が42施設（58.3%）であった。また各月3～6例は11施設，3例以下は7施設であった。

設問2.での使用輸血量の分布については，400～1000mlが34施設，1000～2000mlが32施設，2000～3000mlでは7施設であり，3000ml以上の回答はなかった。すなわち全体の90.4%が400～2000mlの輸血量の範囲であったが，さらにその51.5%は400～1000mlの範囲にあった。

設問3.にあった輸血する決断から実際の輸血開始までの時間は，5分以内が3施設，15分以内が16施設，30分以内では29施設であったが，30分以上とするものも23施設あった。すなわち15分以内とする回答は全体の26.7%で，15分以上が73.2%となっていた。

設問4.の救急部から該当部門への輸血血液供給依頼から実際の使用まで所要時間について，日勤帯と夜勤帯とに差がないと回答したのは37施設であった。夜勤帯で長くなると回答したのは32施設であったが，逆に日勤帯より夜勤帯のほうが迅速に施行できるとの回答が2施設よりあった。

設問5.のinformed consent受領の仕方で，informed consentなしで輸血を施行すると回答した施設が25施設，必ずinformed consentを行って輸血を施行する施設が43施設であった。さらに原則的に患者本人からinformed consentを取ると回答したのは4施設であり，informed consentを家族も含めて取るのは4施設，本人，家族ならびにその他いずれかからとするのは36施設であった。

設問6.の大量輸血での処置および検査項目に関するアンケートでは，膠質液の使用，凝固能の検査，電解質の検査，循環系のモニターを併せて行っているとする施設が15施設，溶血，肝機能，鉄代謝のチェックも含めてすべての検査を行うとする施設が8施設あった。しかし膠質液を使用し，凝固能検査，電解質の検査の3項目を行うとする施設は12施設あり，その他の項目との組み合わせを含めこの3項目は49施設，67.1%で行われていた。

設問7.における輸血後感染症に関する検査は救急部，および他診療科で43施設，63.2%において施行され，患者からの依頼があったときのみ施行する施設は25施設であった。

設問8.の輸血後の不規則抗体の発現に関して，検査しているとする27施設に対して，検査していない施設は44施設（61.9%）であった。

今後臨床に導入されるであろう人工赤血球に関する設問9.で，すでに論文などにて人工赤血球についてなんらかの見識を持っているとの回答は58名（80.5%）であったが，まったく情報を得ていないとする回答14名（19.4%）も認められた。

設問10.に対しては条件しだいでは人工赤血球を使用したいとする回答を含め，使用したいとする回答は20名（25%）であったのに対し，不確定要因を含めて使用しないとする回答が60名（75%）であった。

設問11.の酸素運搬能以外に人工赤血球に必要な条件として循環血液量維持とする回答は60名（78.9%），血液粘度の維持は13名（16.6%）の回答があったが，それ以外に止血機能を妨げない，生体内ラジカルの消去，赤血球同様の変形能，浸透圧の維持，即使用される利便性などがあった。

設問12.での人工赤血球の予想使用量上限に関しては100mlまでとする回答はなく，250mlが3名（4.3%），500mlが20名（28.9%）で，1000ml以上の使用とする回答は46名（66.6%）であった。

もし人工赤血球の臨床使用が可能となっていた場合に緊急輸血に対してどれくらいの頻度で使用される可能性があるかとの設問13.に対して，初期段階では輸液にて対応するので該当症例はないとする回答は5施設（7.1%），毎日使用する症例があるとする回答が4施設，1～6症例/週が27施設，1～6症例/月が34施設であった。すなわち毎月使用する可能性があるとする回答は65施設（92.8%）で，さらに毎週使用する可能性のある施設も31施設（44.2%）あった。

設問14.で赤血球製剤使用に関するinformed consentが受領できるまでの人工赤血球の使用に関しては，informed consent受領と関係なく使用するとの回答が57名（79.1%），晶質液中心にて，あるいはその他の方法にて対応するが15名（20.8%）であった。

病院外での救急救命処置としての設問15.では，呼吸管理のみが6名，呼吸管理と体液管理（晶質液単独，膠質液との併用）が33名，さらにこれらに人工赤血球を併用した管理が33名であり，人工赤血球使用を併用可と回答したのは全体の45.8%に相当していた。

設問16.での人工赤血球の有効半減期に対して，2時間は必要との項目を選択したのは10名，12時間が28名（40.0%），24時間が26名（37.1%），さらに長時間を必要とする回答は6名であった。

設問17.での人工赤血球の網内系への影響については，治療上問題とならないとする回答が29名

(42.0%) であったのに対して，問題となると考えるのは40名 (58.0%) であった。とくに易感染性を懸念する見解が25名 (36%) にみられた。しかし一方，逆に生体からのメディエータの遊離を少なくして反応を緩和する可能性があるとする意見が2名 (3%) に，感染以上に出血死の回避が先決とする意見が3名 (4%) にみられた。

設問18.での人工赤血球への期待には33名から回答が得られたが，膠質・晶質液にはない酸素運搬輸液であること9名 (27.2%)，必要時での即応性について言及した意見8名 (24.2%)，輸血に伴う副作用・合併症の回避を挙げた意見4名 (5.7%) などが主なものであった。

