人工血液

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ARTIFICIAL BLOOD

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PROGRAM

IX International Symposium on Blood Substitutes (9-ISBS)

Keio Plaza Inter-Continental Tokyo
3-5 March 2003

Foreward

Medical science has progressed remarkably in the last century. Transfusion medicine is one of the important therapeutic modality which changes the medical practice.However, we still know that transfusion is not perfect because of potentiality of infection, immunological adverse reaction, and storage limitation.
To cover the defect of transfusion system, research and development of artificial oxygen carrier has continued. Through the research, many findings in chemistry, biochemistry, physiology and pathology have been achieved. And several groups have reached to the point of phase III study.
Now we might say that we have come to the new era of transfusion medicine. To expand the role of artificial oxygen carrier in transfusion medicine, we need to combine our knowledge and wisdom.
On the other hand, through the research of red cell substitutes, a concept of oxygen therapeutics has emerged in these decades. This concept has a large possibility to change whole medical field because oxygen is a fundamental element in the body. In other words, if we can have a better understanding of gas biology such as oxygen in the cells, tissues, and organs, we will open a new perspective in medicine.

Meanwhile, research in blood components lead us to investigate not only red cell substitutes but also proteins and platelets. In this symposium, we also focused on these components. Development of artificial antibody and protein have been vigorously pursued in thise decade. Research into ways of substituting platelet function has recently sprouted, but is moving very rapidly. Studies in both of these areas will inevitably open new fields in medical research.
The Ministry of Health, Labour and Welfare, Japan. has supported the research and development of these artificial blood components. Without this research grant, progress of research in Japan would not have been achieved.
We strongly believe that many fruitful discussions and exchange of information will take place in this symposium, and that this will take us one step closer to clinical applications of these blood substitutes.

Koichi Kobayashi, M.D., Ph.D.
Chairman, 9th ISBS
Professor and Chief, Department of Surgery,
Keio University School of Medicine

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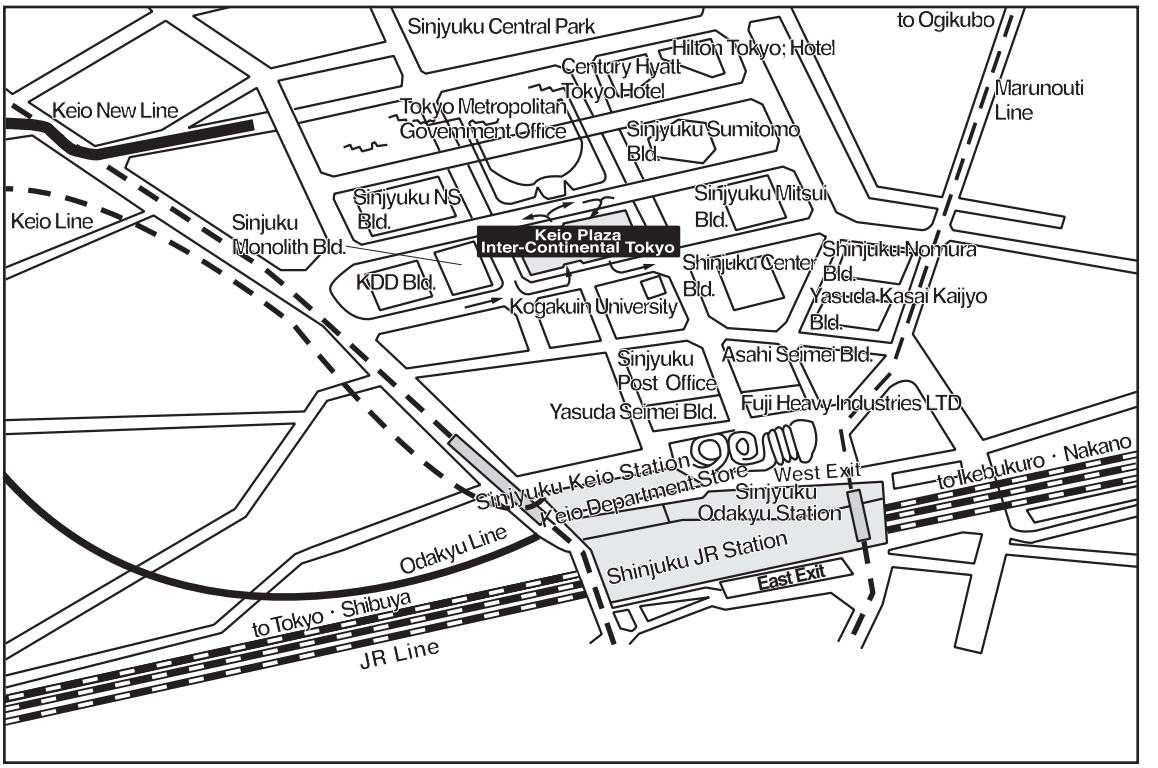

