toyoda T. Artificial blood. *Kagaku* 1965; 35: 7–13 (in Japanese).

12 Kimoto S, Hori M, Toyoda T, Sekiguchi W. Artificial red cells. *Gekachiryou (Surg Ther)* 1968; 19: 324–32 (in Japanese).

13 Bangham AD, Horne RW. Negative staining of phospholipids and their structure modification by surface-active agents as observed in the electron microscope. *J Mol Biol* 1964; 8: 660–8.

14 Djordjevich L, Miller IF. Lipid encapsulated hemoglobin as a synthetic erythrocyte. *Fed Proc* 1977; 36: 567.

15 Rudolph AS, Klipper RW, Goins B, Phillips WT. In vivo biodistribution of a radiolabeled blood substitute: ^{99m}Tc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit. *Proc Natl Acad Sci U S A* 1991; 88: 10976–80.

16 Sakai H, Hamada K, Takeoka S, Nishide H, Tsuchida E. Physical properties of hemoglobin vesicles as red cell substitutes. *Biotechnol Prog* 1996; 12: 119–25.

17 Takeoka S, Ohgushi T, Terase K, Ohmori T, Tsuchida E. Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. *Langmuir* 1996; 12: 1755–9.

18 Sou K, Naito Y, Endo T, Takeoka S, Tsuchida E. Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. *Biotechnol Prog* 2003; 19: 1547–52.

19 Sakai H, Takeoka S, Yokohama H, Seino Y, Nishide H, Tsuchida E. Purification of concentrated Hb using organic solvent and heat treatment. *Protein Expr Purif* 1993; 4: 563–9.

20 Naito Y, Fukutomi I, Masada Y *et al.* Virus removal from hemoglobin solution using Planova membrane. *J Artif Organs* 2002; 5: 141–5.

21 Abe H, Ikebuchi K, Hirayama J *et al.* Virus inactivation in hemoglobin solution by heat treatment. *Artif Cells Blood Substit Immobil Biotechnol* 2001; 29: 381–8.

22 Abe H, Fujihara M, Azuma H *et al.* Interaction of hemoglobin vesicles, a cellular-type artificial oxygen carrier, with human plasma: effects on coagulation, kallikrein-kinin, and complement systems. *Artif Cells Blood Substit Immobil Biotechnol* 2006; 34: 1–10.

23 Wakamoto S, Fujihara M, Abe H *et al.* Effects of hemoglobin vesicles on resting and agonist-stimulated human platelets in vitro. *Artif Cells Blood Substit Immobil Biotechnol* 2005; 33: 101–11.

24 Sakai H, Tomiyama K, Sou K, Takeoka S, Tsuchida E. Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. *Bioconjug Chem* 2000; 11: 425–32.

25 Wakamoto S, Fujihara M, Abe H *et al.* Effects of poly(ethyleneglycol)-modified hemoglobin vesicles on agonist-induced platelet aggregation and RANTES release in vitro. *Artif Cells Blood Substit Immobil Biotechnol* 2001; 29: 191–201.

26 Sou K, Endo T, Takeoka S, Tsuchida E. Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. *Bioconjug Chem* 2000; 11: 372–9.

27 Nakai K, Usuba A, Ohta T *et al.* Coronary vascular bed perfusion with a polyethylene glycol-modified hemoglobin-encapsulated liposome, neo red cell, in rats. *Artif Organs* 1998; 22: 320–5.

28 Sou K, Klipper R, Goins B, Tsuchida E, Phillips WT. Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute. *J Pharmacol Exp Ther* 2005; 312: 702–9.

29 Sakai H, Takeoka S, Park SI *et al.* Surface-modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90%-exchange transfusion in anesthetized rats. *Bioconjug Chem* 1997; 8: 23–30.

30 Sakai H, Tsai AG, Kerger H *et al.* Subcutaneous microvascular responses to hemodilution with red cell substitutes consisting of polyethylene glycol-modified vesicles encapsulating hemoglobin. *J Biomed Mater Res* 1998; 40: 66–78.

31 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* 1997; 43: 289–97.

32 Sakai H, Horinouchi H, Masada Y *et al.* Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* 2004; 32: 539–45.

33 Sakai H, Horinouchi H, Yamamoto M *et al.* Acute 40 percent exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks. *Transfusion* 2006; 46: 339–47.

34 Kerwin BA, Akers MJ, Apostol I *et al.* Acute and long-term stability studies of deoxy hemoglobin and characterization of ascorbate-induced modifications. *J Pharm Sci* 1999; 88: 79–88.

35 Levy A, Zhang L, Rifkind JM. Hemoglobin: a source of superoxide radical under hypoxic conditions. *Oxy-radicals Mol Pathol Proc Upjohn-UCLA Symp* 1988: 11–25.

36 Ringsdorf H, Schlarb B, Venzmer J. Molecular architecture and function of polymeric oriented systems – models for the study of organization, surface recognition, and dynamics of biomembranes. *Angew Chem Int Ed* 1988; 27: 113–58.

37 Tsuchida E, Hasegawa E, Kimura N, Hatashita M, Makino C. Polymerization of unsaturated phospholipids as large unilamellar liposomes at low-temperature. *Macromolecules* 1992; 25: 2007–212.

38 Sakai H, Takeoka S, Yokohama H, Nishide H, Tsuchida E. Encapsulation of Hb into unsaturated lipid vesicles and gamma-ray polymerization. *Polym Adv Technol* 1992; 3: 389–94.

