of 23 mmHg the coencapsulation of SOD is necessary because of the generation of superoxide anion from the Hb.

In the intravenous injection of HbV into rats at a 20 vol % overdose, the rate of metHb formation was about twice that measured under physiological conditions in vitro at a pO_2 of 149 mmHg. Though the coencapsulation of active oxygen scavengers such as SOD and catalase suppress metHb formation, the main reason would be due to the ratio of deoxyHb which is oxidized more easily than oxyHb [25].

Another effective method of metHb reduction is the activation of a metHb reduction system when the concentration of metHb becomes high during the course of the blood circulation [26]. One of the possible ways is to add a reductant from the outer aqueous phase of the vesicles when the metHb ratio becomes high; however the bilayer membrane prevents the reductant from contacting and reacting with the inside metHb. The construction of an electron-transfer pathway across the bilayer membrane is necessary, which takes place in natural systems, for instance, the photosynthetic system in plants, the respiratory chain in mitochondria, ion-channels and so on. Model studies of such systems have been carried out using ubiquinones, cytochrome, dyes, and synthetic lipids.

The possibility of metHb reduction was studied using HbVs in which electron mediators and reductants were incorporated [26]. In the case of the addition of water-soluble reductants such as NADH or ascorbic acid (ASH), no reduction was observed because of their impermeability through the bilayer membrane. When ubiquinone was incorporated into the bilayer membrane as an electron mediator [27] under anaerobic condition, the percentage was steeply decreased less than 10% after 20 min. However, the metHb percentage adversely increased under aerobic condition in the case of the quinone/NADH system. Methylene blue (MB) [28] can exist in both aqueous and hydrophobic phases and is used for the treatment of methemoglobinemia [29,30]. When it was employed as an electron mediator instead of ubiquinone, a high reduction rate of metHb and almost complete reduction under anaerobic condition were observed [31]. Under aerobic condition, the MB/NADH or ASH systems showed the decrease in the metHb percentage though the incorporated amounts became high in comparsion with those under anaerobic condition. Therefore, this system might be useful to reduce the metHb in the HbV by simple injection.

Prevention of aggregation of HbV by surface modification

Because the red cell substitutes are expected to be used as a replacement for a large amount of lost red blood cells, the rheological properties of HbV itself and the mixture with blood are important in relation to hemodynamics. Surface modification of the phospholipid vesicles with some natural or synthetic glycolipids [31–34] or polyethyleneglycol (PEG)-conjugated lipids [35–37] is known to improve the dispersion state of the vesicles and prolong the circulation time in vivo for drug delivery systems (DDS). For HbV, the surface was also modified to improve the

dispersion state of the vesicles in the presence of water-soluble polymers or blood components [7,38]. The PEG-modified HbV (PEG-HbV) have been extensively studied in vitro and in vivo, and its oxygen transporting ability has been evaluated [39,40]; we have paid attention to the physicochemical or rheological aspects such as the modification process and the effect of PEG chains on the solution viscosity [41].

Surface modification of HbV with PEG and characteristics of PEG-HbV

In general, PEG-lipid is incorporated into vesicles by mixing with the other lipid components in organic solvents before dispersing them into an aqueous solution. In this case, both inner and outer sides of vesicular membrane are modified. The PEG chains extending from the inner surface should reduce the encapsulation efficiency of Hb, resulting in a low [Hb]/[Lipid] ratio. We used the modification method with PEG-lipid which is added to the outer aqueous phase of the preformed HbV, and have been studying the thermodynamics of PEG-lipid incorporation into vesicles using the isothermal titration calorimetory, and the equilibrium constant using ^{1}H-NMR, to know the optimal molecular structure of PEG-lipid for stable incorporation and effective function.

The PEG-HbV was dispersed into 5 g/dl human serum HSA (HSA) solution, and the resulting PEG-HbV/HSA data are summarized in Table 1. The diameter was controlled to 0.25 ± 0.08 μm. By increasing the weight ratio of Hb to lipid to 1.75, the lipid concentration was reduced to 5.71 g/dl. The high encapsulation efficiency was due to the control of intermolecular interactions during the assembling and sizing procedure of HbV [12]. The lipid composition of the resulting HbV was DPPC/cholesterol/DPPG/α-tocopherol/PEG-DSPE = 5/5/1/0.1/0.014 by molar ratio (PEG-DSPE = 0.13 mol%). More than 99% of the added PEG-DSPE was incorporated onto the outer surface of the HbV. The oxygen affinity, p_{50} was regulated to 32 mmHg by coencapsulating PLP (18.6 mM). The amount of oxygen release was calculated to be 6.2 ml/100 ml, which was close to the 7.0 ml/100 ml of human blood due to the increased oxygen transporting efficiency of the PEG-HbV, 37% compared to that of human red cells, 28%. No leakage of Hb was observed during and after the introduction of PEG-DSPE. The oxygen affinity of PEG-HbV was almost the same as that of HbV within experimental error, indicating that pH change in the inner aqueous phase and the leakage of coencapsulated small molecules such as PLP, Na^+, and Cl^-, were negligibly small. The densities of the PEG-HbV/HSA and HbV/HSA suspensions were almost the same (1.0336 and 1.0335 g/cm^3), and they were smaller than that of human blood (1.0525 g/cm^3), mainly due to the low concentration of HSA solution (5 g/dl), which is lower than the plasma protein concentration (ca 7.5 g/dl). The concentration of HSA of the suspension is expressed as 3.2 g/dl due to 36% of the total volume being HbV particles.

Table 1

Characteristics of HbV and PEG-HbV compared with XLHb abd RBC

Parameters	XLHb[a]	HbV	PEG-HbV	RBC
Diameter (nm)	5	244 ± 70	251 ± 76	8000
Hb (g/dl)	10.0	10.2	10.0	ca. 15
Lipid (g/dl)	–	5.9	5.7	0.2
[Hb]/[Lipid]	–	1.73	1.75	75 (13[c])
PEG-lipid (mol%)	–	–	0.3	–
p_{50} (mmHg)	32	35	35	28
OTE (%)[b]	32	37	38	28
metHb (%)	< 2	< 3	< 3	< 0.5
HbCO (%)	< 2	< 2	< 2	< 5
pH at 37°C	7.4	7.4	7.4	7.4
Osmolality (mOsm)	300	300	300	300
Oncotic pressure (mmHg)	30	20	20	25
Viscosity (cP) at 37°C	1.6	9.4	3.7	4.0

[a]Intramolecular crosslinked hemoglobin with bis(3,5-dibromosalicyl)fumarate (DBBF).
[b]Oxygen transporting efficiency: the difference in oxygen saturation (%) between pO_2 of 40 and 100 mmHg.
[c]Including membrane proteins (ca 1 g/dl) as lipid.

Effect of PEG conjugation on the aggregation of HbV in HSA solution and in blood mixture

The dispersion states of the PEG-HbV/HSA when mixed with human blood were observed by optical microscopy [43]. In the case of PEG-HbV/HSA, only RBCs were confirmed because of the small diameter of PEG-HbV/HSA, and no aggregate of PEG-HbV was confirmed in TEM photograph. In the case of HbV/HSA, on the other hand, aggregates of the HbV were actually confirmed among the RBCs. The RBCs were neither aggregated nor deformed, suggesting that both HbV and PEG-HbV would not interact with RBCs. The aggregates of HbV were actually observed before mixing with blood. It is quite obvious that PEG chains suppress the HbV aggregation.

Figure 3 shows the solution viscosity of the mixtures of HbV/HSA or PEG-HbV/HSA with blood. When the unmodified HbV was dispersed in PBS, the viscosity of the vesicular suspension became 2.6 cP (shear rate = $230\,s^{-1}$), which is lower than that of blood (3.7 cP). However, when dispersed in a 5 g/dl HSA solution to adjust the colloidal osmotic pressure, the HbV/HSA showed 8 cP viscosity (shear rate = $358\,s^{-1}$), which was substantially higher than that of blood [42]. The HbV/HSA shows a non-Newtonian flow typical for particle suspensions, while PEG-HbV/HSA shows a Newtonian flow and was almost the same as that of human blood at any shear rates. These results indicate that the unmodified HbV aggregates due to the molecular interaction of HSA with the vesicular surface and increase viscosity, while the surface modification of the HbV with PEG chains suppresses HbV aggregation and provides a low viscosity almost the same as that of human blood.