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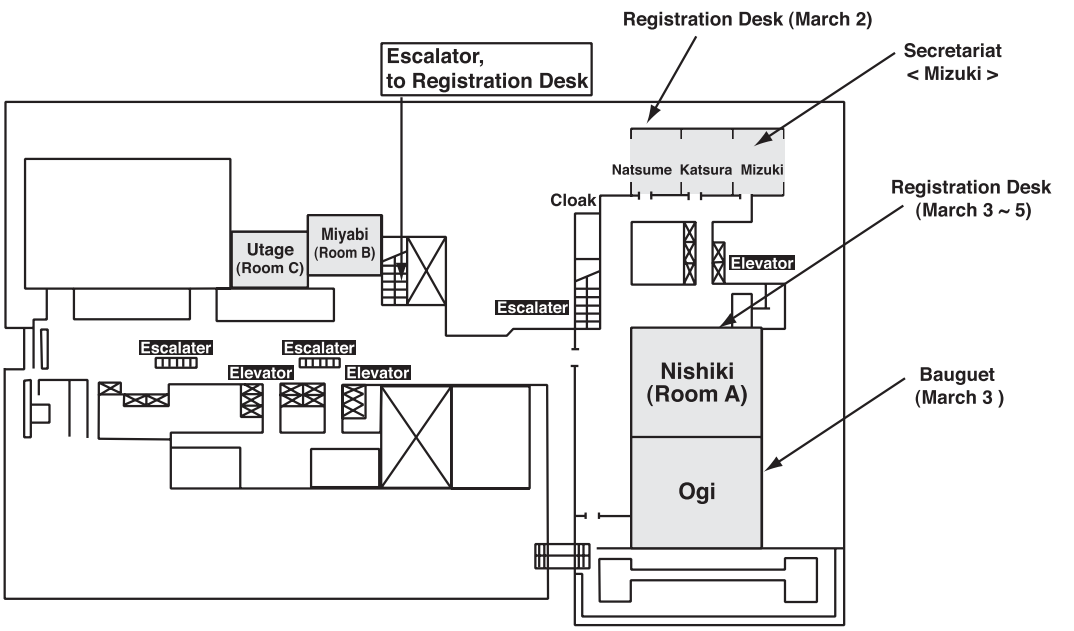

Date	Mar 2 (Sun)	Mar 3 (Mon)		Mar 4 (Tue)			Mar 5 (Wed)	
		Room A (Nishiki)	Room C (Utage)	Room A (Nishiki)	Room B (Miyabi)	Room C (Utage)	Room A (Nishiki)	Room C (Utage)
8:00		Registration						
9:00		Opening Remarks Comment Invited Lecture I		Workshop I	Forum I		SymposiumIII	ForumIII
10:00		Invited Lecture II		Coffee Break			Coffee Break	
11:00		Invited LectureIII Coffee Break President Lecture		Symposium II	Forum II		WorkshopIII	
12:00		Plenary Lecture I				Lunch and Poster II	Conclusion	
13:00			Lunch and Poster I	Plenary LectureIII				
14:00		Symposium I		Special Lecture Coffee Break Invited LectureIV Invited Lecture V Invited LectureVI				
15:00								
16:00								
17:00		Coffee Break Plenary Lecture II		Workshop II				
18:00	Registration and Welcome drink	Special Report						
19:00								
20:00		Banquet						
21:00								

Mar 3 (Mon)

8:50~9:00 ***Opening Remarks (Room A)***

K. Kobayashi *(Chairman of IX International Symposium on Blood Substitutes)*

9:00~9:10 ***Comment (Room A)***

A. Hashizume *(The Ministry of Health, Labor and Welfair, Japan)*
F. Yamada *(Japanese Red Cross Society)*

9:10~9:40 ***Invited Lecture I (Room A)***

Chairperson: M. Suematsu, *Keio Univ., Tokyo*

I-I Rejuvenation of Expired Blood for Blood Transfusion
T. Yonetani, *Pennsylvania Medical Center, Philadelphia*

9:40~10:10 ***Invited Lecture II (Room A)***

Chairperson: P. Keipert, *Alliance, San Diego*

I-II Some Fundamentals about Perfluorocarbons and Perfluorocarbon Emulsions
J. G. Riess, *UCSD, San Diego*

10:10~10:40 ***Invited Lecture III (Room A)***

Chairperson: J. Riess, *UCSD, San Diego*

I-III Clinical Application of Perfluorocarbons for Organ Preservation
S. Matsumoto, *Kyoto Univ., Kyoto*

10:40~11:00 ***Coffee Break (Room A)***

11:00~11:45 ***President Lecture (Room A)***

Chairperson: T.M.S. Chang, *McGill Univ., Montreal*

President Lecture Evaluation of Artificial Oxygen Carriers in vivo
K. Kobayashi, *Keio Univ., Tokyo*

11:45~12:30 ***Plenary Lecture I (Room A)***

Chairperson: E. Tsuchida, *Waseda Univ., Tokyo*

PL-I Expansion of Concept: From Red Blood Cell Substitutes to Oxygen Therapeutics
T.M.S. Chang, *McGill Univ., Montreal*

12:30~14:00 ***Lunch and Poster (Room C)***

14:00~16:30 ***Symposium I (Room A)***
Biocompatibility and Protocols in Oxygen Therapeutics

Chairperson: T. Agisi, *Itabashi Central Hospital, Tokyo*

S-I-1 Targeted O_2 Delivery by Low-p50 Hemoglobin: a New Basis for Hemoglobin-based Oxygen Carriers.
R. Winslow, *Sangart Inc., San Diego*

S-I-2 Hemopure®: Clinical Development and Experience
M. Gawryl, *Biopure Corp., Cambridge*

S-I-3 Clinical Development of a Perfluorochemical Emulsion (Oxygent™) as an Intravascular Oxygen Therapeutic
P. Keipert, *Alliance Pharm. Corp., San Diego*

S-I-4 Clinical Results of Perftoran Application: Present and Future
E I. Maevtsky, *Inst. Theore. Exp. Biophysics, Moscow*