39 Akama K, Gong WL, Wang L, Tokuyama S, Tsuchida E. Stable preservation of hemoglobin vesicles as a blood substitute. *Polym Adv Technol* 1999; 10: 293–8.

40 Akama K, Awai K, Yano Y, Tokuyama S, Nakano Y. In vitro and in vivo stability of polymerized mixed liposomes composed of 2,4-octadecadienoyl groups of phospholipids. *Polym Adv Technol* 2000; 11: 280–7.

41 Szebeni J. Complement activation-related pseudoallergy: a new class of drug-induced acute immune toxicity. *Toxicology* 2005; 216: 106–21.

42 Rabinovici R, Rudolph AS, Yue TL, Feuerstein G. Biological responses to liposome-encapsulated hemoglobin (LEH) are improved by a PAF antagonist. *Circ Shock* 1990; 31: 431–45.

43 Phillips WT, Klipper R, Fresne D, Rudolph AS, Javors M, Goins B. Platelet reactivity with liposome-encapsulated hemoglobin in the rat. *Exp Hematol* 1997; 25: 1347–56.

44 Loughrey HC, Bally MB, Reinish LW, Cullis PR. The binding of phosphatidylglycerol liposomes to rat platelets is mediated by complement. *Thromb Haemost* 1990; 64: 172–6.

45 Doerschuk CM, Gie RP, Bally MB, Cullis PR, Reinish LW. Platelet distribution in rabbits following infusion of liposomes. *Thromb Haemost* 1989; 61: 392–6.

46 Abe H, Azuma H, Yamaguchi M *et al.* Effects of hemoglobin-vesicles, a liposomal artificial oxygen carrier, on hematological responses, complement and anaphylactic reactions in rats. *Artif Cells Blood Substit Immobil Biotechnol* 2007; 35: 157–72.

47 Sakai H, Horinouchi H, Tomiyama K *et al.* Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am J Pathol* 2001; 159: 1079–88.

48 Sakai H, Horinouchi H, Masada Y, Takeoka S, Kobayashi K, Tsuchida E. Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. *Biomaterials* 2004; 25: 4317–25.

49 Sakai H, Masada Y, Horinouchi H *et al.* Physiologic capacity of reticuloendothelial system for degradation of hemoglobin-vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J Pharmacol Exp Ther* 2004; 311: 874–84.

50 Bennett GD, Kay MM. Homeostatic removal of senescent murine erythrocytes by splenic macrophages. *Exp Hematol* 1981; 9: 297–307.

51 Yamaoka T, Tabata Y, Ikada Y. Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. *J Pharm Sci* 1994; 83: 601–6.

52 Kobayashi K. Summary of recombinant human serum albumin development. *Biologicals* 2006; 34: 55–9.

53 Sakai H, Yuasa M, Onuma H, Takeoka S, Tsuchida E. Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. *Bioconjug Chem* 2000; 11: 56–64.

54 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* 1996; 24: 1869–73.

55 Sakai H, Tsai AG, Rohlfs RJ *et al.* Microvascular responses to hemodilution with Hb-vesicles as red cell substitutes: influences of O_2 affinity. *Am J Physiol Heart Circ Physiol* 1999; 276: H553–62.

56 Cabrales P, Sakai H, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. *Am J Physiol Heart Circ Physiol* 2005; 288: H1885–92.

57 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* 2002; 283: H1191–9.

58 Yoshizu A, Izumi Y, Park SI *et al.* Hemorrhagic shock resuscitation with an artificial oxygen carrier hemoglobin-vesicle (HbV) maintains intestinal perfusion and suppresses the increase in plasma necrosis factor alpha (TNFα). *ASAIO J* 2004; 50: 458–63.

59 Terajima K, Tsueshita T, Sakamoto A, Ogawa R. Fluid resuscitation with hemoglobin vesicles in a rabbit model of acute hemorrhagic shock. *Shock* 2006; 25: 184–9.

60 Goto Y, Terajima K, Tsueshita T *et al.* Fluid resuscitation with hemoglobin-vesicle solution does not increase hypoxia or inflammatory responses in moderate hemorrhagic shock. *Biomed Res* 2006; 27: 283–8.

61 Yamazaki M, Aeba R, Yozu R, Kobayashi K. Use of hemoglobin vesicles during cardiopulmonary bypass priming prevents neurocognitive decline in rats. *Circulation* 2006; 1(Suppl.): I220–5.

62 Giarratana MC, Kobari L, Lapillonne H *et al.* Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nat Biotechnol* 2005; 23: 69–74.

63 Plock JA, Contaldo C, Sakai H *et al.* Is the Hb in Hb vesicles infused for isovolemic hemodilution necessary to improve oxygenation in critically ischemic hamster skin? *Am J Physiol Heart Circ Physiol* 2005; 289: H2624–31.

64 Tsai AG, Vandegriff KD, Intaglietta M, Winslow RM. Targeted O_2 delivery by low-P_{50} hemoglobin: a new basis for O_2 therapeutics, *Am J Physiol Heart Circ Physiol* 2003; 285: H1411–9.

65 Wang L, Morizawa K, Tokuyama S, Satoh T, Tsuchida E. Modulation of oxygen-carrying capacity of artificial red cells (ARC). *Polym Adv Technol* 1992; 4: 8–11.

