unique protein having an extremely high binding constant of hemin ($K > 10^{12}$ M^{-1}) (Tolosano, 2002). Crystal structure analysis of the hemopexin-hemin complex revealed that the hemin is tightly bound by double histidine coordinations to the central ferric ion and multiple hydrogen-bondings with the amino acid residue (Paoli, 1999). Nevertheless, the concentration of hemopexin in the plasma is rather low (< 17 μM) and human serum albumin (HSA) may provide a reserve binding capacity of hemin in various conditions, for instance, trauma, inflammation, hemolysis, *etc.* In fact, HSA binds hemin with a relatively high affinity ($K = 10^8$ M^{-1}) (Adams, 1980). If HSA can transport O_2 like Hb, it would be of extreme medical importance not only as a blood replacement composition, but also as an O_2-therapeutic reagent.

We have found that a series of super-structured heme derivatives with a covalently linked proximal-base were incorporated into HSA, and the obtained red-colored albumin-heme hybrids (Figure 1) can reversibly bind and release O_2 under physiological conditions in the same manner as Hb and myoglobin (Mb) (Komatsu, 1999, 2000, 2001a, 2002; Nakagawa, 2004; Tsuchida, 1999). Since recombinant HSA (rHSA) is manufactured on a large scale by yeast expression, the rHSA-heme hybrid has become entirely synthetic hemoprotein and absolutely free of infectious pathogens. Our recent animal experiments demonstrated that rHSA-heme actually works as an "oxygen-carrying plasma protein" in the blood stream (Komatsu 2004; Tsuchida, 2000). Although the NO-binding affinity of rHSA-heme is higher that that of Hb (Komatsu, 2001b), it does not induce unfavorable vasopressor effect at all (Tuschida, 2003). We suspect that the electrostatic repulsion between the albumin surface and glomelular basement membrane around the endotherial cell retards the rapid leakage of the rHSA-heme molecule and quick scavenging of NO. The albumin-heme is now recognized to be one of the promising materials as a new class of RBC substitute. In this chapter, we describe the O_2-transporting efficacy and preclinical safety of this synthetic heme-based O_2-carrier.

Figure 1

O_2-Binding property and physicochemical characteristics

From the thirty super-structured heme compounds, which were all synthesized by the authors, we found that the oxygenated rHSA-FecycP showed a high stability against the autooxidation; the half-lifetime against the ferric form in vitro (9 hrs at 37°C) was close to that of the native Mb (Komatsu, 2002). We have selected rHSA-FecycP with a similar p_{50} value (34 Torr at 37 °C) to RBC as the most suitable material for an artificial O_2-carrier. The physicochemical characteristics and shelf-life of the rHSA-heme solution ([rHSA]: 5 g/dL, heme/rHSA: 4 (mol/mol), isoelectric point: 4.8, COP: 18 mmHg, viscosity: 1.1–1.2 cP, shelf-life: over 2 years) were already reported elsewhere (Komatsu, 1999, 2002; Tsuchida, 2002)

Blood compatibility *in vitro*

The viscosity of the rHSA-heme solution (1.2 cP at a high shear rate of 230 s^{-1}) was much lower than that of whole blood (4.0 cP) and exhibited Newtonian type shear rate dependence just like rHSA itself. After the mixing of the rHSA-heme solution into whole blood at 10~44 % of the volume, the heme concentration in the plasma phase remained constant for 6 hrs at 37 °C, and no significant time dependence was observed in the numbers of RBC, white blood cells, and platelets (PLT) (Huang, 2003). The microscopic observations clearly showed that the shapes of the RBC have not been deformed during the measurement period. These results suggested that the rHSA-heme has no effect on the morphology of the blood cell components *in vitro*. With respect to the blood coagulation parameters (prothrombin time and activated partial thromboplastin time), the coexistence of rHSA-heme had only a negligibly small influence. Moreover, it was also shown that the rHSA-heme solution has no influence to

the complement factors (CH50, SC5b-9) and the PLT activation. Although more functional assay is necessary to firming establish the biocompatibility of rHSA-heme with whole blood, we can conclude that it has a good compatibility with blood cells.