S-I-5 HemoZyme® as Battlefield Hemorrhagic Trauma Protectant
C. Hsia, *Synzyme Technologics LLC, Irvine*

S-I-6 The Development and Preclinical Testing of a Novel Second Generation Recombinant Hemoglobin Solution
K. Burhop, *Baxter Healthcare Corp., Boulder*

16:30~16:50 ***Coffee Break (Room A)***

16:50~17:35 ***Plenary Lecture II (Room A)***

Chairperson: M. Shimizu, *Kyorin Univ., Tokyo*

PL-II Regulation of Blood Substitutes

T. Silverman, *FDA, Bethesda*

17:35~18:20 ***Special Report (Room A)***

Chairperson: H. Ikeda, *Hokaido Red Cross Blood Center*

Special Report Oxygen Therapeutic Hemopure in Breast Cancer Reconstructive Surgery

G. Edwards, *University of Witwatersrand, Johanesburg*

Mar 4 (Tue)

8:30~10:10 ***Workshop I (Room A)***
Current Progress in Research and Development of Artificial Oxygen Carrier

Chairperson: K. Burhop, *Baxter Healthcare Corp., Boulder*

WS-I-1 Hemoglobin Encapusulation - Influence of Preparation of Wall Materials
Zhiguo Su, *National Key Laboratory of Biochemical Engineering, Beijing*

WS-I-2 Kinetics of Ligand Binding to MP4, Polyethylene Glycol-conjugated Hemoglobin
K. Vandegriff, *Sangart Inc., San Diego*

WS-I-3 Serum Albumin Including Synthetic Hemes as an Oxygen-Carrying Hemoprotein
T. Komatsu, *Waseda Univ., Tokyo*

WS-I-4 Highly Stable, Low-Sized Fluorocarbon Emulsion with Fluorocarbon/Hydrocarbon Diblocks as Interfacial Film Reinforcers
M. P. Krafft, *CNRS, Strasbourg*

WS-I-5 Encapusulation Effects of Concentrated Hemoglobin with Phospholipid Membrane
S. Takeoka, *Waseda Univ., Tokyo*

10:10~10:20 ***Coffee Break (Room A)***

10:20~12:00 ***Symposium II (Room A)***
Development of Artifical Antibody

Chairperson: Y. Kurosawa, *Fujita Helth Univ., Nagoya*

S-II-1 Generation of Human Monoclonal Antibodies with a Human Myeloma Cell Line
A. Karpas, *Univ. Cambridge, Cambridge*

S-II-2 Isolation and Preparation of Therapeutic Antibodies with Neutralizing Activities against Viruses, Toxins Secreted by Bacteria and Snake Venom
Y. Kurosawa, *Fujita Health Univ., Nagoya*

S-II-3 Production of Human Monoclonal and Polyclonal Antibodies by Use of TransChromo Animals
I. Ishida, *Kirin Brewery, Tokyo*

S-II-4 Application of Baculovirus Membrane Display to Antibody Production
T. Hamakubo, *The Univ. of Tokyo, Tokyo*

12:00~13:15 ***Lunch and Poster (Room C)***

13:15~14:00 ***Plenary Lecture III (Room A)***

Chairperson: T. Yonetani, *Univ. Pennsylvania, Philadelphia*

PL-III Redox and Signaling Reactions of Hemoglobin: I) Redox Reactions of Hemoglobin
A. I. Alayash, *FDA, Bethesda*

14:00~14:40 ***Special Lecture (Room A)***

Chairperson: K. Kobayashi, *Keio Univ., Tokyo*

Special Lecture Low Concentrations of Nitric Oxide Increase Oxygen Affinity of Sickle Erythrocytes In Vitro and In Vivo
W. N. Zapol, *MGH, Harvard Univ., Boston*

14:40~14:55 ***Coffee Break (Room A)***

14:55~15:25 ***Invited Lecture IV (Room A)***

Chairperson: A. Alayash, *FDA, Bethesda*

IL-IV Modified Hemoglobins Increase Eosinophil Migration into Intestinal Villus Lamira Propria
A. Baldwin, *Univ. Arizona, Tucson*

15:25~15:55 ***Invited Lecture V (Room A)***

Chairperson: R. Winslow, *Sangart Inc., San Diego*

IL-V Approach to Clinical Trial of HbV (Liposome Encapsulated Hemoglobin Vesicle) Considering Medical Ethics and Study Efficacy
M. Takaori, *Higashitakarazuka Hosp., Takarazuka*

15:55~16:25 ***Invited Lecture VI (Room A)***

Chairperson: K. Nishi, *Kumamoro Univ., Kumamoto*

IL-VI Design and Evaluation of Oxygen Therapeutics
A. G. Greenburg, *Brown Univ., Providence*

16:25~17:45 ***Workshop II (Room A)***
Microcirculatory Events and Artifical Oxygen Carrier

Chairperson: M. Intaglietta, *UCSD, La Jolla*

WS-II-1 Effective Oxygen Transport in the Microcirculation after Exchange with Colloidal Plasma Expanders and Hemoglobin Based Blood Substitutes
M. Intagilietta, *UCSD, La Jolla*

WS-II-2 Roles of Platelet-Associated Adhesion Molecules in Regulation of Leukocyte Adhesion to Microvascular Endothelium
M. Suematsu, *Keio Univ., Tokyo*

WS-II-3 From Microcirculation to Metabolism: New Methods to Assess Tissue Ischemia
D. Erni, *Inselspital Univ. Hosp., Bern*