66 Tsai AG, Friesenecker B, McCarthy M, Sakai H, Intaglietta M. Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model. *Am J Physiol* 1998; 275: H2170–80.

67 Sakai H, Sato A, Takeoka S, Tsuchida E. Rheological property of hemoglobin-vesicles (artificial oxygen carriers) suspended in a series of plasma substitute aqueous solutions. *Langmuir* 2007; 23: 8121–8.

68 Intaglietta M, Cabrales P, Tsai AG. Microvascular perspective of oxygen-carrying and -noncarrying blood substitutes. *Annu Rev Biomed Eng* 2006; 8: 289–321.

69 Matheson B, Kwansa HE, Bucci E, Rebel A, Koehler RC. Vascular response to infusions of a nonextravasating hemoglobin polymer. *J Appl Physiol* 2002; 93: 1479–86.

70 Contaldo C, Plock J, Sakai H *et al.* New generation of hemoglobin-based oxygen carriers evaluated for oxygenation of critically ischemic hamster flap tissue. *Crit Care Med* 2005; 33: 806–12.

71 Contaldo C, Schramm S, Wettstein R *et al.* Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity. *Am J Physiol Heart Circ Physiol* 2003; 285: H1140–7.

72 Erni D, Wettstein R, Schramm S *et al.* Normovolemic hemodilution with Hb vesicle solution attenuates hypoxia in ischemic hamster flap tissue. *Am J Physiol Heart Circ Physiol* 2003; 284: H1702–9.

Correspondence: Prof. Eishun Tsuchida, Oxygen Infusion Project, Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan.
(fax: +81 3 3205 4740; e-mail: eishun@waseda.jp).■

Hemostatic Efficacy of a Recombinant Thrombin-Coated Polyglycolic Acid Sheet Coupled With Liquid Fibrinogen, Evaluated in a Canine Model of Pulmonary Arterial Hemorrhage

Yotaro Izumi, MD, Masatoshi Gika, MD, Noriko Shinya, DVM, Sumika Miyabashira, BS, Takayuki Imamura, PhD, Chikateru Nozaki, PhD, Masafumi Kawamura, MD, and Koichi Kobayashi, MD

Background: In thoracic surgery, although infrequent, we encounter unexpected damage to the pulmonary artery (PA). In the present study, we evaluated the hemostatic efficacy of a newly developed fibrin-based sheet material, thrombin sheet, coupled with liquid fibrinogen (TSF), in an experimental model of PA hemorrhage.

Methods: Female beagles (n = 8) were used for the study. Left thoracotomy was performed under general anesthesia. PA injury (approximately 4 × 2 mm) was created, and repaired by TSF (TSF group) or TachoComb (TC group). The animals were allowed to survive, and the repaired site was evaluated 4 weeks after the experiment.

Results: The number of sheet application and compression procedures required for hemostasis was increased in the TC group compared with in the TSF group (TC vs. TSF, 4 ± 1 vs. 1 ± 0.5, $p = 0.01$, unpaired t test). The time required to achieve hemostasis was increased in the TC group compared with in the TSF group (TC vs. TSF, 7 ± 3 vs. 1 ± 0.5 minutes, $p = 0.01$, unpaired t test). The amount of bleeding during the hemostasis procedure was increased in the TC group compared with in the TSF group (TC vs. TSF, 48 ± 22 vs. 3 ± 3 g, $p = 0.01$, unpaired t test). At 4 weeks, rethoracotomy revealed no apparent indication of delayed bleeding, such as intrathoracic hematoma formation or excessive adhesion formation in the vicinity of PA, in either group. Histologically, the vessel lumen was well sustained in both groups, with no apparent stenosis or thrombus formation.

Conclusion: The hemostatic efficacy of TSF was superior to TC in this particular experiment. Single application of TSF was sufficient to achieve hemostasis in all but one animal. Compression time of approximately 1 minute was also very short albeit that the bleeding was from the PA and not an artery. These results were presumably because the adhesion was stronger, faster, and the sheet was more pliable in TSF compared with TC.

Key Words: Recombinant thrombin sheet, Hemostasis, Pulmonary artery.

J Trauma. 2007;63:783–787.

In thoracic surgery, although infrequent, we encounter unexpected damage to the pulmonary artery (PA) resulting in moderate to massive hemorrhage. In most cases, bleeding can be controlled on the spot by manual compression, but compression needs to be released at some point to repair the damage, either to directly suture the vessel, or to sufficiently isolate the vessel, clamp it, and control the bleeding. Significant blood loss can ensue during this period. It will be possible to reduce this blood loss if a ready-to-use hemostatic material, capable of swift hemostasis becomes available.

Hemostatic materials exploit various mechanisms such as absorbing and concentrating components of blood, increasing the enzymatic activity of clotting factors, or activating platelets.[1–3] Topical hemostatic agents utilizing materials such as cellulose or collagen provide mechanical scaffolds on which thrombus forms, but lack any inherent coagulation potential.[4,5] In this regard, fibrin-based materials can mimic thrombus formation and currently seem to be most effective.[6–11]

We have developed recombinant thrombin,[12,13] which was lyophilized onto a bioabsorbable synthetic nonwoven polyglycolic acid fabric (Neoveil, Gunze K. K., Kyoto, Japan) to yield a new fibrin-based sheet material, thrombin sheet (TS).[14] TS combined with liquid fibrinogen (TSF), is capable of swift hemostasis even when applied inside a blood pool. Fibrinogen solution is dripped onto the sheet immediately before application. Neoveil is loosely fabricated, and the sheet thickness is adjusted to 0.15 mm. This makes TSF quite supple so that it is able to securely conform to the contour of the applied site.