Change of blood pressure after the administration

The administration of extracellular Hb-based O_2-carriers often elicits an acute increase in blood pressure by vasoconstriction. At the beginning of this study, our concern was that the small rHSA-heme molecules (8 × 3 nm) injected into the blood vessels would be eliminated from the circulations, and contributes to the significant consumption of NO in the interstitial space between the endothelium and vascular smooth muscle. In fact, rHSA-heme strongly binds NO; the NO-binding affinity (p_{50}^{NO} = 1.8×10^{-8} Torr) is 9-fold higher compared to the Hb's and enough to react 1 μM NO in the wall of the vasculator (Komatsu, 2001b). In order to clarify the hemodynamic behavior after the administration of this entirely synthetic O_2-carrying hemoprotein, we tested a top-load dose of the rHSA-heme solution in anesthetized rats (Tsuchida, 2003). Contrary to our expectations, only a negligibly small change in the mean arterial pressure (MAP) was observed after the administration of the rHSA-heme solution (5 g/dL, 300 mg/kg) [Figure 2(a)]. If anything, the difference from the baseline (ΔMAP) slowly decreased to -6.8 ± 3.4 mmHg within 20 min and remained constant during the monitoring period. The response is completely the same as observed following infusion with an equivalent volume of rHSA (5 g/dL) in this experimental setup. In contrast, the administration of extracellular Hb solution elicited an acute increase in blood pressure (ΔMAP: 16 ± 1.9 mmHg), followed a graduated decrease throughout the 60 min period of observation (Tsuchida, 2003). Why does rHSA-heme not induce the hypertension? The answer probably lies in the negatively charged molecular surface of the albumin vehicle. One of the unique characteristics of serum albumin is its low permeability through the muscle capillary pore, which is less than 1/100 that for Hb due to the

electrostatic repulsion between the albumin surface and the glomerular basement membrane around the endothelial cells. In the blood vessels, rHSA-heme presumably circulates for a longer time compared to Hb without extravasation. The heart rate (HR) responses after the rHSA-heme injection were also negligibly small [Figure 2(b)]. Visualization of the intestinal microcirculatory changes clearly showed that the widths of the venule and arteriole are fairly constant (Tsuchida, 2003).

Figure 2

Exchange transfusion into acute anemia rat model after 70% hemodilution

The physiological responses to a 30% exchange transfusion with rHSA-heme solution after 70% hemodilution with 5 g/dL rHSA were investigated using anesthetized rats (Komatsu, 2004). First, the isovolemic 70% hemodilution was carried out using 5 g/dL rHSA solution. The blood withdrawal via the common carotid artery (2 mL) and the rHSA infusion from the femoral vein (2 mL) (each 1 mL/min) were repeated for nine cycles until Hct was reduced to 13.6% (32% of the initial Hct value: 42.6%). After 10 min, a 30% volume of the circulatory blood was withdrawn, producing severe hemorrhagic shock state. The same volume of the samples was then intravenously injected. As negative- or positive-control groups, the rats were infused with the 5 g/dL rHSA solution (rHSA group) or the shed rat blood ([heme]=5.3 mM, whole blood group). The circulation parameters, blood parameters, renal cortical pO_2 [ptO_2(R)] and muscle tissue pO_2 [ptO_2(M)] were carefully monitored for 60 min after the injection.

By administration of the 5 g/dL rHSA solution, the MAP, HR, respiration rate, ptO_2(R), ptO_2(M), arterial blood O_2-pressure (paO_2), venous blood O_2-pressure (pvO_2), and arterial blood CO_2-pressuren ($paCO_2$) did not recover, leading to death within 32 min (Figure 3). In contrast, the infusion of the whole blood improved these values to their initial levels except for ptO_2(M). In the rHSA-heme group, the animals survived

over 60 min after the infusion, and the HR, respiration rate, ptO_2(R), and pvO_2 showed similar recoveries as observed in the whole blood group (Komatsu, 2004). MAP, ptO_2(M), paO_2, pH, and pCO_2 also significantly returned. We are certain that the albumin-heme solution has the potential to resuscitate the hemorrhagic shock, stabilize the blood circulation, and transport oxygen throughout the body.

Figure 3

Preclinical safety

In order to evaluate the preclinical safety of this synthetic O_2-carrier, we performed a 20% exchange transfusion with rHSA-heme into anesthetized rats and measured the time courses of the circulation parameters (MAP, HR, respiration rate) and blood parameters (paO_2, pvO_2, pH, blood cell numbers) for 6 hrs, which is adequate time to know an acute toxicity (Huang, 2004a). After stabilization of the animal condition, the 20% exchange transfusion was performed by 1 mL blood withdrawal via the common carotid artery and 1 mL rHSA-heme infusion from the femoral vein (each 1 mL/min) with four repeating cycles.

The appearance of the all animals showed absolutely no change for 6 hrs after the exchange transfusion. The physiological responses of the blood circulation, gas equilibria and blood cell numbers in the rHSA-heme group were almost the same as those of the control group (only surgery treatments without infusion) and rHSA groups (Figure 4) (Huang, 2004a). MAP and HR did remain constant after the injection of the rHSA-heme, suggesting again that the albumin-based O_2-carrier does not induce the vasoconstriction. It is also noteworthy that the autooxidation of the ferrous rHSA-heme to ferric state was retarded in the blood stream; the half-lifetime of the oxygenated rHSA-heme in vivo was ca. 4-fold longer than that in vitro (Tsuchida, 2000). It has been found that autooxidated rHSA-hemin was certainly reduced in the whole blood

suspension. A physiological concentration of ascorbic acid continuously provided by RBC probably rereduces the ferric hemin, leading to the apparent long lifetime of the oxygenated species.