WS-II-4 Effect of Artificial Oxygen Carrier on Neutrophil Movement in the Microvasculature of Hamster Check Pouch
S. Hoka, *Kitasato Univ., Sagamihara*

WS-II-5 Studies on Safety of Hb-Vesicles in Brain
M. Okamoto, *Cornell Univ., New York*

Mar 5 (Wed)

8:30~10:10

Symposium III (Room A)
Artificial Platelet

Chairperson: Y. Ikeda, *Keio Univ., Tokyo*

S-III-1 Development of Platelet Substitutes: Scientific and Regulatory Considerations
J. Fratantoni, *Maxcyte Inc., Rockville*

S-III-2 A Novel Approach to Development of Artificial Platelets in Japan
Y. Ikeda, *Keio Univ., Tokyo*

S-III-3 Observation of Microscopic Motion of Artificial Platelets in the Model Arteriole
K. Tanishita, *Keio Univ., Yokohama*

S-III-4 In Vivo Evaluation of New Platelet Substitute Glycoprotein Ib-α Bound on Recombinant Albumin Polymer
Y. Hasegawa, *Univ. Tsukuba, Tsukuba*

10:10~10:20 ***Coffee Break (Room A)***

10:20~12:40

Workshop III (Room A)
Changes after Administration of Artificial Oxygen Carrier

Chairpersons: S. Yuasa, *Saitama Red Cross Blood Center, Saitama*
H. Miyao, *Saitama Medical Center, Saitama*

WS-III-1 Hemoglobin, Transcriptional Activator and Suppressor. How to Tip the Balance?
J. Simoni, *Texas Tech Univ., Lubbock*

WS-III-2 Hemodynamic and Metabolic Changes after Administration of PEG-Hb in Hemorrhagic Shock in Swine
B. Kjellstrom, *Kalorinska Inst., Stockholm*

WS-III-3 Nanometer-sized Oxygen Carrier Alleviates Myocardial Infarction in the Rat
A. T. Kawaguchi, *Tokai Univ., Isehara*

WS-III-4 Daily Repeated Infusion of Hb-Vesicles (HbV) into Wister Rats for Two Weeks: A Preliminary Safety Study

H. Sakai, *Waseda Univ., Tokyo*

WS-III-5 Blood Substitute Resuscitation as a Treatment Modality for Moderate Hypovolemia

A. T. W. Cheung, *UC Davis, Davis*

WS-III-6 Evaluation of Anionic Liposome-Encapsulated Hemoglobin in Rabbits

V. D. Awasthi, *Univ. Texas, San Antonio*

WS-III-7 Effect of Pegylated Hemoglobin on Microvascular Oxygen Transport and Function in Acute Anemia after Isovolemic Hemodilution

A. G. Tsai, *UCSD, La Jolla*

12:40~13:00 ***Conclusion (Room A)***

E. Tsuchida, *ARISE Waseda Univ., Tokyo*

Mar 4 (Tue)

8:30~10:00 ***Forum I (Room B)***

Chairperson: H. Sakai, *Waseda Univ., Tokyo*

F-I-1 Comparison of Platelet Substitutes Made of PolyAlb and Vesicles
S. Takeoka, *Waseda Univ., Tokyo*

F-I-2 Real-Time Visualization of Artificial Platelet Interacting with Immobilized Matrix and Native Platelet Bound on the Matrix Surface under Controlled Blood Flow Conditions
S. Goto, *Tokai Univ., Isehara*

F-I-3 High Level Expression of Recombinant Human Prethrombin-2 in Mammalian Cells
H. Yonemura, *The Chemo-Sero-Therapeutic Research Institute, Kumamoto*

F-I-4 Hemorrhagic Shock in Air Breathing Pigs Treated with Bubble-Forming Intravenous Dodecafluoropentane Emulsion
C. Lundgren, *CRESE, SUNY., Buffalo*

F-I-5 Hemoglobin Mediated Contraction of Isolated Blood Vessels: Why Is Precontraction Necessary?
H.W. Kim, *Brown University/The Miriam Hosp., Providence*

F-I-6 Research on the Purification of Hemoglobin for RBC substitute
Wang Hang, *CAMS, Chengdu*

10:20~12:20 ***Forum II (Room B)***

Chairperson: I. Sakuma, *Hokkaido Univ., Sapporo*
H. Sakai, *Waseda Univ., Tokyo*

F-II-1 Pharmacodynamic Study of Polyethylene Glycol Conjugated Bovine Hemoglobin (PEG-bHb) on Animals
Bi Zhigang, *Beijing Kaizheng Biotech Developing Ltd., Beijing*

F-II-2 Hemoglobin-vesicles as Oxygen Carriers: Influence on Phagocytic Activity and Histopathological Changes in Reticuloendothelial System

H. Horinouchi, *Keio Univ., Tokyo*

F-II-3 The Effect of Liposome-Encapsulated Hemoglobin on Tissue Oxygen Metabolism of Small Intestine Following Hemorrhagic Shock in Rats

K. Onodera, *Nippon Medical School, Tokyo*

F-II-4 Multiangle Laser Light-Scattering Method for the Study of Bovine Hemoglobin Dissociation

Wang Xiaoning, *Beijing Kaizheng Biotech Developing Ltd., Beijing*

F-II-5 Protective Effects of a Novel Perfluorocarbon Emulsion Administered during Cardiopulmonary Bypass