The hemostatic effect of fibrin-based materials in vascular injuries have been evaluated extensively using arterial injury models.[1–3,6,8–11] To our knowledge, control of hemorrhage from the PA has not been adequately assessed.

Submitted for publication February 28, 2006.
Accepted for publication June 26, 2006.

From the Division of General Thoracic Surgery (Y.I., M.K., K.K.), Department of Surgery, School of Medicine Keio University, Tokyo, Japan; Department of Thoracic Surgery (M.G.), Saitama Medical Center, Saitama Medical School, Saitama, Japan.; Pathology Department (N.S.), Therapeutic Protein Products Research Department (S.M.), and Research Department 1 (T.I., C.N.), The Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN), Kumamoto, Japan.

Address for reprints: Yotaro Izumi, MD, Division of General Thoracic Surgery, Department of Surgery, School of Medicine Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; email: yotaro@sc.itc.keio.ac.jp.

DOI: 10.1097/TA.0b013e318151ffdc

Although blood pressure in the artery is much higher in comparison with that of the PA, adequate attachment of the sheet material may be more difficult to achieve in the PA because the vessel wall is more easily deformed by compression. Therefore, materials that control arterial hemorrhage may not necessarily be effective in PA hemorrhage.

In the present study, we evaluated the hemostatic effect of TSF in an experimentally created PA hemorrhage model. The efficacy of TSF was compared in the same model with that of TachoComb (TC) (ZLB Behring Co., Ltd., Bern, Switzerland), a widely used commercially available fibrin-based sheet product. We also measured in vitro fibrin formation in each material as a parameter of hemostatic potential.

MATERIALS AND METHODS

Preparation of Thrombin Sheet

Preparation of TS has been described previously.[14] Briefly, mannitol (Nakarai Kagaku, 213-03, Kyoto, Japan), with a final concentration of 0.5% to 1.5%, and 40 mmol/L of calcium chloride were added to a solution containing 0.5% to 2% of glycerol. Recombinant thrombin[12,13] was added to make the final concentration 1,500 U/mL. This solution was dripped at a rate of 0.05 mL/cm^2 and spread evenly using rubber-tipped rods onto a bioabsorptive synthetic nonwoven fabric (3 cm × 3 cm) made of polyglycolic acid, Neoveil (thickness, 0.15 mm). The sheet was frozen at −80°C for 2 hours and dried to fix the recombinant thrombin. The sheet was trimmed to 1.5 cm × 1.5 cm for use in this experiment. The sheet was dipped in 0.2 mL of liquid human fibrinogen (Bolheal, Chemo-sero-therapeutic Research Institute, Kumamoto, Japan). The liquid fibrinogen seeped evenly into the sheet within seconds. The sheet was then immediately applied.

Animal Experiment

Female beagles (Kitayama Labes Co. Ltd., Nagano, Japan) (n = 8) were used for the study. Body weight was measured before induction of anesthesia. Anesthesia was induced by subcutaneous injection of atropine sulfate (0.25 mg per animal), followed 10 minutes after by intramuscular injection of xylazine (1 mg/kg) and ketamine (10 mg/kg). The radial vein was cannulated. Anesthesia was maintained by continuous infusion of 0.1% ketamine in 5% glucose at a rate of approximately 1 mL/min. After injection of suxamethonium (10 mg per animal), the animal was intubated and mechanically ventilated with 40% oxygen. Tidal volume was approximately 200 mL, and respiratory rate was 14 breaths per minute. Empirically, mean systemic arterial blood pressure was maintained at approximately 100 mm Hg during this anesthesia protocol.

The animal was placed in a right lateral position, and left fourth intercostal thoracotomy was performed. The interlobar portion of the left PA was used for the experiment. A 2-mm plastic catheter with an 18-gauge needle tip was gently inserted into the PA, and PA pressure was measured until the values stabilized (for approximately 1 minute). Bleeding during this procedure was negligible. Next, the vessel wall adjacent to the needle insertion site was held with fine-toothed forceps, and the vessel wall was resected with fine scissors so as to expand the needle hole proximally. Accounting for the thin and soft PA wall, we preferred this procedure to the use of a punch device. In our preliminary experiment, the laceration thus created was approximately 4 mm × 2 mm. The needle catheter was removed, and free bleeding was visually confirmed for approximately 3 seconds, after which it was controlled by manual compression. Blood in the thoracic cavity was thoroughly suctioned. TSF (TSF group, n = 4) or TC (TC group, n = 4), both 1.5 cm × 1.5 cm in size, was prepared for application. Manual compression was released, and immediately the sheet was applied to cover the laceration. Manual compression was applied over the sheet for 1 minute. If bleeding was not controlled, an additional sheet was applied followed by another minute of manual compression. This was repeated until bleeding was visually controlled. Bleeding during this period was absorbed using gauze, and measured in grams. Hemostasis was confirmed by observation for an additional 10 minutes, and then the chest was closed. Ketoprofen (1 mg/kg) and ampicillin sodium (15 mg/kg) was injected intramuscularly. The animals were allowed to recover and then were returned to their cages. After 4 weeks, the animals underwent rethoracotomy under general anesthesia and the left chest cavity was observed. The interlobar portion of the left PA was carefully inspected for traces of secondary bleeding, for the magnitude of tissue adhesion, and for the presence of residual materials. PA pressure measurement was performed as previously, distal to the site of vessel injury. The animals were killed by pentobarbital overdose, and the left PA was resected together with the left lung. The specimens were fixed in 10% buffered formalin, and embedded in paraffin. Three micrometer paraffin sections were stained with hematoxylin and eosin for histologic examinations.