Figure 4

Furthermore, 20% exchange transfusions with rHSA-heme into anesthetized rats were followed by blood biochemical tests of the withdrawn plasma and histopathology observations of the vital organs for 7 days (Huang, 2004b).

In the albumin-heme group, a total of 30 analytes by the blood biochemical tests showed almost the same values as those observed in the reference rHSA group, implying that no significant toxicity by the exchange transfusion with rHSA-heme (Huang, 2004b). Histopathology observations implied that the administration of rHSA-heme did not produce any negative side-effect on the vital organs. All these results showed the preclinical safety of the rHSA-heme solution.

Future researches

As described in this chapter, the results showed the O_2-transporting efficacy and initial clinical safety of the rHSA-heme solution, which allows us to undergo further advanced preclinical testing of this synthetic O_2-carrying plasma protein. Exchange transfusion with rHSA-heme into beagles is now under investigation.

Furthermore, rHSA-heme as a monomolecular O_2-carrier was tested for its ability to increase O_2 tension in the hypoxia of the solid tumor rat model. By the direct administration of the rHSA-heme solution (10 mL/kg) into the ascites hepatoma LY80 tumor on the femur, the O_2 tension of the hypoxic region immediately increased to 3.45 ± 1.43 Torr, which corresponds to a 2.4-fold increase compared to that of the baseline value (Kobayashi, 2003). These high O_2 levels continued for 300 s after the infusion.

While more research is required to consider how rHSA-heme behaves in the tumor blood vessel and is related to the increase in the O_2 partial pressure, the present results obviously indicate that rHSA-heme led to an increased O_2-release in the hypoxic region in the solid tumor. Experiments of a combined treatment with the rHSA-heme administration and radiation therapy are currently underway.

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Figure Legends

Figure 1 Super-structured heme derivatives for the albumin-heme hybrids and the red-colored rHSA-heme solution ([rHSA]= 5 g/dL).

Figure 2 Changes of (a) MAP and (b) HR in anesthetized rats before and after infusion of rHSA-heme solution (n=5) (●; rHSA-heme group and ○; Hb group). MAP is represented as change from the basal value (ΔMAP) just before the infusion with mean ± S.E.M. (n=5) (basal value is 90.1 ± 3.0 mmHg). HR was shown as mean ± S.E.M. (n=5). (Ref. Tsuchida, 2003)

Figure 3 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) pvO_2 and (f) $ptO_2(R)$ in anesthetized rats after 70% hemodilution with rHSA and 30% exchange transfusion with rHSA-heme solution (n=6) [●; rHSA-heme group, ○; whole blood group, △; rHSA group]. MAP, HR, pvO_2 and $ptO_2(R)$ are represented as percent ratios of the basal values with mean ± S.E.M.. Hct, HR and pH were shown as mean ± S.E.M.. HD: hemodilution, B: bleeding, I: sample injection. $^{a}p<0.05$ vs. rHSA group. $^{b}p<0.05$ vs. whole blood group. (Ref. Komatsu, 2004)

Figure 4 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) paO_2 and (f) pvO_2 in anesthetized rats after 20% exchange transfusion with rHSA-heme or rHSA solution (n=6) [◊; control group (only surgery treatments without infusion), △; rHSA group, ●; rHSA-heme group]. MAP, HR, paO_2 and pvO_2 are represented as percent ratios of the basal values with mean ± S.E.M.. Hct, HR and pH were shown as mean ± S.E.M.. (Ref. Huang, 2004a)

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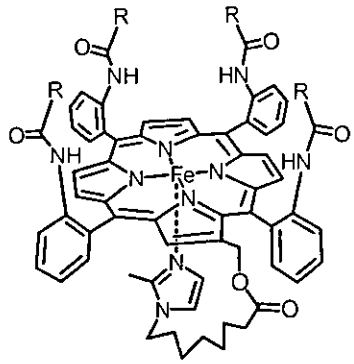
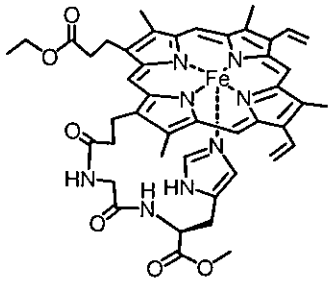