M. Isaka, *Hokkaido Univ., Sapporo*

F-II-6 Engineering Greater Temperature Dependance for O_2 Binding to Hemoglobins and Myoglobins

N. Matsuda, *Rice Univ., Houston*

F-II-7 Effect of NRC on the Oxygen Metabolism in Acute Massive Hemorrhaged Rats

T. Ishizuka, *Terumo Co., Hatano*

F-II-8 Increased Survival Time during Second Hemorrhage When First Hemorrhage is Resuscitated with Hemospan™ Compared to Blood or Colloid

D. Drobin, *Karolinska Inst. at Soder Hosp., Stockholm*

Mar 5 (Wed)

Forum III (Room C)

Chairperson: M. Takaori, *Higashi Takarazuka Satoh Hosp., Takarazuka*
H. Horinouchi, *Keio Univ., Tokyo*

8:30~10:00

F-III-1 Cardiac Output in Hamsters during Progressive Exchange Transfusion with Oxygen and Non-Oxygen Carrying Blood Replacement Fuluids
P. Cabreles, *UCSD, La Jolla*

F-III-2 Microvascular Flow and Tissue Oxygenation after Hemorrhage and Resuscitation with MalPEG-Hb and Polymerized Bovine Hemoglobin in Awake Hamsters
R. Wettstein, *UCSD, La Jolla*

F-III-3 Effect of the Synthetic Amino-lipids Formulating Hemoglobin-Vesicles(HbV) on the Circulation Level of the Blood Cells
K. Sou, *Waseda Univ., Tokyo*

F-III-4 $(PEG_{5k})_6$-Hb: A Non-Hypertensive Hemoglobin Molecule Generated by Conservative PEGylation
A. Acharya, *Albert Einstein College of Medicine, Bronx*

F-III-5 The Influence of Hemodilution and Oxygen Affinity of Hemoglobin Vesicles on the Oxygenation in Ischemic Hamster Flap Tissue
C. Contaldo, *Inselspital Univ. Hosp., Berne*

F-III-6 Ability of Polymorphonuclear Neutrophil to Respond to Infection in Presence of Cell-Free Hemoglobin. An in vitro Study
M. Toussaint-Hacquard, *Univ. Nancy, Nancy*

Poster

Mar 3 (Mon)

P-1 Combining Hemoglobin With Adenosine and Reduced Glutathione Attenuates Its Direct and Indirect Neurotoxic Potential
J. Simoni, *Texas Tech Univ., Lubbock*

P-2 Duration of Efficacy of NRC Administered in Divided Doses
Y. Tsutsui, *Terumo Corp., Tokyo*

P-3 The Oxygen Delivery of Artificial Oxygen Carrier with High Oxygen Affinity for Ischemic Tissues
T. Kimura, *Terumo Corp., Tokyo*

P-4 Pretreatment of Blood Serum Containing Hb-Vesicles for Accurate Clinical Laboratory Tests
H. Sakai, *Waseda Univ., Tokyo*

P-5 Reduction of MetHb via Electron Transfer from Photoreduced Flavin and Restoration of O_2-Binding Ability of Hb-Vesicies as an O_2 Carriers
H. Sakai, *Waseda Univ., Tokyo*

P-6 Compatibility of Albumin-Heme with Blood Cell Components
Y. Huang, *Waseda Univ., Tokyo*

P-7 Exchange Transfusion of Albumin-Heme as an Artifical Oxygen Carrier into Anesthetized Rats: Physiological Responses and Oxygen Delivery
H. Yamamoto, *NIPRO Corp., Kusatsu*

P-8 Human Serum Albumin Hybrids Including Iron Complex of Protoporphyrin IX with a Proximal Base and Their Dioxygenation
A. Nakagawa, *Waseda Univ., Tokyo*

P-9 Size-Exclusion High Performance Liquid Chromatography (SE-HPLC) with UV Absorbance, Light-Scattering and Refractive Index Detectors to Determine the Molecular Weight and Distribution of Molecular Weight of the PEGylated Bovine Hemoglobin (PEG-bHb)

Zhang Xiaowei, *Beijing Kaizheng Biotech Developing Ltd., Beijing*

P-10 Reduction of Methemoglobin Vesicles by Using Membrane Permeation of Reductants

T. Atoji, *Waseda Univ., Tokyo*

P-11 Increased Oncotic Pressure vs. Plasma Viscosity as Determinants of Functional Capillary Density Following Sequential Administration of Dextran 70 kDa and Hetastarch in Extreme Hemodilution

N. Hangai-Hoger, *UCSD, La Jolla*

P-12 Systemic and Microvascular Responses to Hemorrhagic Shock and Resuscitation with Hb-Vesicles

H. Sakai, *Waseda Univ., Tokyo*

P-13 Studies on the Immunogenicity of PEGylated Bovine Hemoglobin (PEG-bHb)

Bi Zhigang, *Beijing Kaizheng Biotech Developing Ltd., Beijing*

P-14 Evaluation of Secondly Hemostasis for Oligopeptide-Conjugated Latex Beads Enhanced Effect as Platelet Subsitutes

Y. Okamura, *Waseda Univ., Tokyo*

Mar 4 (Tue)

P-15 Inflammatory Reactions Induced by an Application of Perfluorooctylbromide Eulsion during Cardiopulmonary Bypass with Moderate Hemodilution

M. Isaka, *Hokkaido Univ., Sapporo*

P-16 Effect of Surfactant and Perfluorocarbon Type on Droplet Size Stability of Oxygen-Carrying Microemulsions