The School of Medicine Keio University Institutional Animal Care and Use Committee approved all animal studies, which were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Academies Press.

Data are shown as mean ± standard deviation. Comparisons were made between groups using unpaired t test and within groups using paired t test (StatView, SAS Institute Inc., Cary, NC). Significance was assumed at $p < 0.05$.

In Vitro Measurement of Fibrin Formation

The fibrin clots were prepared for in vitro analysis as follows. In the TC group, a piece of the sheet (0.5 cm × 0.5 cm) was soaked with 50 μL of saline containing 10 U/mL of factor XIII, 50 U/mL of recombinant thrombin, and 25 mmol/L of $CaCl_2$, and then incubated for 5, 10, and 30 minutes at 37°C. The reaction was stopped by adding 50 μL of stop solution (4 mol/L urea, 5% sodium dodecyl sulfate [SDS], and 10% 2-mercapto ethanol). Fibrin was dissolved overnight.

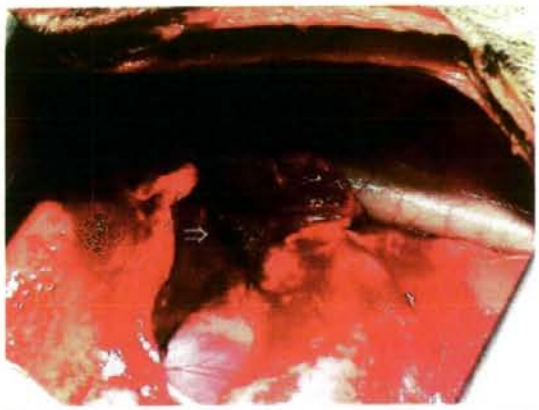


Fig. 1. *The appearance of thrombin sheet immediately after application. The pulmonary artery laceration can be seen through the applied sheet* (arrow).

In the TSF group, 12.5 μL of 20 mg/mL fibrinogen containing 25 mmol/L of $CaCl_2$ was added to TS (size, 0.5 cm $\times$ 0.5 cm; thrombin, 75 U/cm^2), and incubated for 5, 10, and 30 minutes at 37°C. The reaction was stopped by adding of 12.5 μL stop solution as in the treatment for TC.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli.[15] About 1 μg of fibrinogen was subjected to SDS-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel under reducing conditions. The gel was stained with Coomassie brilliant blue R-250.

RESULTS

All the animals survived. Body weight was comparable between groups before the experiment (TC vs. TSF, 10.0 $\pm$ 1.0 vs. 10.4 $\pm$ 0.2 kg, $p = 0.50$) and at 4 weeks after the experiment (TC vs. TSF, 11.6 $\pm$ 0.9 vs. 11.7 $\pm$ 0.6 kg, $p = 0.1$). Within groups, body weight was increased in both groups at 4 weeks after the experiment (TC group $p = 0.03$, TSF group $p = 0.01$).

Mean PA pressure was comparable between groups before vessel laceration (TC vs. TSF, 28 $\pm$ 5 vs. 27 $\pm$ 9 mm Hg, $p = 0.96$) and at 4 weeks after the experiment (TC vs. TSF, 32 $\pm$ 3 vs. 30 $\pm$ 1 mm Hg, $p = 0.2$). Also, within groups, mean PA pressure was comparable in both groups at 4 weeks after the experiment, compared with values before vessel laceration (TC group $p = 0.59$, TSF group $p = 0.21$).

Hemostasis was effectively achieved in both groups after sheet application. In the TSF group, the laceration could be clearly seen through the sheet (Fig. 1, arrow). The number of sheet application and compression procedures required for hemostasis was increased in the TC group compared with in the TSF group (TC vs. TSF, 4 $\pm$ 1 vs. 1 $\pm$ 0.5, $p = 0.01$). The time required to achieve hemostasis was increased in the TC group compared with in the TSF group (TC vs. TSF, 7 $\pm$ 3 vs. 1 $\pm$ 0.5 minutes, $p = 0.01$). The amount of bleeding during the hemostasis procedure was increased in the TC group compared with in the TSF group (TC vs. TSF, 48 $\pm$ 22 vs. 3 $\pm$ 3 g, $p = 0.01$).

At 4 weeks, rethoracotomy revealed no apparent indication in either group of delayed bleeding, such as intrathoracic hematoma formation or excessive adhesion formation in the vicinity of the PA. Macroscopically, adhesion of the lung to the site of sheet application was more apparent in the TC group compared with the TSF group. Residual material was present in both groups, and more prominent in the TC group. Histologically, the vessel lumen was well sustained in both groups, with no apparent stenosis or thrombus formation. However, thickening of the adventitia and the perivascular sheath seemed to be more prominent in the TC group (Fig. 2).