Ⅲ 考 察

アンケートの回収率が39.8%とやや低率であったが，今回の調査で目的とした要点は得られたものと思われた。今回の調査に協力いただいた日本救急医学会評議員の方の多く (80.5%) はすでに人工赤血球に関してなんらかの見識を持っておられた。そしてもし人工赤血球が臨床使用可能となった場合には毎週でも使用する可能性のある施設は31 (44.2%) であることは現在の人工赤血球の有効性 (有効作用時間)，副作用の認容 (網内系への影響) に関する回答率とも一致している。また使用輸血量は400～1000mlとする施設が51.5%となっていたが，このことは最近の日本血液代替物学会が提案した“人工酸素運搬体製造に関する基本的留意事項 (案)”に対して解説した論文[5]で人工赤血球の使用量を一応，20ml/kgと唱えていることとも一致する。すなわち現在開発が進められている人工赤血球で救急患者の半数の輸血に対処できることを示している。一方，人工赤血球使用量への希望は1000ml以上とする回答が全体の66.6%を占めているので，これは今後の人工赤血球開発に対する課題であり，さらなる改良が必要と思われる。

人工赤血球に期待された事項中，もっとも多かったのは確実な酸素運搬能，そして組織への酸素供給であった。またこのような人工赤血球の循環血液内での有効時間については12時間とするものを含め，24時間とする回答が半数を占めているが，現在開発中の人工赤血球の投与後循環血液中滞留半減時間は約30時間[6]と推定されるので，まず多くの現場からの要望に応えられると思われる。しかし人工赤血球内に包埋されているヘモグロビンのメトヘモグロビンへの変化の半減時間に関してはいまだヒトでのデータがなく，ラットでの研究では16～18時間[7][8]となっている。ラットでのデータがそのままヒト，すなわち臨床例には適応されないので，今後の臨床研究で検討しなければならない事項である。

人工赤血球が必要時にただちに使用できることにはとくに大きな期待がある。現行の輸血医療では患者，あるいはその関係者からの輸血施行の informed consent を得るのに時間を要し，さらに輸血発注から実際の輸血までに15分以上の時間を要することが今回の調査で示されている。これに反し現在わが国で開発が進められている人工赤血球は，室温での保存で少なくとも2年間の品質安定性が確認されている[4]。このことは人工赤血球は一般治療薬として用いることができることを示しており，救急部の薬品棚，あるいは救急車の中にも常備することが可能であり，よりよい救命効果が得られると期待される。

さらに人工赤血球への期待には輸血に伴う副作用，合併症を回避したいとの願いが含まれていることも認められた。人工赤血球には血液型がなく，いわゆる universal blood である。したがって血液型の取り違えに伴う輸血事故の発生，あるいは遅発性溶血反応などの免疫性輸血合併症を防止できる。さらに一般輸血に伴う不規則抗体の発生が回避できる。一般輸血でのその発生率は3.1～36.4%[9]~[11]に及ぶと報告されていて，少なくとも5～10%は生じるのではないかと推測されている。輸血が繰り返される場合での合併症を考えると，この数値は軽視できない。ただ今回の調査では輸血後不規則抗体発生に関する一般的な関心は少ないように思えた。

人工赤血球の製造過程においてはウイルス，細菌などの病原体の不活性化も加えられている。そのため輸血性感染症の回避は確実で，この点に関しても人工赤血球への期待が今回の調査に認められた。

現在開発中の人工赤血球はヒトのヘモグロビンをリポソームの二重膜内に包埋したものであるが，これを生体の循環血液中に投与した際には異物と認識され生体の網内系で捕捉される。したがって投与後一時的に生体の網内系の機能が低下し，その後はむしろ亢進する[7]。この点に関してなんらかの危惧を抱く意見もみられる。しかし生体の抵抗力への危惧よりも出血に伴う生命の危機からの脱出を優先すべきであるとの意見もあり，むしろ蘇生を目的とした人工赤血球としての意義が重要視されるべきではないかと思われる。

現在開発中の人工赤血球，すなわちリポソーム包

埋ヘモグロビンを生理食塩液に浮遊させた製品では膠質浸透圧がなく，投与後の血液量維持効果に欠ける。それゆえに使用量の上限が定められている。日本血液代替物学会が提案した“人工酸素運搬体製造に関する基本的留意事項（案）”に対する解説論文[5]でも20ml/kg以上の量を使用する際にはなんらかの膠質液の併用を推奨している。ヘモグロビン包埋リポソーム粒子を膠質液に浮遊させた製品を作製する考えもあるが，臨床の現場では人工赤血球，すなわちヘモグロビン包埋リポソーム粒子投与量と膠質液投与量との比率は各症例ごとに異なる可能性があり，むしろこのように生理食塩液に浮遊させた人工赤血球と膠質液とは別個に投与することの方が現実的ではないかと思われる。

ま と め

今回，日本救急医学会の評議員を対象として救急医療現場での輸血医療の現状と将来開発される人工赤血球への期待についても調査を行った。その結果，現在，救急部での患者管理上，必要とする輸血用血液の少なくとも50％を開発中の人工赤血球で代替できることが認められた。またさらに投与後の酸素運搬機能維持時間についても半数の施設からの期待を満足させることも認められた。そして人工赤血球が速やかに臨床使用されることへの期待が大きいこと，とくにその保存が容易なこと，universal bloodとして使用できること，副作用・合併症の回避ができることへの期待が認められた。

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Effect of hemoglobin vesicle, a cellular-type artificial oxygen carrier, on middle cerebral artery occlusion- and arachidonic acid-induced stroke models in rats