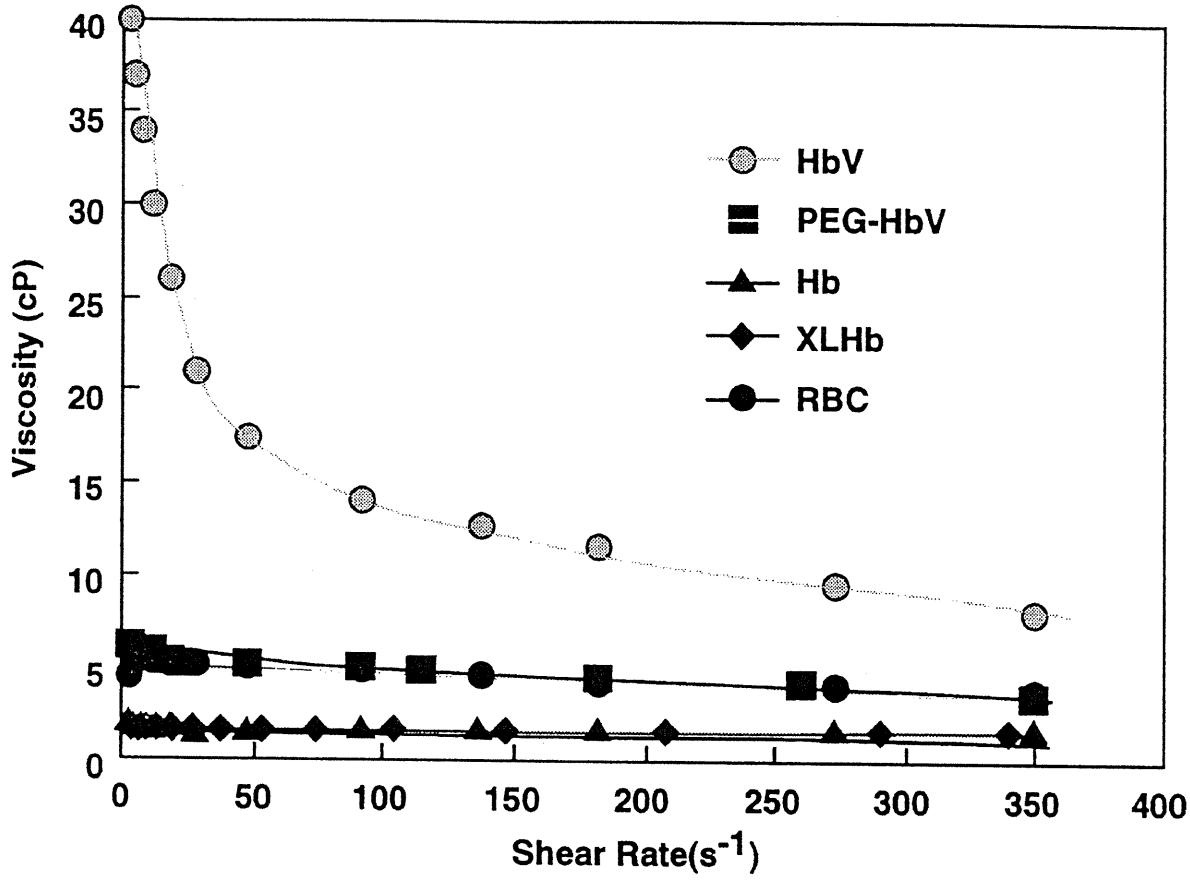

Fig. 3. The profiles of the shear rate dependent solution viscosity of oxygen carriers. The profiles of HbV and PEG-HbV are compared with those of Hb and RBC.

The permeabilities of the PEG-HbV/HSA, HbV/HSA and human blood through isopore membrane filters were studied as a model of the blood flow through capillaries [42]. This method is conventionally used for the RBC deformability measurement. The capillary diameter is usually 5–10 μm. It is generally known that RBC can penetrate through the 5 μm pore size of the membrane filter. A biconcave-shaped RBC with a diameter of 8 μm deforms to a parachute-like configuration and penetrates through the capillaries of ca 5 μm diameter. However, with decreasing pore size of the membranes from 5 to 2 μm, the flow rate decreased for all suspensions, especially for human blood even though the applied pressure is about twice (220 mmHg) that of normal blood pressure. When the pore diameter is 3 μm, blood could hardly penetrate, though both the unmodified HbV/HSA and PEG-HbV/HSA showed high penetration. Due to the small size of HbV (250 nm), both of the suspensions promptly penetrate through the membrane filters with pores of sizes down to 0.4 μm without changes of vesicular size or Hb leakage, and especially the PEG-HbV/HSA, which is not aggregated, penetrates faster than the unmodified HbV/HSA. The HbV/HSA showed aggregation in the optical microscopy; nevertheless, the aggregates dissociated at higher shear rates and penetrated more promptly

than expected. Both the HbV and PEG-HbV can penetrate through sterilizable filters of 0.22 μm in pore size for preparation.

In vivo evaluation of the efficacy of HbV

Ninety percent exchange transfusion tests

Ninety-percent of the estimated circulatory volume of rats was exchanged with 5 g/dl human serum albumin (HSA group, $n = 6$), ratRBC/HSA ($n = 6$) or PEG-HbV/HSA ($n = 6$) at 2 ml withdrawal (via the common carotid artery)/infusion (via the right artrium) cycles at a rate of 2 ml/min [43]. A needle-type polarographic oxygen electrode was placed in the cortex of the left kidney for the continuous measurements of renal cortical tissue oxygen tension. Blood samples for arterial blood gas analyses were taken on the first withdrawal as baseline values, and thereafter at exchange ratio of 10, 40, 60, 70, 80, 90% and at 30 min after the completion of the exchange transfusion. The results were summarized in Fig. 4. All data are shown as percentage of the baseline values and are expressed as mean ± standard deviation (S.D.).

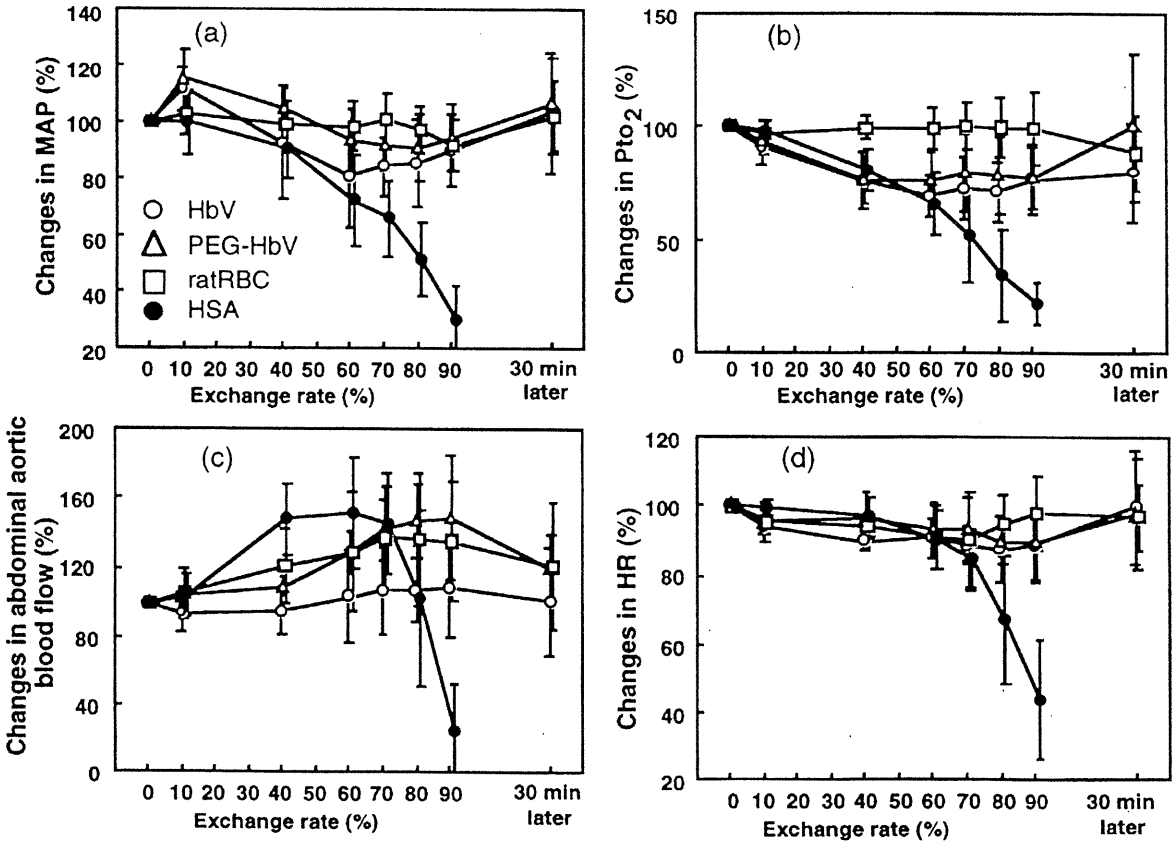

Fig. 4. Changes in (a) mean arterial pressure (MAP); (b) renal cortical tissue oxygen tension (p_tO_2), (c) abdominal aortic blood flow, and (c) heart rate (HR) during the exchange transfusion tests. They are expressed as percentages of the baseline values.