In vitro fibrin formation was more prominent in the TSF group compared with in the TC group. In the TSF group, γ-γ cross-linking was formed within 10 minutes, and α-α polymer was observed. On the other hand, we identified only a trace amount of γ-γ cross-linking in TC group (Fig. 3).

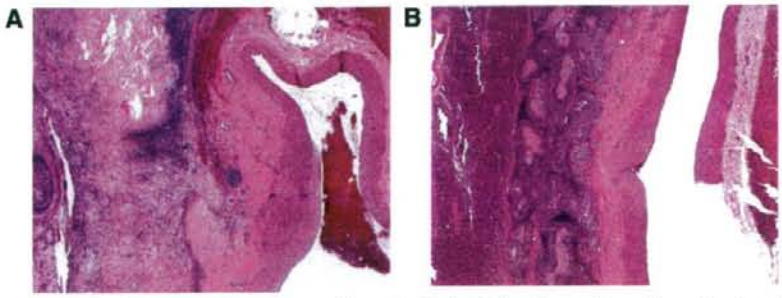


Fig. 2. *Histologically, the lumen of the repaired vessel was well sustained in both TacoComb* (A), *and thrombin sheet* (B) *groups. However, thickening of the adventitia, and the perivascular sheath seemed more prominent in the TacoComb group* (A), *compared with the thrombin sheet* (B) *group (Hematoxylin and eosin stain; original magnification,* $\times$2).

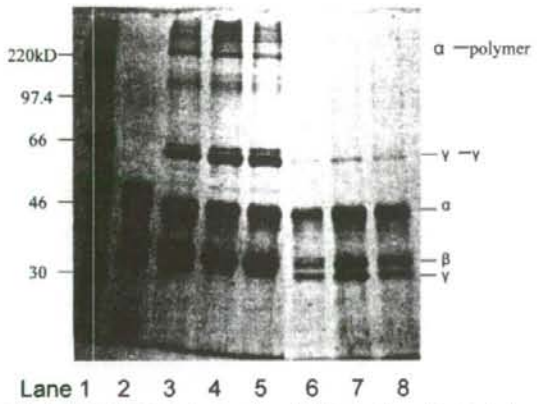


Fig. 3. *SDS-PAGE of in vitro formed fibrin in thrombin sheet plus liquid fibrinogen (TSF group), and TacoComb (TC group). Lane 1, molecular weight marker; lane 2, fibrinogen; lane 3, TSF group reaction for 5 minutes; lane 4, TSF group reaction for 10 minutes; lane 5, TSF group reaction for 30 minutes; lane 6, TC group reaction for 5 minutes; lane 7, TC group reaction for 10 minutes; lane 8, TC group reaction for 30 minutes. In vitro fibrin formation was more prominent in the TSF group compared with in the TC group. In the TSF group, γ-γ cross-linking was formed within 10 minutes, and α-α polymer was observed. On the other hand, we identified only a trace amount of γ-γ cross linking in TC group.*

DISCUSSION

Fibrin-based sealants have been commercially available in Europe and Japan for many years. Most formulations come as solutions of dissolved thrombin and fibrinogen, which are mixed on application. This form of liquid application is obviously not well suited for hemostasis in vessel injuries where there is significant outflow of blood. Solid material is more suitable because it can withstand the initial outflow of blood, and can be held with pressure after application. To this end, dry fibrin-based hemostatic materials are being developed extensively for potential use, particularly in trauma.[8,9,11] These materials should optimally contain both components, thrombin and fibrinogen, lyophilized onto a sheet material ready for immediate use. Dry materials are also easier to store, particularly for use in combat settings. TC is one such material commercially available in Europe and Japan.[16] Similar dry-sheet type fibrin-based sealants have been extensively evaluated in a variety of hemorrhage models. The results are promising.

Compared with dry materials, TSF is semidry, and may not be ideal for use in trauma settings because liquid fibrinogen needs to be separately prepared. But our attempt to include dry fibrinogen into a sheet material considerably increased the rigidity of the sheet, as is the case with TC. To this end, the TSF is quite supple and conforms considerably better to the contour of the applied site in comparison with how well TC conforms. We considered that this may be advantageous for bleeding during surgery, particularly from the PA, which is distributed three dimensionally in the thoracic cavity with branching from short segments and is surrounded by lung tissue, which inflates and deflates during ventilation.

In the present study, we investigated the efficacy of fibrin-based sheet type materials in a PA injury model. Hemostasis in vessel injury has been evaluated quite extensively in swine aortic models.[2,8–11] Considering the size of the animals used, approximately 10 kg dogs versus approximately 40 kg pigs, we consider the laceration size in the present study (4 mm × 2 mm) to be sufficiently large, relative to the laceration size in the widely reported swine model of aortic bleeding (4 mm × 4 mm). In our preliminary experiment, PA injury could not be controlled by manual compression alone.

Both TSF and TC were capable of adequately controlling PA hemorrhage in this study. Because PA pressure is significantly lower compared with systemic arterial pressure, reduction in blood flow caused by stenosis may more readily ensue compared with the response of arteries of similar caliber. Based on the PA pressure measurements and histologic observations, vessel stenosis or intraluminal thrombosis was not apparent with the use of either material in this study 4 weeks after application, despite the relatively large laceration size. Histologically, thickening of the adventitia and the perivascular sheath was suspected in the TC group, which may in part be because of the increased number of sheet applications.