J. C. Briçenõ, *Univ. Los Ande., Bogota*

P-17 Resuscitation from Hemorrhagic Shock with Hemoglobin-Vesicles Suspended in Recombinant Human Serum Albumin

H. Sakai, *Waseda Univ., Tokyo*

P-18 Oxygen Release from Hb-Vesicles:Comparison with Red Blood Cells and Acellular Hemoglobin Solution Using an Artficial Oxygen Permeable Narrow Tube with 28 μm Inner Diameter

H. Sakai, *Waseda Univ., Tokyo*

P-19 Effects of Hemoglobin Vesicles on Blood Cells and Complement in Rat

H. Abe, *Hokkaido Red Cross B. C., Sapporo*

P-20 Prolongation of the Oxygen-Carrying Ability of Catalase-Encapsulated Hb-Vesicles in vivo

Y. Teramura, *Waseda Univ., Tokyo*

P-21 Detection of Lipopolysaccharide in Hemoglobin-Vesicles by Limulus Amebocyte Lysate Test with Pretreatment of Surfactant

S. Hisamoto, *Waseda Univ., Tokyo*

P-22 Pharmacokinetics of the Hemoglobin-Vesicles (HbV) in Rats

K. Sou, *Waseda Univ., Tokyo*

P-23 40% Blood Exchange-Transfusion with Hb-vesicles in Rats and Observation of Hematological and Serum Clinical Laboratory Tests for 2 weeks

M. Yamamoto, *Keio Univ., Tokyo*

P-24 Tumor Oxygenation Using the Hemoprotein (rHSA-FeP) and the Hemoglobin - Vesicle (HbV) in a Rat LY80 Tumor Model

A. Iwamaru, *Keio Univ., Tokyo*

P-25 Nano-size Oxygen Transporter Alleviates Cerebral Infarction in the Rat

A. Kawaguch, *Tokai Univ., Isehara*

P-26 NRC as a Blood Substitute: Duration of Efficacy in Various Species

S. Kaneda, *Terumo Corp., Tokyo*

P-27 Effect of Pegylation on Stability, Toxicity, and Pharmacokinetics of Perfluorocarbon Emulsion as Blood Substitutes

S. Fukushima, *Kobegakuin Univ., Kobe*

Secretariat

The secretariat will be open in room *Mizuki* on the 4th floor of Keio Plaza Inter-Continental Tokyo.

March 2 18:00 - 20:00 (Welcome drink is served at registration room)
March 3-4 8:00 - 18:00
March 5 8:00 - 13:00
Tel: 03-3344-0111 Fax: 03-3345-8269 (during symposium)

Registration and General Information Desk

The registration and general information desk will be located in the robby on the 4th floor of Keio Plaza Inter-Continental Tokyo.(Only March 2, the desk located in the room *Natsume* on the 4th floor of Keio Plaza Inter-Continental Tokyo.)

The desk hours will be as follows:

March 2 18:00 - 20:00 (Welcome drink is seeved at registration room)
March 3-4 8:00 - 17:00
March 5 8:00 - 10:00

Official Language

The official language of the meeting is English. No simultaneous translation will be provided.

Name Badges

Your name badge is your entry to all social and scientific functions. Please wear your name badge at all times.

Certificate of Attendance

A certification of attendance for preregistered participants will be included in the envelope with your name badge at the registration desk.

Proceedings

Please check a homepage ('Proceedings Submission')

URL http://www2.convention.co.jp/9thisbs

Messages

A message board will be located near the registration desk. Messages will be displayed on this board only.

Travel Desk

Hotel provides you tourism information.

Lunch

Lunch and Poster session will provide lunch boxes, however, please note that the prepared number of lunch boxes is limited. There are several restaurants in the Hotel. Please ask the hotel concierge for their recommendations or stop by the general information desk to see the guides there.

Banquet

Banquet will be held on March 4, at room *Ogi*. This will be an excellent time to enjoy this congress.

Date: March 3

Time: 19:00-21:00

Location: *Ogi*, 4th floor, Keio Plaza Inter-Continental Tokyo

Secretariat Before Symposium

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Oral Presentation

Please be your session room at least 30 minutes prior to the time set for presentation.
Time Allocated for Presentation (including discussion)

Plenary Lecture	45 min
Invited Lecture	30 min
Symposium	25 min
Workshop	20 min
Forum	15 min

—Audio-visual Materials—

Only digital presentation will be available. You are able to bring your own notebook computer or CD-ROM for your presentation. The computer must be a DOS/V machine running Windows 98 or later or an Apple Power Macintosh.
You should have your data backed up on CD-ROM in case of computer trouble. The version for backup should be anything later than PowerPoint 2000/XP for Windows and Power Point 2001 for Macintosh. Also the OS should not be anything later than Windows and 2000/ME/98SE for Windows and OS9.x for Macintosh.

Poster Presentation

You are requested to contact the poster reception desk in front of the your session room before mounting your poster to receive thumbtacks. Please mount your poster on the panel assigned by the Organizing Committee. The poster size will be 120cm wide by 160cm high. You are expected to be present at your poster display during the poster session hour for discussion. The Presentation style is free discussion.