Hirotsugu Komatsu[a,*], Toshiyuki Furuya[a], Natsue Sato[a], Katsuji Ohta[a], Akihiro Matsuura[b], Takao Ohmura[b], Satoshi Takagi[b], Masaki Matsuura[c], Mitsuru Yamashita[c], Makiko Itoda[c], Jiro Itoh[c], Hirohisa Horinouchi[d], Koichi Kobayashi[d]

[a] *Yokohama Research Center, Oxygenix Co. Ltd., Leading Venture Plaza 2-401, 75-1, Ono-cho, Tsurumi-ku, Yokohama 230-0046, Japan*
[b] *Oxygenix Co. Ltd., Toranomon Pastoral Main Tower 6F, 4-1-1, Toranomon, Minato-ku, Tokyo 105-0001, Japan*
[c] *Gifu Institute, Japan Biological Science Co. Fukue 52, Kaizu-cho, Kaizu 503-0628, Japan*
[d] *Department of Surgery, School of Medicine, Keio University, Shinanomachi 35, Shinjuku-ku, Tokyo 160-8582, Japan*

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Abstract

Hemoglobin vesicle (HbV), which is also called liposome-encapsulated hemoglobin, functions as a hemoglobin-based oxygen carrier and is expected to be utilized in emergency situations including hemorrhagic shock and several kinds of ischemic diseases. In the present study, we evaluated the efficacy of HbV for improving stroke-related symptoms induced by middle cerebral artery (MCA) occlusion/reperfusion and an intra-internal carotid arterial injection of arachidonic acid (AA) in rats. When HbV (10 mL/kg, i.v.) was administered to rats immediately after the MCA occlusion, it reduced the cerebral infarct volumes of the cortex and total of the cortex plus sub-cortex significantly as compared with saline as a vehicle. In AA-induced stroke model, HbV (10 mL/kg, i.v.) improved the motor dysfunction score and inhibited the increase in cerebral water content suggesting it could suppress cerebral edema. These results strongly suggest that HbV would provide a novel beneficial option for the treatment of stroke, especially acute ischemic stroke.

Keywords: Hemoglobin vesicle; Oxygen carrier; Red blood cell substitute; Artificial; Liposome; Stroke

Hemoglobin vesicle (HbV) is a cellular-type hemoglobin-based oxygen carrier encapsulated by liposome. Since HbV has been already demonstrated to deliver oxygen to organs in the same manner as normal red blood cells (RBCs), it is expected to play an important role as RBC substitute (artificial RBC). Its long stability at room temperature and compatibility to all blood types make it specifically attractive for use in emergency situations. Main characteristics of the HbV that we employed in the present study are as follows [21,13,22]: median particle diameter, 262–269 nm; Hb concentration, 10.0–10.6 g/dL; p50, 23–25 Torr; lipid concentration, 6.9–7.2 g/dL; lipid components, 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylecholine (DPPC)/cholesterol/1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamine (DHSG)/1, 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-polyethylene glycol 5000 (PEG5000-DSPE). The biological activity of HbV has been previously studied in several kinds of animal models. For instance, Izumi et al. demonstrated in a rat exchange-transfusion model that HbV has an oxygen transporting capability almost equivalent to rat RBCs and can be considered as a potential artificial oxygen carrier [12]. As another potential advantage, HbV has been projected to improve ischemia-related symptoms since it can penetrate into ischemic areas through incompletely occluded arteries, arterioles and/or collateral vessels because the particle size is much smaller than that of normal RBC. In fact, HbV improved oxygenation in acutely ischemic hamster flap tissue [8] and augmented oxygen delivery trough transiently occluded arterioles in a hamster window model [17]. However, the effects of HbV in stroke and myocardial infarction models, which are typical ischemic

* Corresponding author. Tel.: +81 45 521 1785; fax: +81 45 521 1786.
E-mail address: komatsu@oxy-genix.com (H. Komatsu).
URL: www.oxy-genix.com (H. Komatsu).

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models, have not been fully investigated. In the present study, we evaluated the effect of HbV on stroke episodes using a transient middle cerebral artery (MCA) occlusion model and an arachidonic acid (AA)-induced stroke model in rats.

All animal study protocols were confirmed to be in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the institutional animal care and use committee of Oxygenix Co. Ltd.

In the study of MCA occlusion/reperfusion [4,27], 63 male Wistar rats (Japan SLC, Shizuoka, Japan) weighing 209–245 g (9 weeks of age) were included. Under halothane anesthesia (2–3%, 0.5–1 L/min), rats underwent transient focal cerebral ischemia using an intraluminal suture. Body temperature was controlled at 37 ± 1 °C with a body temperature controller (Neuroscience, Tokyo, Japan). The right carotid artery bifurcation was exposed, and the common external and internal carotid arteries were mildly ligated transiently. The external carotid artery was incised at the 2-mm distal from the junction of internal carotid artery and a 4–0 monofilament suture (18 mm in length) coated beforehand by silicon was inserted via this incision and advanced through the internal carotid artery to the origin of the MCA. The suture was left in place for 1 h and then pulled out about 10 mm to allow reperfusion. HbV (10 mL/kg) or saline was intravenously administered immediately after the MCA occlusion at an injection speed of 2 mL/min. Twenty-three hours after the reperfusion, the neurological symptom score was determined by the following criteria: 0, no symptoms; 1, adduction of left forelimb; 2, adduction of left forelimb and decrease in response to transversal stimulus; 3, adduction of left forelimb, decrease in response to transversal stimulus, and circling movement; 4, unable to walk (abasia). Immediately after the evaluation of neurological symptoms, rats were sacrificed to excise their brains, which were then sliced into 7 sections (2-mm thick) and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Wako Pure Chemical Ind. Ltd., Tokyo, Japan) at 37 °C for 30 min with shaking, then immersed in 10% formaldehyde neutral buffer solution (Wako Pure Chemical Ind. Ltd.) for preservation. Photograph of each section was taken with a digital camera (Fujifilm, Tokyo, Japan) and the infarct area was measured with a personal computer (iBook, Apple) and software (Adobe Photoshop® ver. 5.5 and NIH image ver. 1.62). Infarct volume was calculated from the data of infarct area according to the following formula: infarct volume $= A + G + 2 \times (B + C + D + E + F)$, where A–G refer to the areas of 7 sections. The infarct volume was