During the exchange transfusion, the hematocrit of blood in both the HbV/HSA and PEG-HbV/HSA groups decreased from about 50 to 5%. This indicates that almost 90% exchange transfusion with HbV/HSA was actually performed. After the hematcrit measurement, the HbV layer was confirmed on the RBC layer in the glass capillary; while PEG-HbV was dispersed in the supernatant not forming a layer. The changes in mean arterial pressure (MAP) showed a slight transient increase in the HbV/HSA and PEG-HbV/HSA groups, then decreased to 90% of the baseline values, and sustained at that level throughout the experiment. There was no significant difference in MAP between HbV/HSA and PEG-HbV/HSA groups. On the other hand, MAP in the HSA group declined to 66.0%, which was significantly lower than the other groups, and from there it continued to decline to zero within 20 min after the completion of the exchange transfusion, meaning death. The renal cortical oxygen tension (p_tO_2) for the HbV/HSA and PEG-HbV/HSA groups decreased to approximately 70% of the baseline values at 90% exchange, and the values were significantly lower than that of ratRBC/HSA group, though they were significantly higher than that of HSA group.

Aortic blood flow in the HSA group increased with exchange ratio and reached to 160% of the baseline up to a 40% exchange ratio, and then showed a significant decrease to 25% at an exchange ratio of 90%. Exchange transfusion with 5 g/dl HSA, a non-oxygen carrying colloid induces isovolemic anemia. At 40% exchange, in the HSA group, cardiac output increased in order to sustain oxygen delivery, which was depicted as an increase in aortic blood flow. However, when the exchange rate exceeded 70%, this compensation could no longer sufficiently function because of the impairment in cardiac function caused by decreased oxygen delivery to the cardiac muscles themselves. On the other hand, PEG-HbV/HSA and ratRBC/HSA groups showed 135–150% of baseline, while the HbV/HSA group sustained a baseline value. Regarding the parameters of heart rate, pH, and base excess, no significant differences were confirmed among those three groups, PEG-HbV/HSA, HbV/HSA, and ratRBC/HSA and the baseline values were sustained. In the HbV/HSA group, oxygen delivery tended to be lower and oxygen consumption tended to be higher those of the PEG-HbV/HSA and ratRBC/HSA groups without significant differences.

In conclusion, hemodynamic and blood gas parameters as well as tissue oxygen tension measurements were well sustained in the HbV/HSA and PEG-HbV/HSA groups during and after the exchange transfusion. This indicates that oxygen transport was satisfactorily maintained by HbV in these animals. The PEG-HbV/HSA group showed higher aortic blood flow than HbV/HSA and ratRBC/HSA groups. This was considered to be the result of the lower viscosity of the PEG-HbV/HSA.

Rabbit shock model

In a state of shock, intestinal ischemia and its impaired barrier function may result in the precipitation of multiple organ failure. When solutions of oxygen carriers are infused in states of ischemia for resuscitation, the differences in the oxygen transporting capability of the solutions would cause the differences in the recovery of intestinal

ischemia. In order to evaluate the oxygen transporting capability of HbV which encapsulates concentrated hemoglobin, a rabbit shock model was used and oxygen transport to the small intestine was measured.

A 2 mm ultrasonic flow probe was placed around the superior mesenteric artery for a measurement of arterial flow. The catheter was advanced 5–10 cm proximally until the tip was located in the superior mesentric vein for sampling of venous blood. A sigmoid tonomitor was positioned in the duodenum 2–3 cm from the pylorus for a measurement of intestinal mucosal pH. A needle type polarographic oxygen electrode was inserted in the submucosa of the small intestine for continuous intestinal tissue oxygen tension measurements.

Shock was induced by withdrawal of 40% of the estimated circulatory blood volume followed by isovolemic infusion of fluids; PEG-HbV dispersed in 5 g/dl HSA (PEG-HbV/HSA), 5 g/dl HSA (HSA), and washed rabbit red cells dispersed in 5 g/dl HSA(RBC/HSA). The hemoglobin concentration of those samples was adjusted to 10 g/dl. The procedure was repeated twice.

From the results concerning on MAP, HR, arterial blood O_2 tension (p_aO_2), and blood flow in the superior mesenteric artery, there were no significant differences between groups. In other words, volume resuscitation with HSA alone was sufficient to sustain these parameters. On the other hand, systemic BE, intestinal mucosal pH, and intestinal tissue O_2 tension (p_tO_2) were sustained significantly higher in the PEG-HbV/HSA and RBC/HSA groups compared to the HSA group. Concerning venous blood O_2 tension (p_vO_2), the higher value in the HSA group probably resulted from shunting of the tissues due to the collapse of peripheral circulation. The small intestine is one of the most vulnerable organs in shock and from these results we can assume that intestinal perfusion was more optimal in the PEG-HbV/HSA and RBC/HSA groups due to their similar oxygen transporting capabilities.

Conclusions

Concentrated hemoglobin could be encapsulated in the phospholipid vesicle by the control of molecular interaction between hemoglobin and phospholipids. The oxygen affinity and the rate of methemoglobin formation are controlled by the coencapsulation of allosteric effectors, reductants, and by the solution pH. Construction of more sophisticated artificial methemoglobin reduction systems would be possible if the kinetics of autoxidation including the generation of active oxygens and the reduction of methemoglobin through the bilayer membrane were studied. The increase in the viscosity of the mixture of blood and HbV was due to the aggregation of vesicles. It was suppressed by the surface modification with PEG chains. The effect of the PEG modification of the HbV were also observed in vivo experiments as the increased blood flow, and the resulting stable gas parameters. The PEG-HbV transported oxygen almost as well as red blood cells and can be considered as a promising candidate for an artificial oxygen carrier.

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References

[1] T.M.S. Chang, Blood substitutes based on modified hemoglobin prepared by encapsulation or crosslinking: an overview. Biomater. Artif. Cell Immobil. Biotechnol., 20 (1991) 159–182.

[2] R.M. Winslow, Blood substitutes: a moving target. Nature Med., 1 (1995) 1212–1215.

[3] E. Tsuchida, Introduction: overview and perspective. In: Artificial Red Cells, E. Tsuchida (Ed.), John Wiley and Sons, New York, 1995, pp. 1–20.

[4] L. Djordjevich, I.F. Miller, Synthetic erythrocytes from lipid encapsulated hemoglobin. Exp. Hemat., 8 (1980) 584–592.

[5] C.A. Hunt, R.R. Burnette, R.D. MacGregor, A.E. Strubbe, D.T. Lau, N. Taylor, H. Kawada, Synthesis and evaluation of a prototypal artificial red cell. Science, 30 (1985) 1165–1168.

[6] A.S. Rudolph, Encapsulation of hemoglobin in liposomes. In: Blood substitutes: Physiological Basis of Efficacy, R.M. Winslow, K.D. Vandegriff, M. Intaglietta (Ed.), Birkhäuser, Boston, 1995, pp. 90–104.

[7] E. Tsuchida, S. Takeoka, Stabilized hemoglobin vesicles. In: Artificial Red Cells, E. Tsuchida (Ed.), John Wiley and Sons, New York, 1995, pp. 35–64.

[8] K.D. Vandegriff, R.M. Winslow, The theoretical analysis of oxygen transport: A new strategy for the design of hemoglobin-based red cell substitutes. In: Blood Substitutes: Physiological Basis of Efficacy, R.M. Winslow, K.D. Vandegriff, M. Intaglietta (Ed.), Birkhäuser, Boston, 1995, pp. 143–154.