R: FepivP

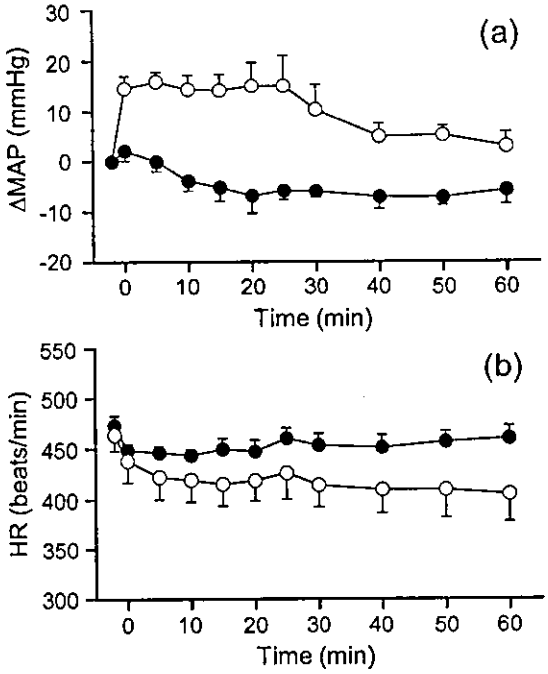
FecycP

FePP

rHSA

rHSA-heme

Figure 1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ΔMAP (mmHg)
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60
Time (min)
(b)
HR (beats/min)
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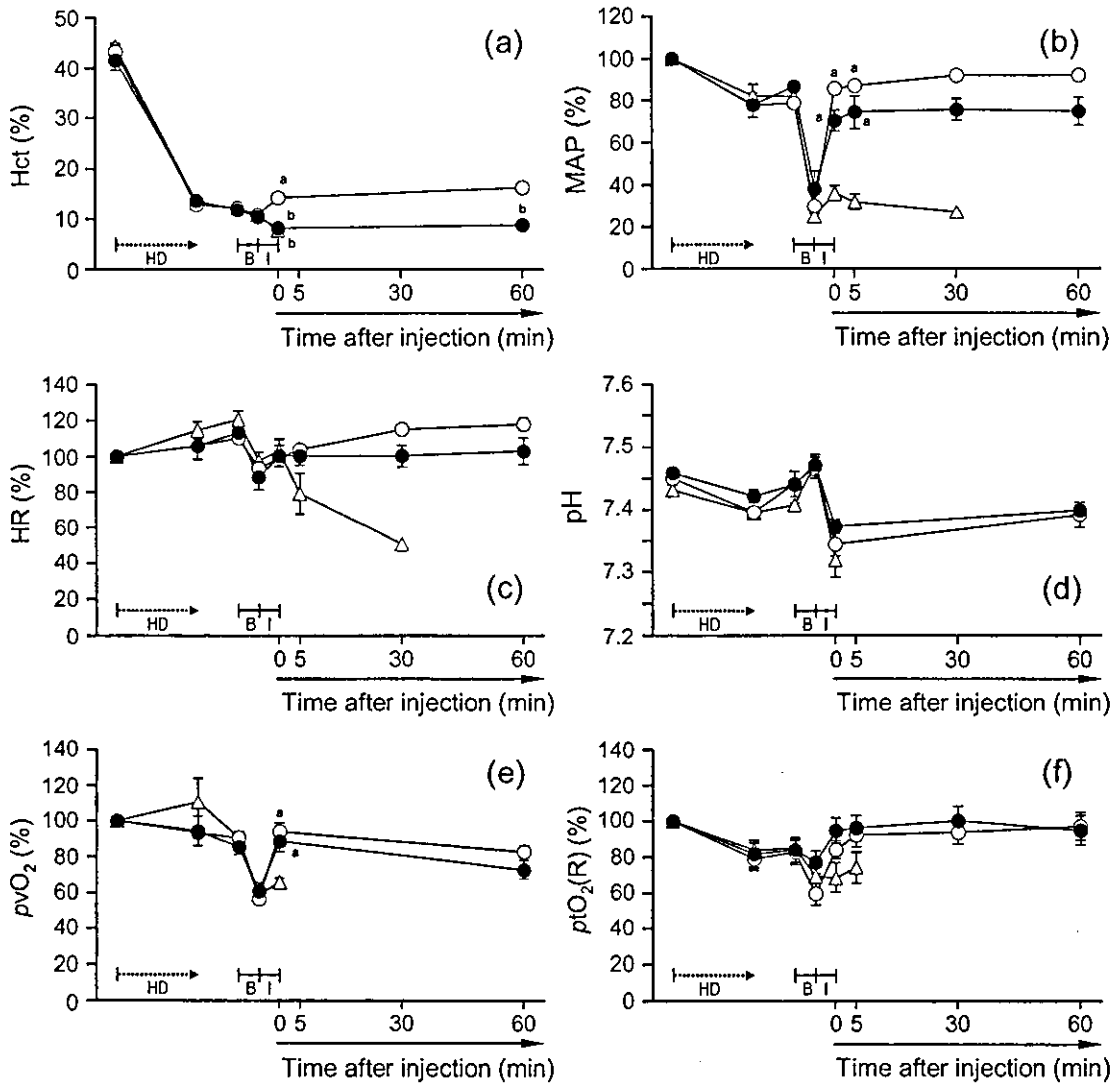
Time (min)