Overall, the efficacy of TSF was superior to that of TC in this particular experiment. A single application of TSF was sufficient to achieve hemostasis in all but one animal in which the sheet was misplaced, and the vessel laceration was only partially covered on the first application. Compression time of approximately 1 minute was also very short albeit that the bleeding was from the PA and not an artery. These results were observed presumably because the adhesion was stronger, faster, and the sheet was more pliable in TSF compared with in TC.

Fibrinogen is a multidomain protein composed of three polypeptide chains termed Aα, Bβ, and γ. Thrombin binds to fibrinogen and cleaves fibrinopeptide A and fibrinopeptide B, and assembles to form fibrin. In the presence of factor XIII and Ca^{2+}, fibrin undergoes intermoleculelar covalent cross-linking, which was not as prominent in the TC group compared with in the TSF group. The stronger and faster adhesion may have been at least in part a result of the facilitated formation of fibrin in TSF as shown in vitro, although in vivo relevance may be less because tissue-derived coagulation factors exist. TSF also allowed for visualization of the laceration through the applied sheet, which was not possible with TC. This enables suturing of the laceration through the TSF for a more definitive hemostasis (data not shown). This property can be considered comparable with what is reported for the American Red Cross fibrin dressing.[8,9] Although we do

not have any experience with this material because of lack of access, the reports indicate that the American Red Cross dressing also achieves superior hemostasis compared with that of TC. Adhesion of TC to the surgical glove during manual compression was another problem. TC had to be gently scraped off the glove with forceps to leave the sheet in place, which was not the case with TSF. Furthermore, after 4 weeks, TSF induced less adhesion compared with TC, suggesting better biocompatibility, although this difference may just be because of the number of applications required. It is true that both materials sufficiently controlled bleeding in this experiment. However, we think that TSF would be much less stressful to use in a setting of unexpected PA bleeding, considering the multiple applications required for TC, as well as the additional blood loss that occurred during this period.

Despite its efficacy, there are certain constraints associated with fibrin-based materials such as availability and cost. It is also true that fibrin-based materials carry a risk of pathogen transmission, although today this is considered to be minimal as a result of improved screening and purification techniques. To alleviate some of these problems, we used recombinant thrombin for TS and development of recombinant fibrinogen is in progress. Ongoing preliminary studies in rabbit aortic injury models also seem to be promising. Studies are necessary to further clarify the hemostatic efficacy of this material in other organ trauma models and in coagulopathic animals.

ACKNOWLEDGMENTS

We thank Dr. Yuji Nishiwaki, Department of Preventive Medicine and Public Health, for his helpful comments on statistical analysis.

REFERENCES

1. Rothwell SW, Fudge JM, Chen WK, Reid TJ, Krishnamurti C. Addition of a propyl gallate-based procoagulant to a fibrin bandage improves hemostatic performance in a swine arterial bleeding model. *Thromb Res.* 2002;108:335–340.
2. Vournakis JN, Demcheva M, Whitson AB, Finkielsztein S, Connolly RJ. The RDH bandage: hemostasis and survival in a lethal aortotomy hemorrhage model. *J Surg Res.* 2003;113:1–5.
3. Connolly RJ. Application of the poly-*N*-acetyl glucosamine-derived rapid deployment hemostat trauma dressing in severe/lethal Swine hemorrhage trauma models. *J Trauma.* 2004;57:S26–S28.
4. Masova L, Rysava J, Krizova P, et al. Hemostyptic effect of oxidized cellulose on blood platelets. *Sb Lek.* 2003;104:231–236.
5. Sirlak M, Eryilmaz S, Yazicioglu L, et al. Comparative study of microfibrillar collagen hemostat (Colgel) and oxidized cellulose (Surgicel) in high transfusion-risk cardiac surgery. *J Thorac Cardiovasc Surg.* 2003;126:666–670.
6. Jackson MR, Friedman SA, Carter AJ, et al. Hemostatic efficacy of a fibrin sealant-based topical agent in a femoral artery injury model: a randomized, blinded, placebo-controlled study. *J Vasc Surg.* 1997; 26:274–280.
7. Jackson MR. New and potential uses of fibrin sealants as an adjunct to surgical hemostasis. *Am J Surg.* 2001;182:36S–39S.
8. Sondeen JL, Pusateri AE, Coppes VG, Gaddy CE, Holcomb JB. Comparison of 10 different hemostatic dressings in an aortic injury. *J Trauma.* 2003;54:280–285.
9. Pusateri AE, Modrow HE, Harris RA, et al. Advanced hemostatic dressing development program: animal model selection criteria and results of a study of nine hemostatic dressings in a model of severe large venous hemorrhage and hepatic injury in Swine. *J Trauma.* 2003;55:518–526.
10. Rothwell SW, Reid TJ, Dorsey J, et al. A salmon thrombin-fibrin bandage controls arterial bleeding in a swine aortotomy model. *J Trauma.* 2005;59:143–149.
11. Kheirabadi BS, Acheson EM, Deguzman R, et al. Hemostatic efficacy of two advanced dressings in an aortic hemorrhage model in Swine. *J Trauma.* 2005;59:25–35.
12. Soejima K, Mimura N, Yonemura H, Nakatake H, Imamura T, Nozaki C. An efficient refolding method for the preparation of recombinant human prethrombin-2 and characterization of the recombinant-derived alpha-thrombin. *J Biochem (Tokyo).* 2001; 130:269–277.
13. Yonemura H, Imamura T, Soejima K, et al. Preparation of recombinant alpha-thrombin: high-level expression of recombinant human prethrombin-2 and its activation by recombinant ecarin. *J Biochem (Tokyo).* 2004;135:577–582.
14. Uchida T, Shinya N, Kaetsu H, Imamura T, Nozaki C. Thrombin-carrying bioabsorbable synthetic nonwoven fabric, WO 2004/043503.
15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680–685.
16. Agus GB, Bono AV, Mira E, et al. Hemostatic efficacy and safety of TachoComb in surgery. Ready to use and rapid hemostatic agent. *Int Surg.* 1996;81:316–319.