[9] A.G. Tsai, H. Kerger, M. Intaglietta, Microcirculatory consequences of blood substitution with α,α-hemoglobin. In: Blood Substitutes: Physiological Basis of Efficacy, R.M. Winslow, K.D. Vandegriff, M. Intaglietta (Ed.), Birkhauser, Boston, 1995, pp. 155–174.

[10] S.B. Olsen, D.B. Tang, M.R, Jackson, E.R. Gomez, B. Ayala, B.M. Alving, Enhancement of platelet deposition by cross-linked hemoglobin in a rat carotid endarterectomy model. Circulation, 93 (1996) 327–332.

[11] W. Kaca, R.I. Roth, K.D. Vandegriff, G.C. Chen, F.A. Kuypers, R.M. Winslow, J. Levin, Effects of bacterial endotoxin on human cross-linked and native hemoglobins. Biochemistry, 34 (1995) 11176–11185.

[12] S. Takeoka, T. Ohgushi, T. Ohmori, K. Terase, H. Nishide, E. Tsuchida, Layer controlled hemoglobin vesicles by Interaction of hemoglobin with phospholipid assembly. Langmuir 12 (1996) 1755–1759.

[13] S.I. Park, T. Kose, M. Hamasaki, S. Takeoka, H. Nishide, E.Tsuchida, Effect of the pH-controlled hemoglobin vesicles by CO_2 gas. Artif. Cells Blood Substit. Immobil. Biotechnol. 1998, in press.

[14] T. Yang, K.W. Olsen, The effect of crosslinking by bis(3,5-dibromosalicyl) fumarate on the autoxidation of hemoglobin. Biochem. Biophys. Res. Commun., 163 (1989) 733–738.

[15] L.P. Stratton, A.S. Rudolph, W.K. Knoll. Jr., S. Bayne, M.C. Farmar, The reduction of methemoglobin levels by antioxidants. Hemoglobin, 12 (1988) 358–368.

[16] A. Tomoda, T. Yoneyama, A. Tsuji, Changes in intermediate haemoglobins during autoxidiation of haemoglobin. Biochem. J., 195 (1981) 485–492.

[17] W.J. Wallace, R.A. Houchens, J.C. Maxwell, W.S. Coughey, Mechanism of autoxidation for hemoglobins and myoglobins. J. Biol. Chem., 257 (1982) 4966–4977.

[18] J.A. Watkins, S. Kawanishi, W.S. Caughey, Autoxidation reactions of hemoglobin a free from other red cell components: a minimal mechanism, Biochem. Biophys. Res. Commun., 13 (1985) 742–748.

[19] Y. Ogata, T. Okamoto, K. Suzuki, T. Kamitani, The Development of Neo Red Cells (NRC) with enzymatic reduction system of the methemoglobin. Artif. Blood, 2 (1994) 62–66.

[20] H. Sakai, S. Takeoka, H. Yokohama, Y. Seino, H. Nishide, E. Tsuchida, Purification of concentrated hemoglobin using organic solvent and heat treatment. Protein Expression Purif., 4 (1993) 563–569.

[21] V. Sampath, W.S. Caughey, Prooxidant effects of glutathione in aerobic hemoglobin solutions. Superoxide generation from uncoordinated dioxygen. J. Am. Chem. Soc., 107 (1985) 4076–4078.

[22] P. Eyer, H. Hertle, M. Kiese, G. Klein, Kinetics of ferrihemoglobin formation by some reducing agents, and the role of hydrogen peroxide. Mol. Pharmacol, 11 (1975) 326–334.

[23] H. Sakai, S. Takeoka, Y. Seino, E. Tsuchida, Suppression of methemoglobin formation by glutathione in a concentrated hemoglobin solution and in a hemoglobin vesicle. Bull. Chem. Soc. Jpn., 67 (1994) 1120–1125.

[24] S. Takeoka, H. Sakai, T. Kose, Y. Mano, Y. Seino, H. Nishide, E. Tsuchida, Methemoglobin formation in hemoglobin vesicles and reduction by encapsulated thiols. Bioconjugate Chem., 8 (1997) 539–544.

[25] R.E. Brantley, S.J. Smerdon, A.J. Wilkinson, E.W. Singleton, J.S. Olson, The mechanism of autoxidation of myoglobin. J. Biol. Chem., 268 (1993) 6995–7010.

[26] S. Takeoka, T. Ohgushi, H. Nishide, E. Tsuchida, Reduction of methemoglobin via electron transfer across the bilayer membrane of Hb vesicles. Bull. Chem. Soc. Jpn., 70 (1997) 1171–1178.

[27] E.L. Ulrich, M. Girvin, W.A. Cramer, J.L. Markley, Location and mobility of ubiquinones of different chain lengths. Biochemistry, 24 (1985) 2501–2508.

[28] E.R. Jaffe, G.A. Neumann, Comparison of the effect of menadine, methylene blue and ascorbic acid on the reduction of methemoglobin in vivo. Nature, 202 (1964) 607–608.

[29] C.C. Yang, S.F. Hwang, M.M. Chou, A.J. Zbehlik, Metobromuron/metolachlor ingestion with late onset methemoglobinemia in a pregnant woman successfully treated with methylene blue. J. Toxicol. Clin. Toxicol., 33 (1995) 713–716.

[30] L.F. Rodrigues, L.M. Smolik, A.J. Zbehlik, Benzocaine-induced methemoglobinemia: report of a severe reaction and review of the literature. Ann. Pharmacother, 28 (1994) 643–649.

[31] P.-S.Wu, G.W. Tin, J.D. Baldeschwieler, T.Y. Shen, M.M. Ponpipom, Effect of surface modification on aggregation of phospholipid vesicles. Proc. Natl. Acad. Sci. USA, 78 (1981) 6211–6215.

[32] T.M. Allen, A. Cohn, Large unilamellar liposomes with low uptake into the reticuloendothelial system. FEBS Lett., 223 (1987) 42–46.

[33] A. Gabizon, D. Papahadjopoulos, Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc. Natl. Acad. Sci. USA, 85 (1988) 6949–6953.

[34] S. Takeoka, H. Sakai, M. Takisada, E. Tsuchida, Inhibition of intervesicular aggregation of phospholipid vesicles by incorporation of dialkyl oligosaccharide glycerol. Chem. Lett., 1992 (1992) 1877–1880.

[35] A. Kilbanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphipatic polyoxyethylenes effectively prolong the circulation time of liposomes. FEBS Lett., 268 (1990) 235–237.

[36] M.C. Woodle, D.D. Lasic, Sterically stabilized liposomes. Biochim. Biophys. Acta. 1113 (1992) 171–199.

[37] T.O. Harasym, P. Tardi, S.A. Longman, S.M. Ansell, M.B. Bally, P.R. Cullis, L.S. Choi, Poly(ethylene glycol)-modified phospholipid prevent aggregation during covalent conjugation of proteins to liposomes. Bioconjugate Chem., 6 (1995) 1043–1802.

[38] H. Yoshioka, Surface modification of haemoglobin-containing liposomes with polyethylene glycol prevents liposome aggregation in blood plasma. Biomaterials, 12 (1991) 861–864.

[39] A. Usuba, M. Miyazawa, R. Motoki, K. Sakaguchi, K. Suzuki, T. Kamitani, A. Takahashi, Oxygen transport capacity and hemodynamic effect of newly developed artificial blood Neo Red Cells (NRC). Intern. J. Artif. Organs, 16 (1993) 551–556.

[40] S. Zheng, R. Beissinger, R.L. Sherwood, D.L. McCormick, D.D. Lasic, F.J. Martin, Liposome-encapsulated hemoglobin: A red blood cell substitute. J. Liposome Res., 3 (1993) 575–588.

[41] H. Sakai, K. Hamada., S. Takeoka, H. Nishide, E. Tsuchida, Physical characteristics of hemoglobin vesicles as red cell substitutes Biotechnol. Prog., 12 (1996) 119–125.

[42] H. Sakai, S. Takeoka, S.I. Park, T. Kose, H. Nishide, Y. Izumi, A. Yoshizu, K. Kobayashi, E. Tsuchida, Surface modification of hemoglobin vesicles with poly (ethyleneglycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anethetized rats. Bioconjugate Chem., 8 (1997) 23–30.