Table 1
Criteria of motor dysfunction score

1. Rolling test

Score	Symptom
0	Rat does not fall down and run away without resistance and/or turn its face to opposite direction in response to pushing right and left flanks.
1	Rat makes resistance or move to left side in response to pushing right flank.
2	Rat does not fall down in response to pushing left flank and do roll in a clockwise direction in response to pushing right flank.
3	Rat falls down stretching hind limbs out sideways but not forelimbs in response to pushing left flank.
4	Rat falls down stretching four limbs out sideways in response to pushing left flank and rise in 4 s.
5	Rat falls down stretching four limbs out sideways in response to pushing left flank and rise in 5–29 s.
6	Rat falls down stretching four limbs out sideways in response to pushing left flank and cannot rise for 30 s or longer.
7	Rat falls down rightward without stimulus and can not rise for 30 s or longer.

2. Posture test (when neck of rat was picked up)

Part	Score	Symptom
Head	0	Rat does not lean its head to the right or left and keep its eyes horizontally.
	1	Rat leans its head to the right but keep its eyes horizontally.
	2	Rat leans its head to the right and turns slightly its right eyes downward.
	3	Rat leans its head to the right and turns markedly its right eyes downward.
Right forelimb	0	Rat does not put down its right forelimb.
	1	Rat slightly puts down its right forelimb.
	2	Rat puts down its right forelimb but does not place it on the abdomen.
	3	Rat remarkably put down its right forelimb and place it on the abdomen.
Right hind limb	0	Rat does not put down its right hind limb.
	1	Rat slightly puts down its right hind limb.
	2	Rat remarkably put down its right hind limb but keeps its adduction slightly.
	3	Rat remains down the tip of its right hind limb just underneath.

3. Hemiplegia test

Score	Symptom
0	Rat makes a strong resistance and pulls back its right hind limb immediately when it is lifted with a pen.
1	Rat makes a slight resistance and pulls back its right hind limb slowly when it is lifted with a pen.
2	Rat remains its right hind limb stretched without resistance when the limb is stretched backward.

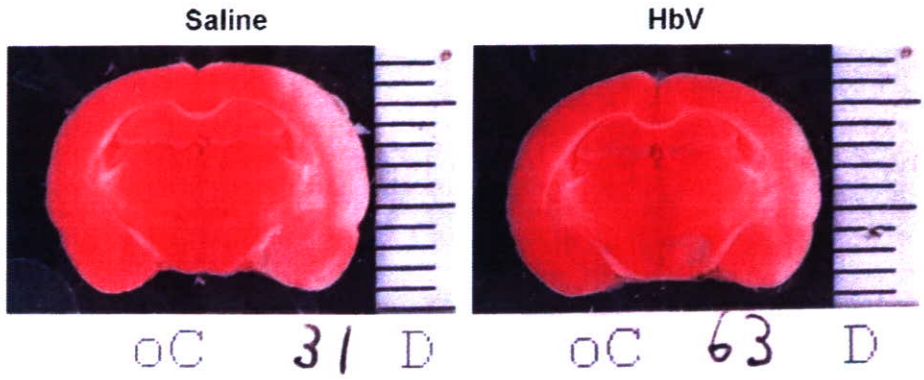

Fig. 1. Representative photographs of TTC-stained cerebral sections taken from MCA-occlusion/reperfusion-induced stroke rats. Left and right panels are photographs of saline- and HbV-treated rats, respectively. The individual rats shown here approximate mean values for the infarct volume in each group (infarct volumes of left and right panels were 194.1 and 119.0 mm^3, respectively; the mean values of saline- and HbV-treated groups were 174.8 and 114.1 mm^3, respectively).

determined separately in the cortex, sub-cortex, and the total of cortex plus sub-cortex.

For the experiment of AA-induced stroke model [23], 42 male Wistar rats (Japan SLC) weighing 223–248 g (9 weeks of age) were used. Under halothane anesthesia, rats underwent the cervical median incision. A polyethylene cannula was inserted into the left external carotid artery and advanced through it to the origin of the internal carotid artery. AA (2 mg/kg, Sigma–Aldrich, Japan) was injected trough the cannula at an injection speed of 0.4 mL/min. HbV (10 mL/kg) or saline was intravenously administered immediately after the injection of AA. Survival of rats was confirmed 3 h after the AA injection, and surviving rats were subjected to a motor dysfunction test in accordance with the criteria shown in Table 1 (maximal total score: 18). The score of a deceased rat was counted to be 19. Then, each rat was sacrificed by decapitation to obtain the whole brain. Cerebellum, medulla oblongata, ponticulus, and bulbus olfactorius were removed from the whole brain, and the remaining organ (cerebrum) was divided into right and left hemispheres along the corpus callosum. After both the hemispheres were measured for wet weight (W), they were dried in an oven heated at 80 °C for 3 days or longer and weighed again to obtain the dry weight (D). The cerebral water content was calculated as follows: cerebral water content (%) = $(W - D)/W \times 100$.