Figure 2


(a)
Hct (%)
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Time after injection (min)
(b)
MAP (%)
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100
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60
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20
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(c)
HR (%)
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100
80
60
40
20
0
(d)
pH
7.6
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7.2
(e)
pvO_2 (%)
(f)
ptO_2(R) (%)

Figure 3

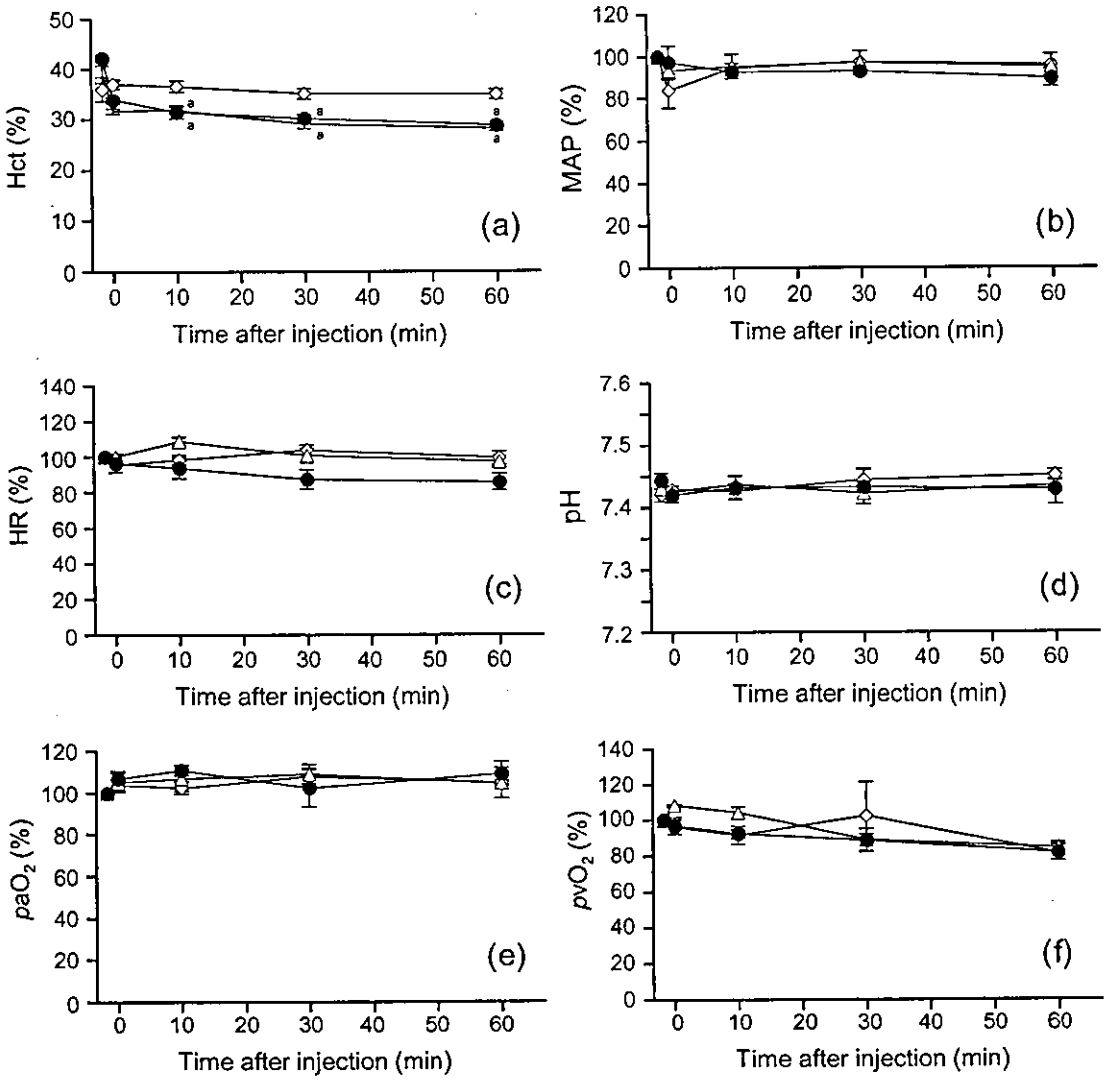
Hct (%)
(a)
Time after injection (min)
MAP (%)
(b)
Time after injection (min)
HR (%)
(c)
Time after injection (min)
pH
(d)
Time after injection (min)
paO2 (%)
(e)
Time after injection (min)
pvO2 (%)
(f)
Time after injection (min)