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

Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 15

Microvascular Responses to Hemodilution with Hb-Vesicles: Importance of Resistance Arteries and Mechanisms of Vasoconstriction

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Introduction

Phospholipid vesicles encapsulating concentrated hemoglobin (Hb vesicles, HbV) have the potential of becoming industrially produced red cell substitutes. They most closely reproduce the characteristics of natural blood including the red cell membrane function of physically preventing direct contact of Hb with the cellular components of circulation [1–3]. The desirability of this barrier function is evident in considering the side effects found in the use of acellular Hb solutions such as chemically-modified Hb and recombinant Hb which are now in clinical trials.

The principal systemic side effect consistently reported in the administration of a red cell substitute based on Hb solutions is a pressor response [4,5]. This has been widely regarded to be due to the nitric oxide (NO) scavenging effect of Hb, caused by the intrinsic high affinity of NO to Hb, a process presumed to evoke vasoconstriction [6]. Even though the pressor effect has been proposed to be beneficial as a remedy for hypotension in endotoxin shock, vasoconstriction is deleterious to the downstream microvascular function and tissue oxygenation. Conversely, it has been confirmed that NO-related vasoconstriction by the liposome-encapsulated hemoglobin (Hb-vesicles, HbV) does not occur in an ex vivo experiment using a rabbit aortic strips [7]. Direct microcirculatory observation, using a conscious hamster fitted with a dorsal skin window, has shown that arterioles (diameter, less than ca. 50 μm) do not constrict [8]. Since local biochemical events interact with systemic regulation, the understanding of phenomena such as Hb induced vasoconstriction requires the combined microscopic and systemic analysis of vascular function [9].

The presence of red cell substitutes that utilize Hb as an oxygen carrier induces vasoconstriction by variety mechanisms in addition to the NO–Hb reaction, all of which affect the "resistance vessels" that regulate peripheral blood flow [10]. At first this chapter summarizes the non-invasive technique to observe the microvascular perfusion and the responses to the hemodilution with HbV. Then, we discuss

proposed mechanisms which may cause vasoconstriction and reduced downstream blood flow, and discusses applications to the design of red cell substitutes to improve microcirculation and tissue oxygenation.

Microhemodynamic measurements and hemoglobin vesicles

Method to observe microhemodynamics and tissue oxygenation

Types of the animal preparations for microvascular observation which are often used are, sartorius, cheek pouch, cremaster, conjunctiva, cremaster, mesentery, pia mater, skeletal muscle, etc., or in special case bat wings. An ideal preparation should have the following properties: no anesthesia, no trauma, immobility, transparency, accessibility and undisturbed environment of the tissue (no irrigation, plastic films, air, etc.) [11,12]. Hamster dorsal skin fold window chamber preparation is currently used in our experiment of hemodilution with red cell substitutes because of no need of anesthesia during observation, less trauma, enough transparency, and long term potency for observation [8,9]. A cover glass (diameter, 12 mm) was surgically installed on the exposed skin allowing intravital observation of the microvasculature and tissues as shown in Fig. 1. Polyethylene catheters were implanted in the jugular vein

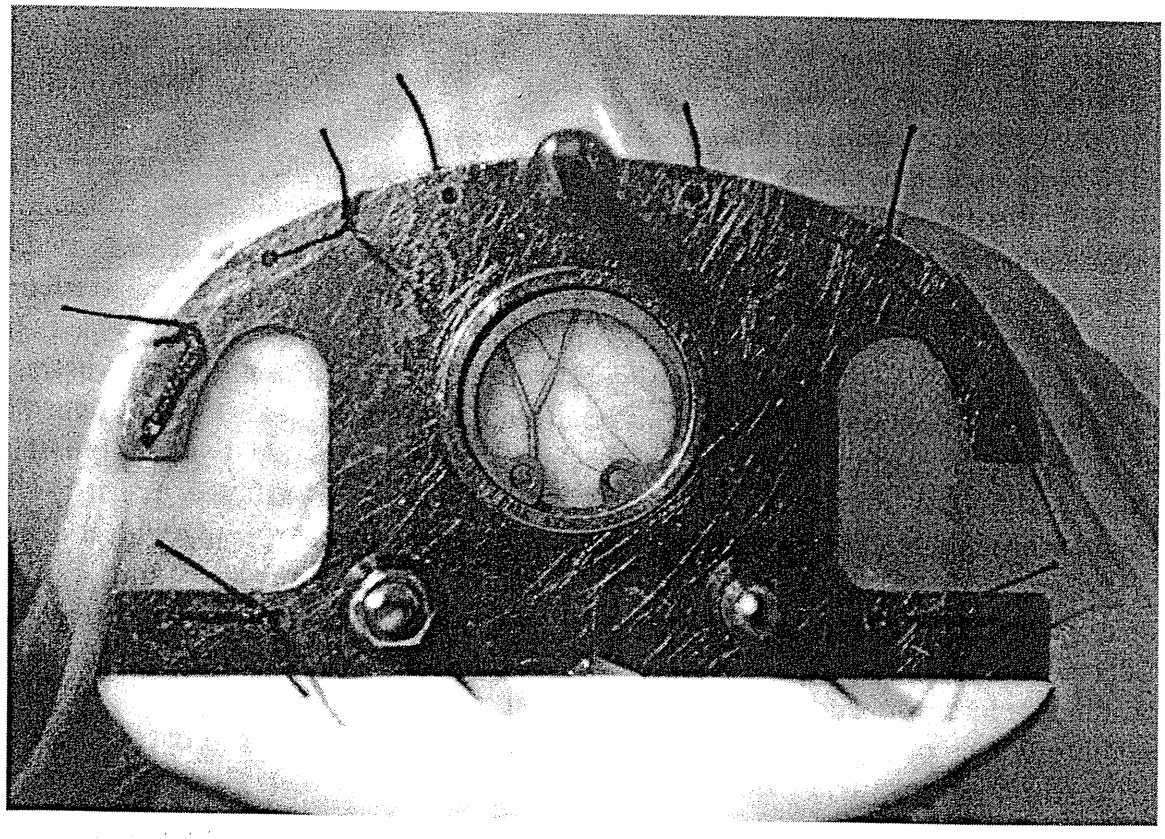

Fig. 1. Dorsal skinfold chamber in a Syrian golden hamster used to visualize microvessels in the subcutaneous tissue.

and the carotid artery. They were passed subcutaneously from the ventral to the dorsal side of the neck and exteriorized through the skin at the base of the chamber. During the measurement, the animals were placed in a porous plastic tube from which the window chamber protruded to minimize animal movement without impeding respiration.

Mean arterial pressure (MAP), heart rate, blood gas, and hematocrit were measured from arterial line. Microvessels in the subcutaneous tissue and the skeletal skin muscle were observed with an inverted microscope with a trans-illumination technique (Fig. 2). Microvascular diameter was measured with an image-shearing