Data are indicated as the mean ± standard error. Statistical significance was determined with Excel 2003 or 2004 (Microsoft), SAS System (ver.8.2, SAS Institute, Tokyo, Japan), and EXSAS (ver.7.14, Scientist, Tokyo, Japan). Differences in the neurological symptom and the motor dysfunction score were tested by the Aspin–Welch t-test. Differences in infarct size and cerebral water content were tested by the F-test followed by either the Student's t-test or the Aspin–Welch t-test, when the variances of both groups were similar or dissimilar, respectively. Difference in survival rates was analyzed by the Chi-square test. These differences were regarded as statistically significant when p-values were less than 0.05.

In the MCA-occlusion/reperfusion-induced stroke model, neurological symptom scores (mean ± S.E.M.) of the saline- and HbV-treated groups were 2.3 ± 0.2 and 1.9 ± 0.1, respectively. The difference between the groups was not statistically significant although HbV showed a tendency to decrease the score. Fig. 1 shows representative photographs of TTC-stained cerebral sections derived from MCA-occlusion/reperfusion-induced stroke rats. The left panel (saline-treated rat) shows that about half of the area (infarct area) of the right hemisphere did not stain with TTC. On the other hand, an area that is not stained with TTC in a HbV-treated rat (right panel) reduced remarkably as compared with that of a saline-treated rat. The infarct volumes of rats corresponding to left and right panels were 194.1 and 119.0 mm^3, respectively. These rats shown in the images were selected to represent mean values for infarct volume in each group (saline-treated group, 174.8 mm^3; HbV-treated group, 114.1 mm^3) as described below. The results of quantitative analysis on the infarct volumes are summarized in Fig. 2. Infarction volumes of the cortex and the cortex plus sub-cortex in HbV-treated rats were significantly smaller than those in saline-treated rats. Infarction volumes of the sub-cortex was also decreased to 81.4% of saline-treated rats by HbV treatment although the difference was not statistically significant ($P = 0.0572$).

In response to the injection of AA into the internal carotid artery, 3 of 19 saline-treated rats (15.8%) died within 3 h (Table 2). On the other hand, all rats treated with HbV sur-

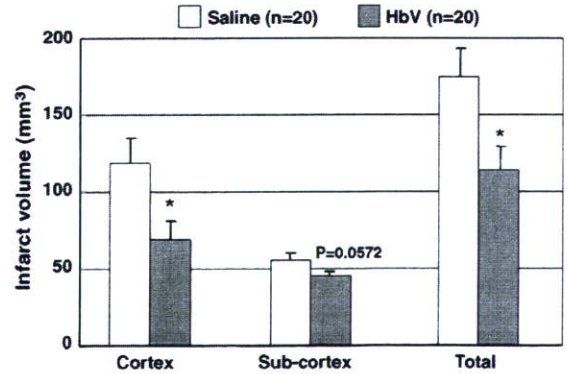

Fig. 2. Effect of HbV on MCA-occlusion/reperfusion-induced cerebral infarction in rats. Columns and bars indicate the mean and standard error of cerebral infarct volume, respectively. *$P < 0.05$, significantly different from the saline group (Student's t-test).

Table 2
Survival rate and motor dysfunction score

Test material	Survival rate	Rolling test	Posture test			Hemiplegia test	Total (mean ± S.E.)
			Head	Right forelimb	Right hind limb		
Saline	16/19 (84.2%)	4.6	2.4	2.7	1.7	1.3	12.8 ± 0.9
HbV	20/20 (100.0%)	3.5	1.8	2.6	0.9	0.7	9.4 ± 0.7[a]

[a] $P < 0.01$ vs. saline (Aspin–Welch t-test).

vived the injection of AA although the difference in survival rate between saline- and HbV-treated groups was not statistically significant. The motor dysfunction score of the HbV-treated group was significantly lower than that of the saline-treated group. As a result of AA injection, cerebral water content of left hemisphere (AA-injected side) was increased, indicating that cerebral edema was induced, and the administration of HbV significantly inhibited that increase (Fig. 3).

In the present study, HbV has been demonstrated to have protective effects on ischemic brain both in rat MCA-occlusion/reperfusion- and AA-induced models. MCA occlusion/reperfusion model has been well characterized and frequently used to evaluate drug efficacy on cerebral ischemia and infarction [24,26,2,11,1]. Treatment with HbV immediately after MCA occlusion significantly reduced infarct size (34.7% reduction), which is comparable to t-PA in the embolic model with blood clot (34% reduction) [6]. Edaravone, a radical-scavenging neuroprotectant that is in routine use for acute phase stroke in Japan, inhibited cerebral infarction by 20–25% in MCA occlusion models [16,14]. Although different experimental conditions were employed for each of these products, HbV was concluded to be effective in a model of stroke, as compared to well-known anti-stroke drugs.