Schedule

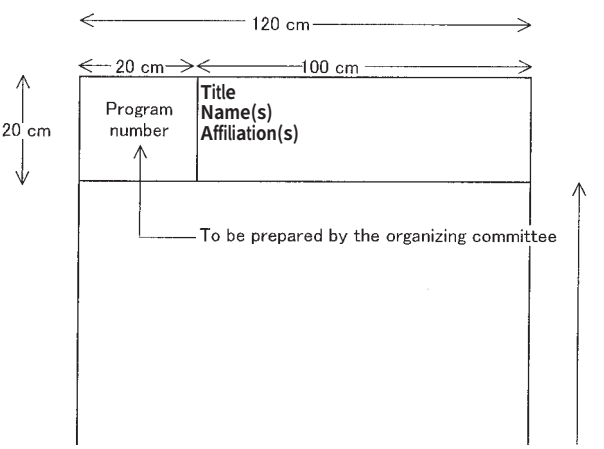
【Poster No. 1～14】
March 3

Mounting Time:	8:00-10:00
Session Time:	12:30-14:00
Removal Time:	17:00-19:00

【Poster No. 15～27】
March 4

Mounting Time:	8:00-10:00
Session Time:	12:00-13:15
Removal Time:	17:00-19:00

(poster sample)



Chairpersons Instruction
Please be in your session room at least 10 minutes prior to the start of session.
All Chairpersons are requested to strictly observe the time schedule.

ABSTRACTS

IX International Symposium on Blood Substitutes (9-ISBS)

Keio Plaza Inter-Continental Tokyo
3-5 March 2003

PL— 1

Expansion of Concept: From Red Blood Cell Substitutes to Oxygen Therapeutics

Thomas Ming Swi Chang, OC, MD, CM, PhD, FRCPC

Director, Artificial Cells & Organs Research Centre
Director, MSSS-FRSQ Research Group on Blood Substitutes in Transfusion Medicine
Professor of Physiology, Medicine and Biomedical Engineering
Faculty of Medicine, McGill University, Montreal, Quebec, Canada

Transfusion using whole blood is no longer a common practice. It is now used mostly in the form of its different cellular components and plasma protein components. In the same way, it was thought that red blood cell substitutes must have the same safety and efficacy as red blood cells before they can be used in routine clinical medicine. Research and clinical trials now also show that it may be more useful to fractionate the various functions of red blood cells (oxygen carrier, antioxidant activity, carrier of SNO and nitric oxide, prevention of metHb formation and others) into specific oxygen therapeutics. The oxygen carrying function by itself can have useful therapeutic functions in those conditions that only required oxygen carriers. For example, polyHb, conjugated Hb and PFC now in clinical trials have adequately fulfilled the function of oxygen carrier in elective surgery. The comparatively short circulation time and the exposure to reducing agents in the blood mean that there is no problem with metHb formation in polyHb and conjugated Hb. In well control elective surgery there is no need for antioxidants to make the oxygen carrier more complicated. However, in other conditions with potentials for ischemia-reperfusion injuries, one would need oxygen therapeutics with oxygen carrying capacity plus antioxidant properties. Examples include PolyHb-SOD-CAT, Hb with chemical antioxidants and others. In other cases where longer circulation time is needed, Hb lipid vesicles and Hb biodegradable polymeric nanocapsules are being developed. These later examples are oxygen therapeutics closer to red blood cell. One can also use oxygen therapeutics for those conditions that are not suitable for red blood cells. Thus, in ischemia due to stroke or other causes, water soluble oxygen carriers like polyHb can more easily transverse the obstructed vessels to supply oxygen. Usually, this oxygen therapeutic would need an antioxidant component. In regard to the antioxidant component, the concentrations of SOD and CAT can be made much higher than those in the red blood cells resulting in even better antioxidant activity than RBC. In radiotherapy for tumor, modified Hbs have been used to increase the oxygen supply to the less perfused tumor. A further extension is our recent use of PolyHb-tyrosinase to both increase oxygen supply for radiotherapy and remove tyrosine needed for the growth of a skin cancer, melanoma. Our recent studies on the use of PolyHb-SOD-CAT in global cerebral ischemia; biodegradable polymeric Hb nanocapsules and the new PolyHb-tyrosinase oxygen therapeutics will be discussed in more details (www.artcell.mcgill.ca).

PL—II

Regulation of Blood Substitutes

Toby A. Silverman, M.D.

Clinical Review Branch, Office of Blood Research and Review, CBRR, FDA, Rockville, Maryland, USA

The development of oxygen therapeutics for a variety of clinical applications has progressed rapidly in the past few years. The potential benefits are many and include universal compatibility, immediate availability, and long term storage. Use of oxygen therapeutics as substitutes for red blood cells has been the predominant focus of recent clinical trials, both in elective surgery and in trauma. Other uses for oxygen therapeutics have been considered, but are not yet the subject of clinical development. A number of new and as yet unresolved problems have been found during preclinical and clinical development. Although these toxicities are the subject of active research, the mechanisms of the various toxicities have not been fully elucidated. The impact of these findings on clinical trial design, and general considerations of efficacy and safety evaluation in clinical trials will be discussed. An overview of pertinent United States regulations will be provided.

PL—III

Redox and Signaling Reactions of Hemoglobin: I) Redox Reactions of Hemoglobin

Abdu I. Alayash, Ph.D.

Division of Hematology, CBER, FDA, Rockville, Maryland, USA

Uncontrolled heme-mediated oxidative reactions of cell-free hemoglobin (Hb) and its reaction with various oxidant and antioxidant systems have emerged as an important pathway of toxicity. Oxidative processes, which are either enhanced or suppressed when chemical and/or genetic modifications are introduced that lower oxygen affinity, can impact the safety of these proteins. Direct cytotoxic effects associated with Hbs have been ascribed to redox reactions between Hb and biological oxidants. Biochemical changes at the cellular, tissue, and organ levels have been documented to occur in response to Hb oxidative reactions. Protective strategies designed to overcome Hb's "radical" nature must take into account the type of chemistry introduced to stabilize and functionally modify the protein in order to limit its vulnerability to undergo oxidative self-destructive side reactions.