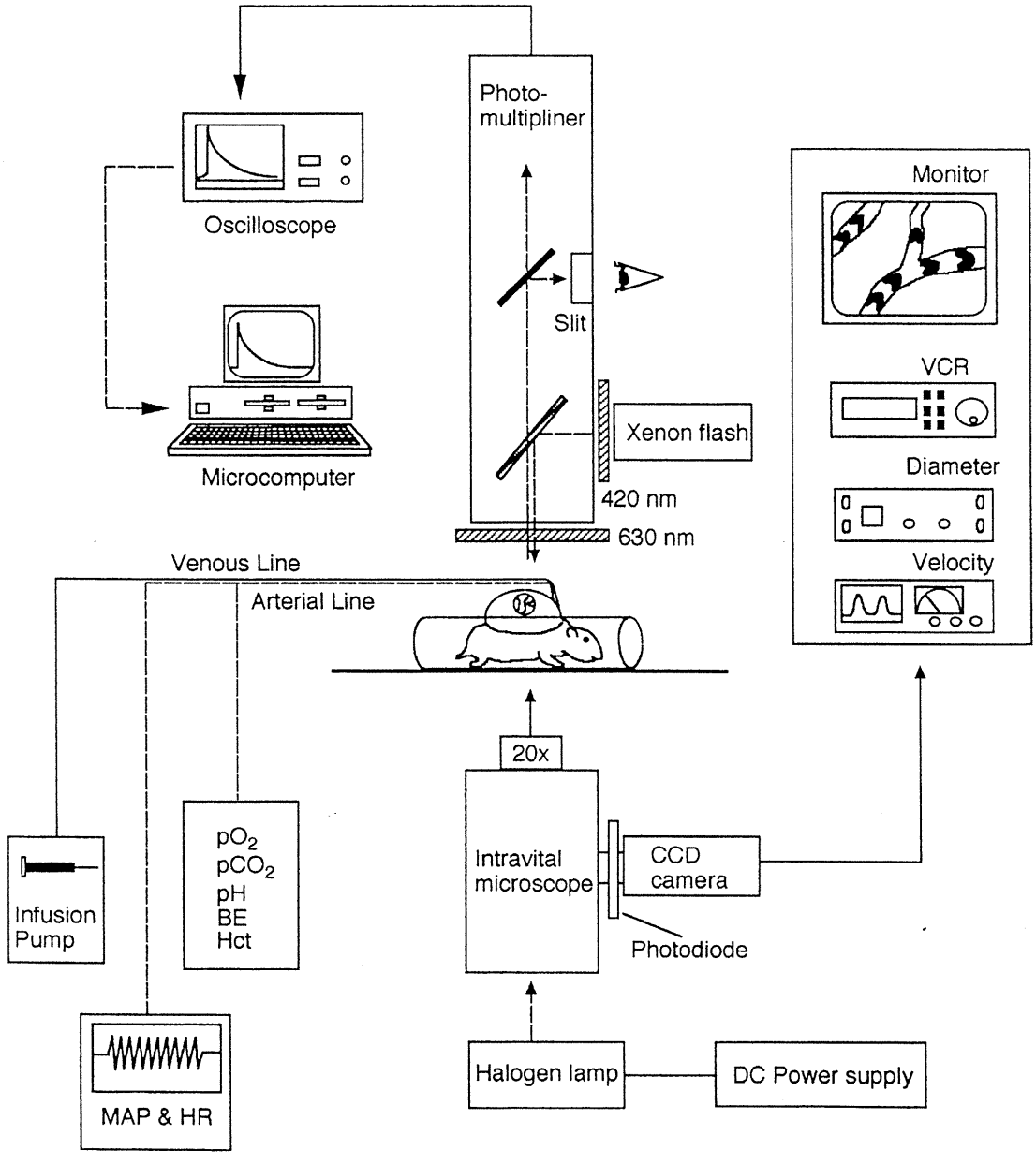

Fig. 2. Schematic diagram of system for the measurement of hemodynamic parameters and oxygen tensions in microcirculation.

system (Digital Video Image Shearing Monitor 908, I.P.M. Inc.), while RBC velocity was analyzed with photodiodes and the cross-correlation technique (Velocity Tracker Mod-102 B, I.P.M. Inc.) [13,14]. Blood flow rates (Q) were calculated using the RBC velocity and the diameter. Functional capillary density was analyzed on-line by counting the number of capillaries stemming from one A_3 arteriole.

Subcutaneous microvascular and interstitial pO_2 values were determined with the O_2 dependent quenching of phosphorescence emitted by bovine serum albumin bound paradium-*meso*-tetra(4-carboxyphenyl)porphyrin complexes after pulsed light excitation [15]. The method allows non-invasive assessment of intravascular pO_2 and determination of interstitial oxygenation, since intravascularly injected porphyrin-albumin extravasate into the interstitium over time. Phosphorescence was excited by xenon strobe arc, while pO_2 measuring sites were microscopically vignetted by an adjustable slit, and the signals were captured by a photomultiplier. One hundred and twenty eight decay curves were averaged, visualized and the pO_2 was obtained with computer fitting to a single exponential, using the Stern-Volmer equation.

Hemodilution with hemoglobin vesicles modified with polyethyleneglycol

Using the intravital microscopic method of hamsters mentioned above, the function of HbV as a blood replacement was tested during severe hemodilution where 80% of the red blood cell mass was substituted with suspensions of the vesicles in 5% human serum albumin (HSA) solution [8]. The characteristics of HbV were, diameter $= 258 \pm 57$ nm, oxygen affinity = 31 mmHg, and [Hb] = 10 g/dl. Vesicles were tested with membranes that were unmodified (HbV/HSA) or conjugated with polyethyleneglycol (PEG) on the vesicular surface (PEG-HbV/HSA). The viscosity of 10 g/dl HbV/HSA is 8 cP at 358 s^{-1} due to the intervesicular aggregation, while that of 10 g/dl PEG-HbV/HSA is 3.5 cP since PEG chains inhibit aggregation. Both materials yielded normal mean arterial pressure, heart rate, and blood gas parameters at all levels of exchange that could not be achieved with HSA alone. Subcutaneous microvascular studies showed that PEG-HbV/HSA significantly improved microhemodynamic conditions (flow rate, functional capillary density, vessel diameter, oxygen tension) relative to unmodified HbV/HSA. PEG-HbV was homogeneously distributed in the plasma phase, while aggregates of the unmodified HbV were clearly observed in capillaries and venules where the flow rate were low (Fig. 3). Even though it is confirmed in vitro that the aggregates dissociate reversibly at higher shear rates, it is unlikely that they will dissociate in vessels where the flow rate or shear rate is low. Aggregation and decreased flow rate may constitute a vicious circle that reinforces negative effects on blood flow. Thus PEG reduced vesicular aggregation and viscosity improving microvascular perfusion relative to the unmodified type.

In this experiment, it has been clarified that arterioles (diameter, less than ca. 50 μm) do not constrict. However, we speculated that the upstream vessels such as small arteries and thoracodorsal arteries, not visible in the preparation, were also