Figure 4

総　説

完全合成型人工酸素運搬体の開発

Development of Totally Synthetic Artificial Oxygen Carrier

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和文抄録

我々は，遺伝子組換えヒト血清アルブミン分子中に合成ヘム誘導体を最大で８分子包接させた，全く新しい完全合成型人工酸素運搬体であるアルブミン－ヘムの開発を進めている．アルブミン－ヘムはカプセル化の必要がなく，それ自身がアルブミン由来の膠質浸透圧を保有しており，また一酸化窒素（NO）捕捉による血圧上昇等の副作用も示さない．アルブミン－ヘムの大量製造方法を既に確立しており，その概要を示すとともに製剤特性をまとめた．またラットを用いた交換輸血試験の結果，アルブミン－ヘムは明らかな酸素運搬効果を示した．

さらに，ヒトヘモグロビンカプセル型人工酸素運搬体（Hb-V）についても，㈱オキシジェニクスとの共同開発を進めている．現在のところ，最も臨床試験に近い段階に進んでいる人工酸素運搬体であるが，献血由来のヘモグロビンを使用しているため，感染の危険性や安定供給の問題が完全には解決されていない．そこで，遺伝子組換えヒトヘモグロビン（rHb）の開発にも着手した．既にrHbの発現を確認しており，現在大量製造方法の検討に着手している．

これらの人工酸素運搬体の製造施設を当社注射剤工場と併設して設置するために準備を進めているところである．

Abstract

Development of an innovative and original artificial oxygen carrier, albumin-heme, which consists of recombinant human serum albumin (rHSA) and synthetic heme derivative, has been promoted in our company. Albumin-heme, in which maximally eight molecules of synthetic heme are incorporated into albumin molecule, has a suitable colloidal osmotic pressure itself and also do not have side effects such as hypertension due to depletion of nitric oxide (NO). Large-scale production process has already been established. Characteristics of albumin-heme are described in this paper. Furthermore, the exchange transfusion experiments in rats revealed that albumin-heme had oxygen carrying properties.

We also have been developing a hemoglobin vesicle as another type of artificial oxygen carrier in collaboration with Oxygenics Inc. This type of oxygen carrier is the most promising preparation close to clinical use. However, some risks of infection and uncertainty of stable blood supply still exist because of use of donated human blood as raw material. So, we decided to develop recombinant human hemoglobin (rHb). A purified rHb has already been obtained by our original expression method. At the present, we have been preparing for large-scale production facilities in our pharmaceutical factory in Japan to produce these artificial oxygen carriers as mentioned above.

Keywords

synthetic oxygen carrier, albumin-heme, synthetic heme, hemoglobin vesicle, recombinant human hemoglobin, recombinant human serum albumin

緒言

米国を中心にヘモグロビン修飾型の人工酸素運搬体（修飾Hb）が開発中であるが，いずれもヒトあるいはウシのヘモグロビンを原料としており，その安全性と安定供給に懸念が残る．

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また，これらの修飾Hbは，ヘモグロビン（Hb）濃度の上昇により膠質浸透圧及び粘度が上昇するため，生体に投与できるHb量が限られており，結果として酸素運搬能に限界が認められる．また，天然の赤血球のように細胞膜に覆われていないため，Hbが容易に逸脱し，NO捕捉による血管収縮といったHb自体の毒性の発現やHb機能維持のための解糖系酵素やメトHb還元酵素等の各種酵素系の保持ができないため安定性が悪いといった問題点が依然として指摘されている[1,2]．これに対して日本国内ではリポソームにヘモグロビンを内包した，細胞型人工酸素運搬体の開発が進められている．これらの製剤はヘモグロビンの副作用を低減し，より安全で有効性の高い人工酸素運搬体として最も臨床に近い段階に開発が進んでいる．一方でこれらの人工酸素運搬体は献血由来の原料に依存している点で，国内使用に限定されるとともに安定供給にも不安が残る．

我々は，遺伝子組換えヒト血清アルブミン（rHSA）の高純度，高生体適合性，非感染性，量産性などの特徴に着目し，rHSAに可逆的酸素配位能を有する合成ヘムを包接させた新規の完全合成ヘム蛋白質（アルブミンーヘム）の開発を行っている．このアルブミンーヘムは修飾ヘモグロビンで認められるNO捕捉による血管収縮作用は有していない．既に合成ヘムの選定が終了し，ラット，イヌを用いた交換輸血実験で有効な酸素運搬能を確認している．本稿では，アルブミンーヘムの製剤としての安定性，製造方法，動物での評価結果について報告する．

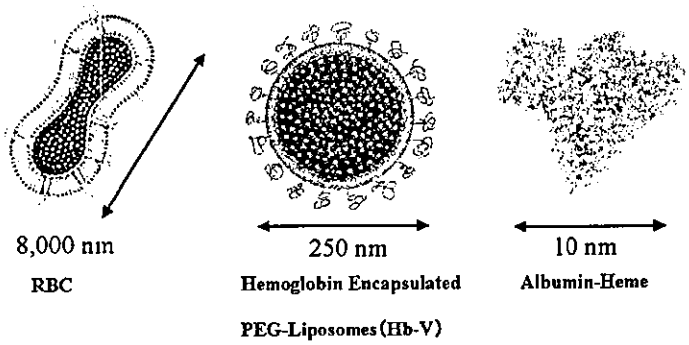


Fig. 1. Two types of artificial oxygen carrier under developing in NIPRO.