AA-induced model has been used as an experimental thrombo-embolic stroke model, which is characterized by inducing platelet aggregation, endothelial damage and cerebral edema [23,9], and as a peripheral vascular disease model, e.g. gangrene in rats [25]. In addition to platelet activation and aggregation resulting in the formation of vascular occlusive blood clot, AA induces a rupture of the blood brain barrier and an increase in vascular permeability. In this model, HbV suppressed the increase in cerebral water content, a parameter representing the cerebral edema, of which inhibition was closely related to the improvement of motor dysfunction score seen in the HbV-treated group.

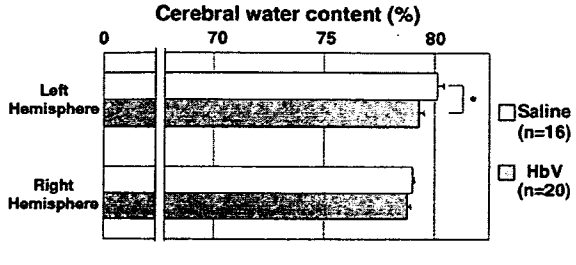

Fig. 3. Effect of HbV on AA-induced cerebral edema in rats. Columns and bars indicate the mean and standard error of cerebral water content, respectively. *$P < 0.05$ (Aspin–Welch t-test).

Thus, HbV has been found to be effective in the treatment of stroke as shown in the present experiments, but its mode of protective action has not been fully clarified. Protection of ischemic cerebral tissues by medical and drug interventions is mainly attributable to the oxygen supply to ischemic regions during ischemia, and the increased oxygen supply during ischemia seems to contribute to the inhibition of injury after reperfusion. It is possible that HbV can penetrate into ischemic areas through incompletely occluded arteries, arterioles and/or collateral vessels thanks to its small particle size, consequently deliver oxygen to the area, inhibit cerebral hypoxia, and decrease infarct size. The direct analysis of blood flow and oxygen metabolism/consumption in the ischemic brain after HbV administration remains to be investigated.

Although re-oxygenation is requisite for salvaging ischemic brain tissue, reactive oxygen and/or nitrogen species generated during reperfusion play important roles in further deterioration of ischemic brain. A nonselective nitric oxide (NO)-synthase inhibitor, L-nitro-arginine methyl ester (L-NAME) is reported to reduce cerebral infarct volume by abolishing the increase in brain NO production in a model of transient focal cerebral ischemic mice [7]. Diaspirin cross-linked hemoglobin (DCLHb) ameliorated ischemic cerebral injury in rats by binding NO, which has been implicated as neurotoxic [5]. HBOC-201, glutaraldehyde-polymerized bovine hemoglobin, reduced ischemia-reperfusion injury in canine myocardial ischemia in part by delivering more oxygen to ischemic tissue, and moreover, increased regional perfusion and blood pressure by scavenging NO [10]. These findings suggest that NO scavenging by cell-free hemoglobin is one of the possible mechanisms to mitigate ischemic injury. On the other hand, in the preclinical safety evaluation of DCLHb, myocardial lesions were observed following administration of DCLHb to certain species, and it was suggested that reduction in NO level was an important mechanistic factor for the myocardial lesions [3], indicating that the NO scavenging effect has both faces of advantage and disadvantage. Because the NO scavenging activity is an intrinsic property of hemoglobin, hemoglobin-based oxygen carriers are generally possible to capture NO, diminish NO-induced vasodilation, and cause myocardial lesions. However, it is considered that cell-free hemoglobin such as DCLHb extravasates through endothelial layer due to its small size (less than 50 nm in diameter) and trap NO, on the other hand, HbV, a first cellular-type oxygen carrier, does not extravasate owing to its moderate size (around 260 nm in diameter) [15]. In fact, HbV was generally safe in a single-dose and multiple (14 days-repeated)-dose toxicity studies as demonstrated by Sakai et al. [19,18,20], presuming that it would not induce such myocardial lesions, though more detailed

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.