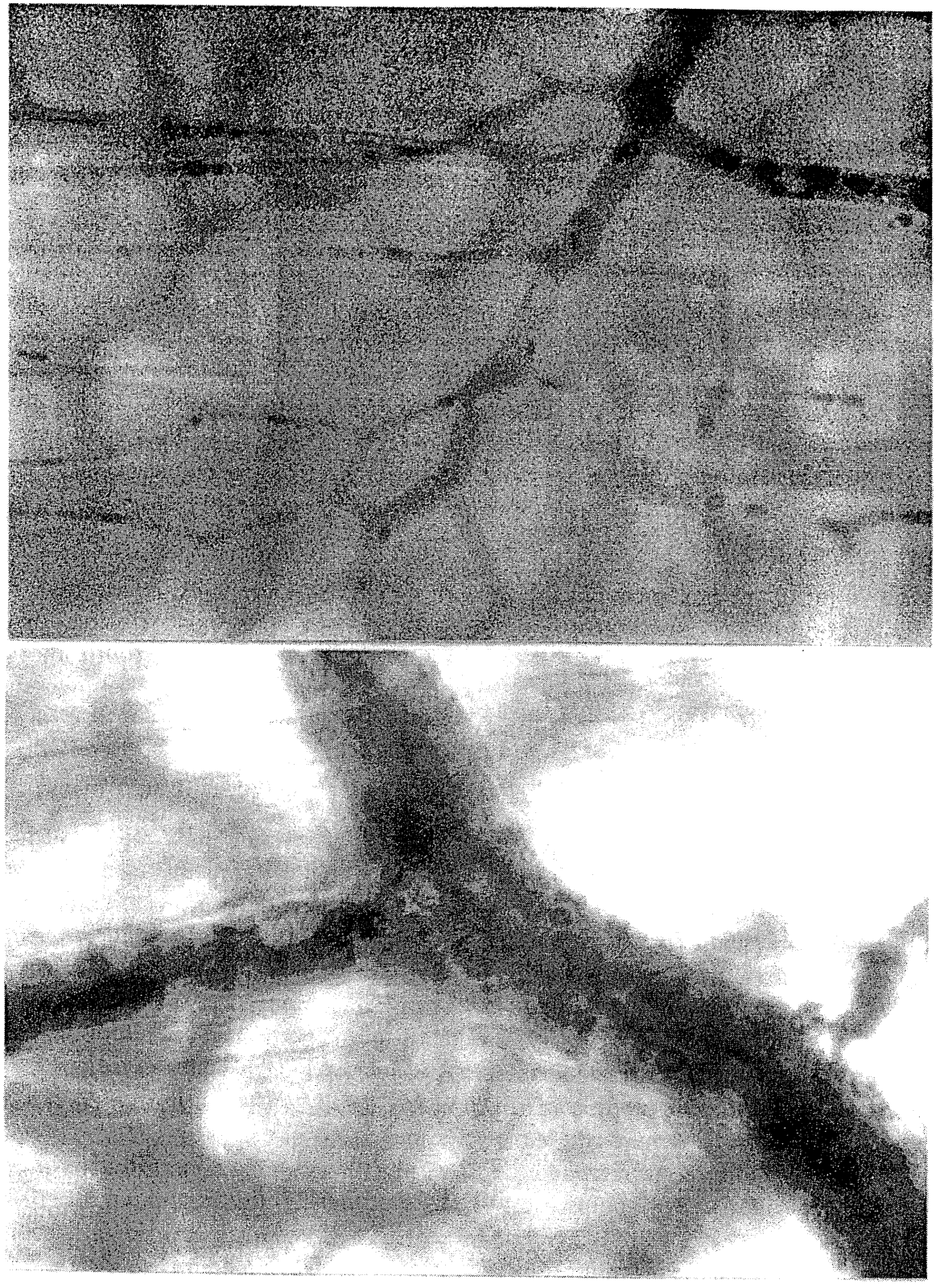

Fig. 3. Micrographs of microvasculature after 80% exchange with PEG-HbV/HSA and unmodified HbV/HSA. (Top) Microvasculature are blackened owing to the homogeneous distribution of PEG-HbV in the plasma phase. (Bottom) The aggregates of HbV form blocks in collecting venules.

important to observe to elucidate the mechanism of controlling downstream microcirculation.

Importance of arterioles or small arteries (resistance vessels) for the microcirculation

Approximately a half of the total blood pressure drop across the microvasculature occurs in the small arteries and arterioles, termed "resistance vessels" in vascular networks such as the mesentery, pia mater, skeletal muscle, cremaster, and cheek pouch as shown by Davis et al. [16] (Fig. 4). Vascular resistance is dominated by adrenergic constriction and intrinsic smooth muscle tone of metabolic, myogenic and autacoid origin [17–19]. However, most of the microcirculatory studies after infusion of red cell substitutes did not cover these vessels because of the difficulty in access especially in unanesthetized in situ condition [8,15,20].

Non-invasive observation of resistance vessels

The behavior of the microvasculature and corresponding larger feeding arteries and capacitance veins was analyzed for the first time in the conscious hamster dorsal skinfold window preparation by shifting the position of the window to include the vessels [21]. These vessels correspond to circumflex scapula in humans. The diameter of the artery is of the order of 150 μm and the vein diameter is in the range of 300–400 μm, and they run in parallel together with the major nerves of this tissue. These vessels exhibited the most significant responses during hemodilution with human serum albumin (HSA), both reducing their diameter to 70% of control, while the

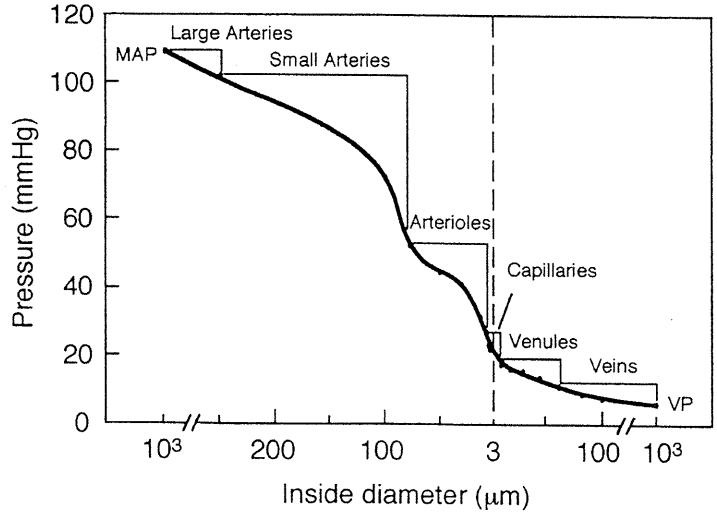

Fig. 4. Pressure drop across the vascular system in the hamster cheek pouch. MAP, mean arterial pressure; VP, venous pressure (cited from Davis et al., Am. J. Physiol., 250, H291, 1986).

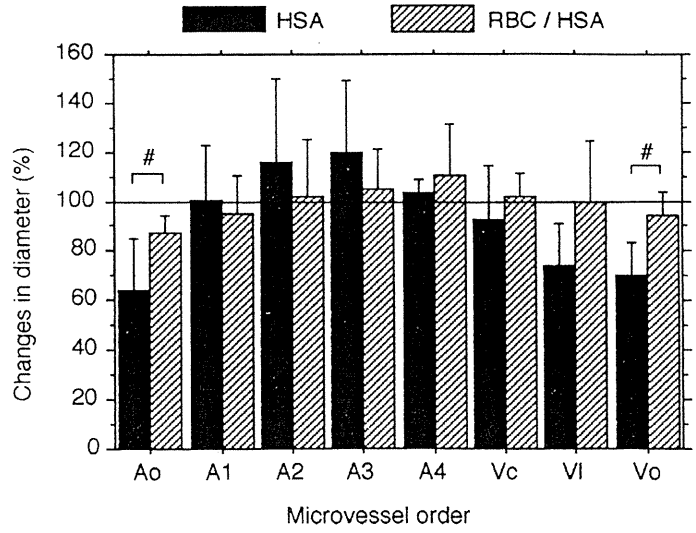

Fig. 5. Diameter changes of microvasculature in conscious hamster dorsal skinfold preparation after 80% hemodilution with 8% human serum albumin (HSA) and washed RBCs suspended in HSA (RBC/HSA) with hemoglobin concentration of 10%. Baseline diameters (in μm); Ao (150); A_1 (50); A_2 (20); A_3 (10); A_4 (8); Vc (30); V_1 (80); Vo (300). Values are mean ±stanadard deviation. # significant difference ($p < 0.05$).

smaller arterioles usually investigated in microvascular studies, with diameters in the range of 50–70 μm did not exhibit any significant change (Fig. 5). The significant constriction of the feeding arteries may help to maintain blood pressure while constriction of the capacitance veins may contribute to redistribute blood from skin to vital organs.

The small arterioles showed a nonsignificant tendency to dilate, however, blood flow was not affected as a consequence of the upstream arterial constriction, and the microvasculature became hypoxic. Hemodilution with washed RBC suspended in HSA did not show such vasoconstriction, and blood flow was maintained with higher microvascular oxygen tensions. The two reperfusion media differ physically both in terms oxygen carrying capacity and viscosity, the latter probably inducing changes in shear stress at the vascular wall. Our recent observation showed that immediately after the 10 vol% toploading of acellular α,α-crosslinked Hb (XLHb, [Hb] = 5 g/dL) into hamsters, they started to show hypertension (+30 mmHg) with simultaneous vasoconstriction of A_0 (−23%) as shown in Fig. 6, but not with A_1. On the other hand, cellular HbV group (data not shown here), as well as HSA group did not show such dramatic changes. The difference may be mainly explained with the nitric oxide scavenging effect of acellular XLHb, which can easily approach to the smooth muscle of the artery and binds NO, while the larger HbV particles with diameter of about 200–250 nm can not come close to the smooth muscle. Nakai et al. has shown that the molecular size of Hb products influences the permeability of endothelial cell layer and accessibility to the smooth muscle [Chapter 20, ref 7]. These tests indicate the importance of observing the resistance vessels that bridge the systemic vasculature and the microvasculature in evaluating efficacy and side effects of red cell substitutes.