一方，Fig. 1.に示すように，ヘモグロビンを封入した細胞型人工酸素運搬体（Hb-V）の開発も併行して進めており，㈱オキシジェニクス，早稲田大学，慶応義塾大学とともにHb-VのGMPに準拠した製造施設の設置を準備中である．さらに内包する献血由来のヘモグロビンを遺伝子組換えヘモグロビン（rHb）に置き換るためにrHbの開発にも着手している． これらの概要についても紹介する．

これらの完全合成型人工酸素運搬体は，原料供給の不安が払拭されるだけでなく，感染の危険性も回避できるため，酸素運搬能を有する薬剤としての全く新しい利用展開も可能となる．本稿で紹介する2種類の完全合成人工酸素運搬体は赤血球より極めて小さいという特長を生かし（Hb-Vで250 nm，アルブミンーヘムで10 nm），腫瘍の酸素化，虚血部位の酸素化による治療効果の向上についても臨床応用を目指した評価を進めている．これらの市場は赤血球市場（国内約300億円，日米欧で約2,400億円）と同等以上の市場が予測されており，新たな治療手段として今後の期待が大きい．

開発概要

1．アルブミンーヘム

(1) アルブミンーヘムの概要

アルブミンーヘムはFig. 2.に示したように，rHSAの分子中に合成ヘム誘導体を包接した全く新しい概念の人工酸素運搬体である．ヘムの結合サイトはサブドメインⅠb，Ⅱa，Ⅲbなどが推定されており，rHSA 1分子当たり最大8分子のヘムが取り込まれる[3,7]．アルブミンーヘムのCDスペクトルはrHSA単独の場合とほぼ重なっており，α-helix の含量（約67%）も変わらない．我々のグループは世界に先駆けて開発に成功したrHSAを保有しており[8]，合成ヘムとの組み合わせにより，感染の危険性のない完全合成型の人工酸素運搬体として開発を進めている[9]．

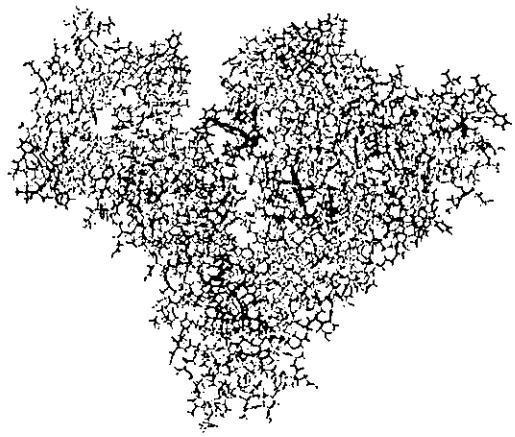


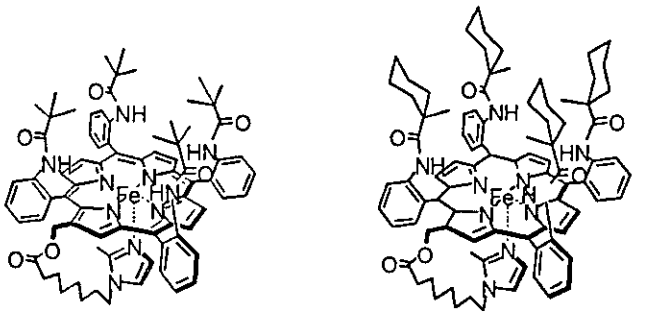
Fig. 2. Stereo view of albumin-heme
Maximally, 8 molecules of synthetic heme can be incorporated into albumin molecule.

酸素配位最小単位分子であるヘム鉄を血漿蛋白質であるアルブミンの内部に包接させた構造のアルブミンーヘムはカプセル化の必要がなく，そのまま投与できることが最大の利点であり，加えて，膠質浸透圧の調節能を有し，調製が容易，低コストなど，優れた特徴を持つ．また，欧米で開発が進行している修飾Hb製剤は，平滑筋近傍への逸脱とNO捕捉に伴う血管収縮・血圧上昇が問題視されているが[1,2]，アルブミンーヘム製剤を生体内へ投与しても，そのような副作用は全く観測されない[10]．アルブミンーヘムもHbと同様にNOを強く結合するものの，アルブミンの等電点が4.8と低いために，血管内皮細胞を覆う基底膜（厚さ50 nm）との間に静電反発を生じ，Hb粒子に比べて

血管外へ漏出しにくいためと考えている．現状は，ヘム誘導体の選定作業を終了し，輸血代替のみならず，腫瘍の酸素化による放射線治療効果の向上，虚血部位の酸素化等の新しい適応症へ向けた評価を行っているところである．