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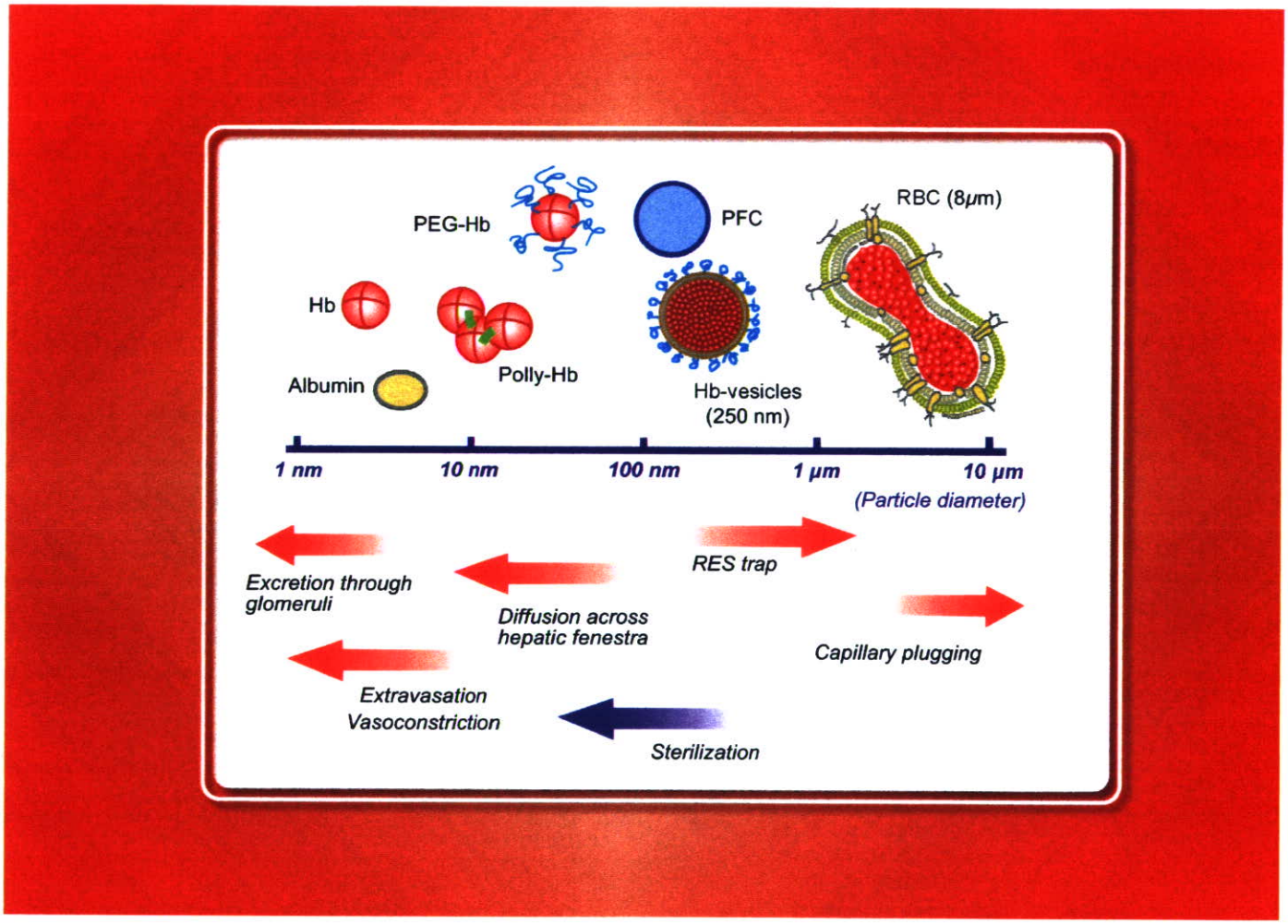

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Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions

● 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 Landsteiner 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];

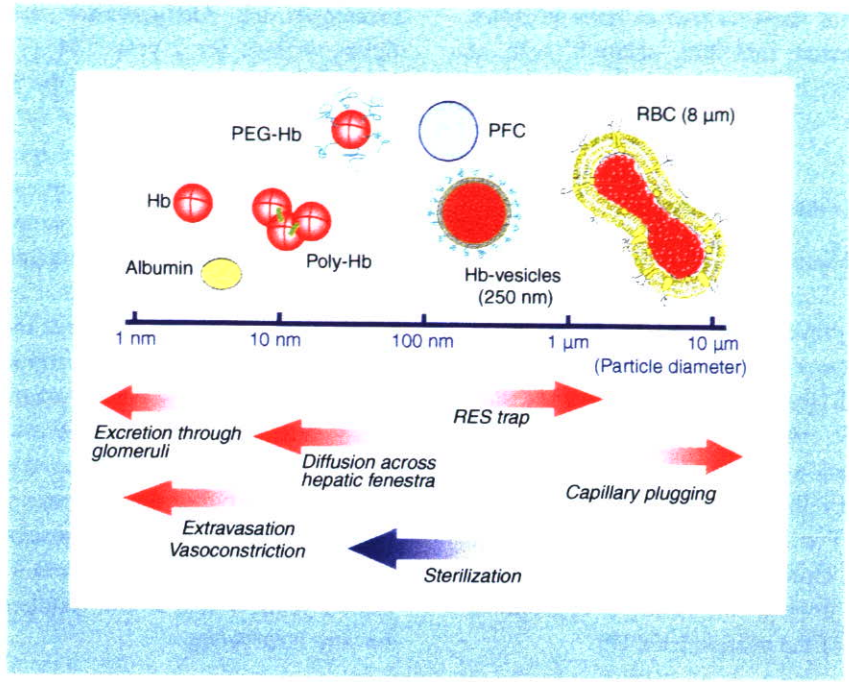

Fig. 1 What is the optimal dimension of artificial oxygen carriers? There is an upper limitation, below the capillary diameter, to prevent capillary plugging, and for the sterilization by membrane filters. On the other hand, the much smaller ones show higher rates of renal excretion and vascular wall 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).

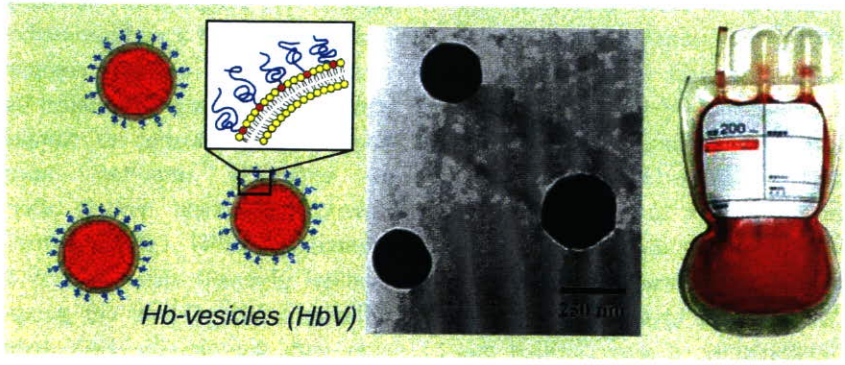

Fig. 2 (Left) Schematic representation of Hb-vesicle (HbV). One particle contains about 30 000 Hb molecules. The surface of one HbV is modified using polyethylene glycol chains that ensure the dispersion stability of HbV during storage and during circulation in the bloodstream. (Middle) The transmission 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