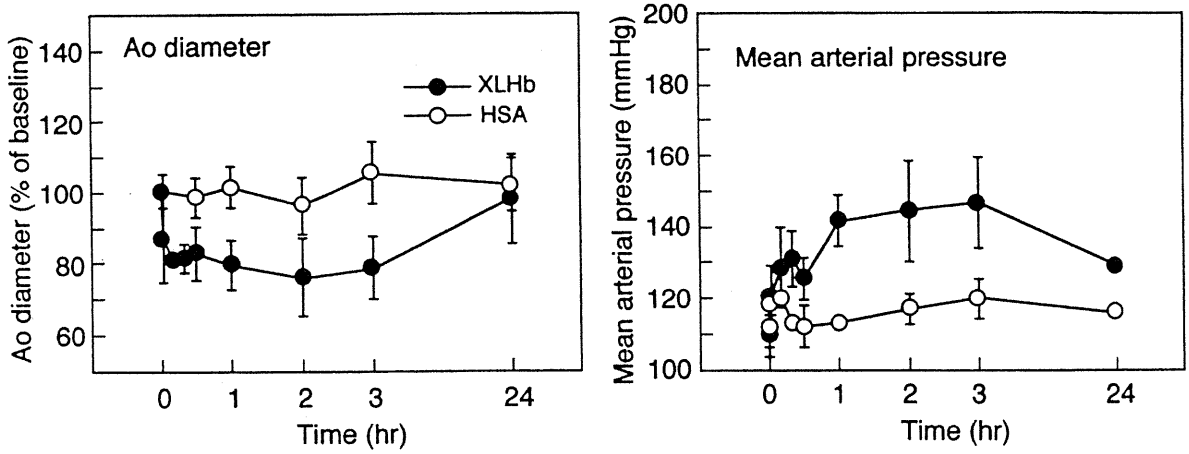

Fig. 6. Time course of A_0 diameter and mean arterial pressure after 10% toploading of intramolecularly cross-linked Hb (XLHb, 5 g/dL) and human serum albumin (HSA, 5 g/dL).

Oxygen dependent vasoconstriction

Blood flow toward many organs appear to be closely regulated such that the tissue receives an adequate supply of O_2 to satisfy the requirements of oxidative metabolism under a wide variety of circumstances. Arterial constriction and/or the decreased functional capillary density in conditions of increased pO_2 have been observed in several studies [22–26], where the vasoconstriction has been attributed to the lowered production of endothelium-derived prostacyclin. Recently, the cellular O_2 sensor was identified as a cytochrome P450 enzyme that catalyzes the arachidonic cascade to produce constrictor hydroxyarachidonic acid [27]. Thus, several blood flow regulatory mechanisms appear to prevent an overabundance of O_2 delivery to tissues.

Transfer of O_2 from acellular Hb or cellular HbV to the vascular wall is faster than from RBCs as shown by stopped flow in vitro analysis (k_{off}; Hb : 80 s^{-1}, HbV : 30 s^{-1}; RBC : 4 s^{-1}) [28]. This is due to morphological differences arising from the diffusion of the O_2 molecule form the viscous and concentrated Hb solution in RBCs and the plasma barrier present between RBCs and the vascular wall [29], a situation markedly different from the rapid release of O_2 from the homogeneous dispersion of Hb in plasma. Although HbVs have a cell-like structure, they release O_2 at a faster rate than RBCs due to their smaller size (250 nm) and consequent greater dispersion homogeneity. Although the faster release of O_2 could be advantageous, it is also possible that it may lead to the autoregulatory vasoconstriction [9,30,31].

Vasoconstriction has also been attributed to the O_2 binding-dissociation equilibrium of the O_2 dissociation curve of Hb rather than the rate of O_2 release. Transmembrane introduction into RBCs of a synthetic allosteric effector such as inositol hexaphosphate reduces the O_2 affinity of Hb, which increases arterio-venous O_2 saturation difference and increases the amount of O_2 released from RBCs. Reduction of cardiac output with maintenance of constant systemic pressure was found after infusion of an allosteric effector, or the introduction of blood with

right-shifted RBCs, indicating the increase of peripheral resistance by arterial vasoconstriction in response to overabundant O_2 supply [32–34]. The pressor reaction was not confirmed and not detrimental to tissue oxygen tension with the use of modified RBCs.

Our recent study of conscious hamster dorsal skinfold microcirculation during hemodilution with a low O_2 affinity HbV (p_{50} = 53 mmHg) showed that microvascular perfusion was reduced even though arterioles (diameter < 50 μm) slightly dilated. However, upstream small arteries (diameter, ca. 150 μm) constricted significantly. Comparing the shape of O_2 dissociation curve of HbVs with that of hamster RBCs, shows that the slopes at pO_2 = 60 mmHg is steeper for HbVs than for RBCs. Since this oxygen partial pressure was found in the same arteriolar vessels for both types of solutions, it is likely that HbVs release a larger amount of O_2 in these vessels than the RBCs, which may induce vasoconstriction. Conversely, higher O_2 affinity HbVs (p_{50} = 16 mmHg) had a tendency to cause higher microvascular perfusion.

These findings suggest an important concept in design of a red cell substitute: in normal conditions the O_2 dissociation curve of RBCs is regulated not to release a lot of O_2 before entering the microcirculation. Furthermore, infusion of red cell substitutes with a right shifted O_2 dissociation curve does not necessarily enhance microvascular perfusion. These considerations lead to the hypothesis that there may be an optimal oxygen affinity of red cell substitutes for each clinical setting.

Other mechanisms of vasoconstriction caused by red cell substitutes

Adrenoreceptors and endothelin

αα-crosslinked Hb (XLHb) has been reported to induce vasoconstriction and resulting hypertension by NO-trapping and also in part by adrenergic responses or endothelin production [35–37]. There is evidence that the pressor effect of XLHb originates in the peripheral vascular system and is not mediated by the central nervous system, since XLHb potentiates the pressor responses of norepinephrine, phenylephrine and clonidine, indicating increased sensitivity of peripheral vascular α-adrenoreceptors. The pressor effect of XLHb could be reversed by administration of prazosin and yohimbine, an α_1- and α_2-adrenoreceptor antagonists, respectively. Phosphoramidon, an inhibitor of proendothelin conversion to endothelin also attenuates the pressor effect of XLHb.

Although the location of these receptors and their mechanism of action is not well understood, resistance arteries are known to be rich in adrenoreceptors [38] which regulate the downstream microcirculation. Understanding of these mechanisms will provide the opportunity of controlling or taking advantage of the pressor effect.

Shear stress on the vascular wall and EDRF

It has been reported that increased blood flow acts as an important signal for the coordination of vasodilation along the arterial tree. The ability of small and large

vessels to dilate in response to elevated blood flow has been demonstrated [39]. This flow-induced dilation is endothelium-dependent and primarily mediated by nitric oxide, and in some cases by prostacyclin [40].

It has been proposed that the viscous drag exerted on the endothelial cells by the flowing blood, wall shear stress, triggers flow induced dilation. Wall shear stress is expressed as $8V_m\eta/D$, where, V_m is the mean red blood cell velocity, η is the viscosity, and D is the vessel diameter. Hemodilution with plasma expanders inevitably reduces blood viscosity leading to lowered shear stress. To maintain wall shear stress and diameter, viscosity should be increased. Studies from our and another groups tend to confirm the beneficial effect of perfusion with high molecular weight dextran solutions as a viscous plasma expander [41,42], therefore acellular Hb solutions, which have a lower viscosity than blood, may induce vasoconstriction due to lower shear stress (See the corresponding Chapter 10 in this text, [43].

Control of microvascular function by ATP release from RBCs

Human RBCs have been shown to release adenosine 5′-triphosphate (ATP) in response to the combined effect of hypoxia and hypercapnia. ATP is known to bind to receptors localized on the luminal surface of the endothelium in the peripheral circulation [44]. The binding of ATP to these receptors has been shown to induce the production of NO and prostacyclin, both of which are strong vasodilators of the microvasculature.

Ellsworth et al. [45] demonstrated that ATP production is enhanced at low pO_2 and pH, indicating that these factors associated with an impaired O_2 supply increases the release of ATP from RBCs. Intraluminal loading of ATP to small arteries with diameter 140–60 μm, namely resistance vessels, showed increased diameter and blood supply.

Most of the glycolytic enzymatic system is removed from Hb-related red cell substitutes during Hb extraction from RBCs and purification, preventing the production of ATP and vasodilatory activity. The process of the Terumo Co. (Tokyo, Japan) purifies Hb without removing the enzymatic systems [46], in such a fashion that the product maintains metHb reducing enzymatic activity in liposome-encapsulated Hb, which would facilitate the production of ATP and the maintenance of its vasidilatory activity.

Active oxygen species and vasoactivity

Endothelial cells contain xanthine dehydrogenase/oxidase leading to the production of active oxygen species [47], which are reduced by the cell's superoxide dismutase, catalase, etc. Low intracellular levels of active oxygen species stimulate cyclooxygenase. Superoxide radicals can induce vasodilation in several tissues which is in part mediated by the release of prostacyclin from endothelial cells. In normal condition, a balance exists between the production of prostaglandin (dilator), and thromboxane A_2 (constrictor), and between NO and active oxygen species that allows for the