EDITORIAL COMMENT

The authors have demonstrated that a new fibrin-based sheet material that is combined with liquid fibrinogen immediately before use (TSF) provides superior hemostasis when compared with a commercially available fibrin-based sheet material, TachoComb (TC), in a canine model of hemorrhage from an acute pulmonary artery injury. They reference a similar study showing hemostatic superiority of an American Red Cross dry fibrin-based sheet dressing for obtaining hemostasis from acute injury. They, also, reference studies showing excellent hemostasis for systemic arterial hemorrhage, which, of course, occurs at a much higher pressure than that seen from pulmonary artery hemorrhage. Although the authors point out that there are subtle differences in the physics of the injured low-pressure pulmonary artery compared with the high-pressure aortic perforation, the enhanced efficacy of the TSF in this lower arterial pressure system is predictable. Furthermore, the injury described, herein, might be encountered by a thoracic surgeon doing extirpative surgery for intrathoracic tumors but would be rarely encountered in the injured patient who, typically, would have associated lung injury. Thus, the application of this technique to the injured patient is limited.

This reviewer eagerly awaits subsequent reports defining the benefits of this product in the hands of thoracic surgeons.

Charles E. Lucas, MD
Wayne State University
Detroit, MI

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 364 (2007) 790–795
BBRC
www.elsevier.com/locate/ybbrc

Effects of endogenous ligands on the biological role of human serum albumin in S-nitrosylation ☆

Yu Ishima [a], Takaaki Akaike [b], Ulrich Kragh-Hansen [c], Shuichi Hiroyama [a], Tomohiro Sawa [b], Toru Maruyama [a], Toshiya Kai [a], Masaki Otagiri [a,*]

[a] Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan
[b] Department of Microbiology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-0811, Japan
[c] Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

Received 9 October 2007
Available online 26 October 2007

Abstract

Many proteins have been identified as targets for S-nitrosylation, including structural and signaling proteins, and ion channels. S-nitrosylation plays an important role in regulating their activity and function. We used human serum albumin (HSA), a major endogenous NO traffic protein, and studied the effect of mediators on S-nitrosylation processes which control NO bioactivity. By using NOC-7, S-nitrosoglutathione, and activated RAW264.7 cells as NO-donors we found that high-affinity binding of endogenous ligands (Cu^{2+}, bilirubin and fatty acid) can affect these processes. It is likely that the same effects take place in many clinical situations characterized by increased fatty acid concentrations in plasma such as type II diabetes and the metabolic syndrome. Thus, endogenous ligands, changing their plasma concentrations, could be a novel type of mediator of S-nitrosylation not only in the case of HSA but also for other target proteins.

Keywords: Human serum albumin; Nitric oxide; Type II diabetes; Metabolic syndrome; Cysteine; S-Nitrosylation; Ligand binding; Fatty acids; Copper; Bilirubin

Post-translational modifications are essential in their functional regulation. Among these, changes of the redox state of cysteine residues are of great importance. The sulfhydryl moiety can interact with nitric oxide (NO) and thereby form S-nitrosothiols (RS-NO) [1–3]. RS-NOs may function as NO reservoirs and preserve the antioxidant and other activities of NO [4,5]. For example, it has been reported that S-nitroso human serum albumin (SNO-HSA) may serve in vivo as a circulating reservoir for NO produced by the endothelial cells [6]. The reservoir function was also reported to be operative when application of SNO-HSA to animals suffering from ischemia-reperfusion injury minimized the extent of tissue damage associated with reperfusion [7,8]. However, several pieces of evidence propose that RS-NOs are more than simply NO reservoirs [4]. Thus, the antibacterial and cytoprotective properties of SNO-HSAs [9] are most probably the results of S-transnitrosylation.

HSA is a single, non-glycosylated polypeptide that organizes to form a heart-shaped protein with approximately 67% α-helix but no β-sheet [10]. All but one (Cys-34) of the 35 cysteine residues are involved in the formation of

Abbreviations: HSA, human serum albumin; SNO-HSA, S-nitroso HSA; RS-NOs, S-nitrosothiols; GSH, glutathione; GS-NO, S-nitrosoglutathione; NOC-7, 1-hydroxy-2-oxo-3-(*N*-3-methyl-aminopropyl)-3-methyl-3′-triazene; OA, oleic acid; BR, bilirubin; DTT, 1,4-dithiothreitol; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; NEM, *N*-ethylmaleimide.

☆ This work was supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (18390051), Japan, and by Fonden af 1870.

* Corresponding author. Fax: +81 96 362 7690.
E-mail address: otagirim@gpo.kumamoto-u.ac.jp (M. Otagiri).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.10.094