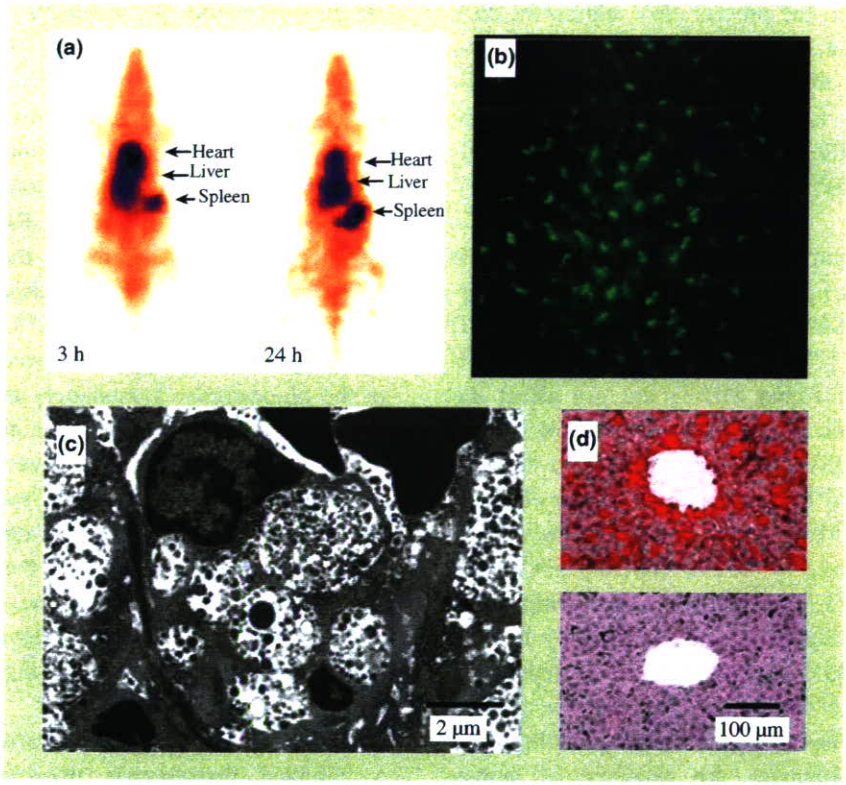

Fig. 3 Biodistribution and fate of Hb-vesicle (HbV). (a) Gamma-camera images of the distribution of ^{99m}Tc-labelled HbV in rats. At 3 h after injection, the heart and the liver showed a strong intensity because of the large blood volume. However, 24 h later, the intensity increased in the liver and 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].

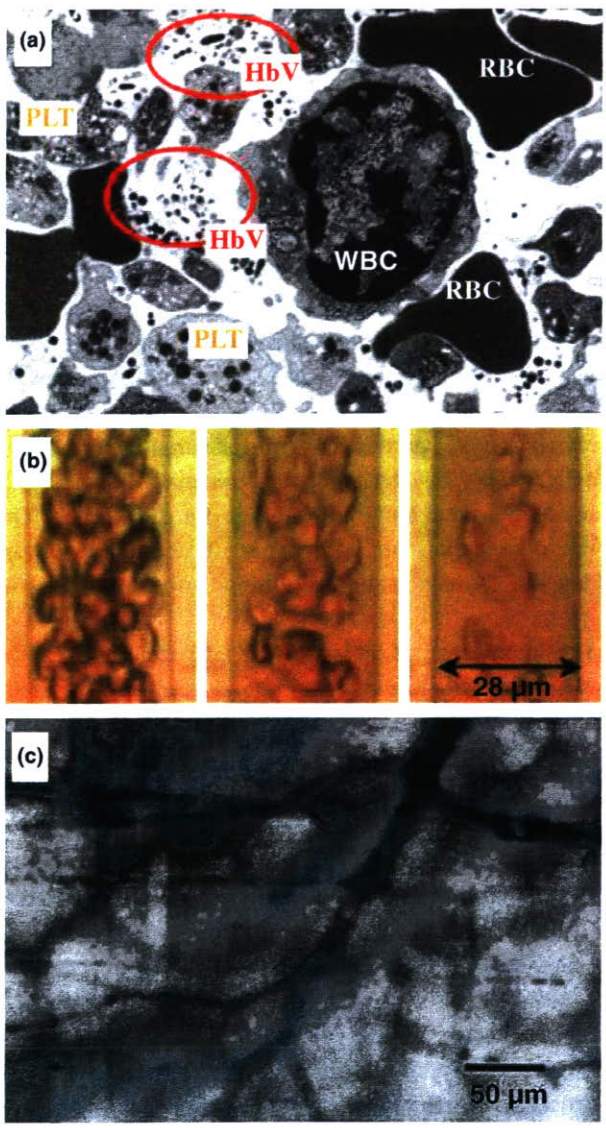

Fig. 4 How small is Hb-vesicle (HbV)? (a) The transmittance electron micrograph of rat blood 1 day after infusion of HbV. The buffy coat, obtained by a centrifugation of blood, was fixed using a 2.5% glutaraldehyde solution. Many HbV particles are visible in the red circles. They are much smaller than 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).

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