Fig. 3.に選定したヘム誘導体の構造を示した．ポルフィリン骨格を有したピケットフェンス型と呼ばれるポルフィリン誘導体（テトラアミノフェニルポルフィリン誘導体）であるが，分子内に軸塩基として作用する側鎖を有しており，ポルフィリン環の図中下側から第5座配位子として配位することにより，酸素分子の結合力を調整することができるという特徴を有している．種々の誘導体の中から有効性や安全性を中心に評価した結果，図中右に示したシクロヘキサノイルアミノフェニル誘導体を選択した[11]．

(2) 製造方法



Pivalamidophenyl deriv　　Methylcyclohexanamido-phenyl deriv.

Fig. 3. Molecular structures of synthetic hemes.

アルブミンーヘムの製造方法概略をFig. 4.に示した．アルブミン水溶液とヘム誘導体エタノール溶液を至適条件化で精密に混合撹拌することにより，アルブミン分子中にヘム誘導体が包接されたアルブミンーヘムが出来上がる．包接させるヘム誘導体の数は化学量論的な添加量を変化させることで容易に調整できる．不純物としてのエタノールを除去することを目的として透析（定容量限外濾過）の後，塩濃度の調節並びに濃縮を行い，最終的にアルブミン濃度5％としてアルブミンーヘム溶液を得る．

なお，製造中のヘム誘導体の安定性を維持するために，アスコルビン酸添加によって予め鉄を還元しておいたヘム誘導体溶液をCOガス通気し，CO結合体として最終アルブミンーヘム製剤を得る．得られたアルブミンーヘムの特性をTable 1.に示した．

一方，Fig. 5.に示したようにCO結合体は安定であるが，使用に先立ち，酸素化の操作が必要であるという欠点を有しており，以下に述べるCOを用いない製造方法の検討を併行して進めてきた．

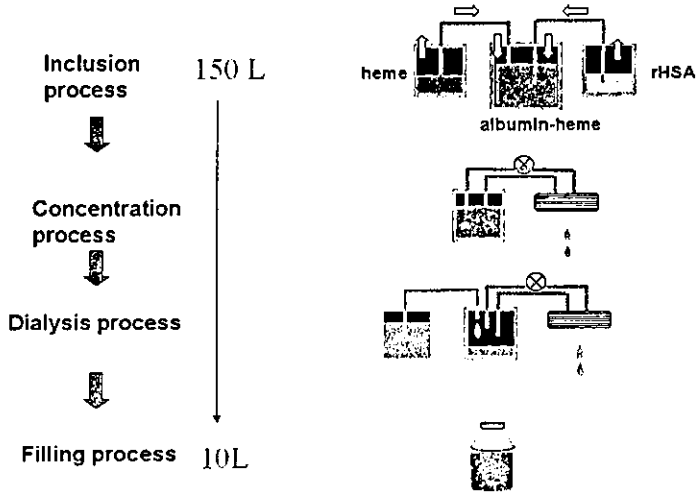


Fig. 4. Outline of manufacturing process of albumin-heme.

Table 1. Physico-chemical characteristics of albumin-heme.

Items	Standard values
[rHSA] (g/dL)	5
[heme] (mM)	3-6
heme/rHSA (mol/mol)	4-8
P_{50} (Torr)	28-38
rHSA pI	4.8
rHSA -helix content (%)	67
Stretching vibration of coordinated O_2 (cm^{-1})	1158
Stretching vibration of Fe-O_2 (cm^{-1})	561
Met-heme (%)	<3
Viscosity (cP at 230s^{-1})	1.1
Specific gravity (g/cm^3)	1.01
Crystal osmotic pressure (mOsm)	300
Colloidal osmotic pressure (Torr)	19
pH (37°C)	7.4
Endotoxin (EU/mL)	<0.2

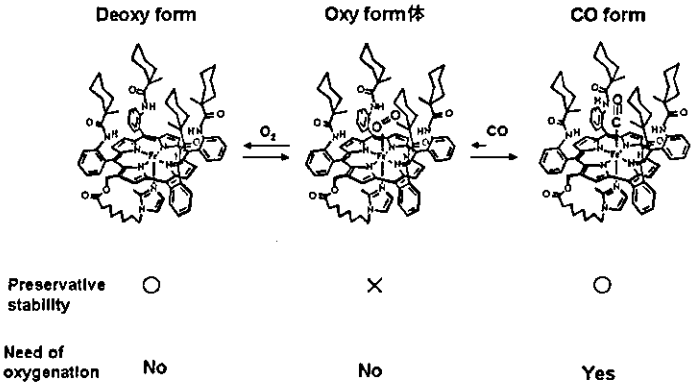


Fig. 5. Comparison of preservative stability and need of prior oxygenation among 3 forms.