Special Lecture

Low Concentrations of Nitric Oxide Increase Oxygen Affinity of Sickle Erythrocytes In Vitro and In Vivo

C. Alvin Head, M.D*., Warren M. Zapol, M.D.**

*Current address:*Department of Perioperative Medicine, Medical College of Georgia, Augusta, Georgia, USA*

***Department of Anesthesia and Critical Care,Massachusetts General Hospital, Boston, Massachusetts, USA*

The hallmark of sickle cell disease (SCD) is the polymerization of deoxygenated sickle hemoglobin (HbS). In SCD patients, one strategy to reduce red blood cell (RBC) sickling is to increase HbS oxygen affinity. Our objective was to determine if low concentrations of nitric oxide (NO) gas would augment the oxygen affinity of RBCs containing homozygous HbS (SS). Blood containing normal adult hemoglobin (AA) or SS RBCs was incubated in vitro in the presence of varying concentrations of NO up to 80 ppm, and oxygen dissociation curves (ODCs) were measured. In addition, blood was obtained from three AA and nine SS volunteers, before and after breathing 80 ppm NO in air for 45 min, and the ODCs were measured. Exposure of SS RBCs to 80 ppm NO in vitro for 5 min or longer decreased the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen (P_{50}), and average of 15% (4.8 $\pm$1.7 mmHg mean $\pm$ SE; $P < 0.001$). The increase in SS RBC oxygen affinity correlated with the NO concentration. The P_{50} of AA RBCs was unchanged ($P > 0.1$) by 80 ppm NO. In SS volunteers breathing 80 ppm NO for 45 min, the P_{50} decreased ($P < 0.001$) by 4.6 (2.0 mmHg. 60 min after NO breathing was discontinued, the RBC P_{50} remained decreased in five of seven volunteers in whom the ODC was measured. There was no RBC P50 change ($P > 0.1$) in AA volunteers breathing NO. Methemoglobin (Mhb) remained low in all subjects breathing NO (SS Mhb 1.4 $\pm$ 0.5%), and there was no correlation ($r = 0.02$) between the reduction in P_{50} and the change in Mhb. Thus, low concentrations of NO augment the oxygen affinity of sickle erythrocytes in vitro and in vivo without significant Mhb production. These results suggest that low concentration of NO gas may offer an attractive new therapeutic model for the treatment of SCD.

President Lecture

Evaluation of Artificial Oxygen Carriers in vivo -History and Perspective of Keio-Waseda Joint Project of Oxygen Infusions-

Koichi Kobayashi, M.D., Ph.D.

Department of Surgery, Keio University, School of Medicine, Tokyo, Japan

Keio-Waseda Joint Project of 'Oxygen Infusions' has started since 1982 when Professor E. Tsuchida had developed totally synthetic 'liposome-heme'. We have been evaluating several types of artificial oxygen carriers since then. Ministry of Health, Labor, and Welfare, Japan has supported us for 6 years since 1997, making good progress in this field. In summary, three materials are vigorously investigated.

Lipidheme-vesicle is a phsopholipid liposome embedding anphiphilic heme (lipidheme). We evaluated its oxygen transporting capability using (1) peritoneal ravage models and (2) exchange transfusion models. Through these studies, we had proved its capability and efficacy as totally synthetic oxygen carrier. Based on these findings, we further developed lipidheme- microsphere and proved its characteristics. They both can be used as resuscitation fluids in a shock state.

Hb-vesicle encapsulates a purified and conc. human Hb solution with a lipid bilayer membrane. We have investigated this material for over 15 years and many improvements were made to refine the characteristics more biocompatible. For example, establishment of purification process of Hb, and molecular assembling technique to form vesicles, and surface modification with PEG. We have evaluated oxygen carrying efficacy and capability both in vitro and in vivo, metabolism of Hb and lipid that constitute Hb-vesicle, pathological change after daily repeated infusion, immunological reaction, microvascular responses in liver, lung, and skeletal muscle, and long term survival test. We have a solid feeling that Hb-vesicle is one of the potential materials which we can proceed to a clinical trial.

Albumin-heme is recombinant human serum albumin incorporating hemes as a totally synthetic oxygen-carrying hemoprotein. From the result of in vivo examinations, we learned that it becomes a new class of acellular oxygen carriers without any vasoconstriction and hypertensive action. We have found that it can be used as a resuscitation fluid in a shock state and has a potential to oxygenate the hypoxia regions in solid tumor. From the footsteps we have traced, we think there are potential role of artificial oxygen carrier in not only in transfusion medicine but also in the broader medical therapeutics. We have to work further to expand the potency of these materials and by doing so we are convinced that we can contribute to the progress of research on artificial oxygen carriers.

Special Report

Oxygen Therapeutic Hemopure in Breast Cancer Reconstructive Surgery

Edwards G, Levien L, Benn C

Department of Surgery, University of Witwatersrand, Johanesburg, South Africa

INTRUDUCTION

HBOC-201 (Hemopure) is a cell-free polymerized haemoglobin solution that not only carries oxygen in the plasma, but also enhances the ability of native red blood cells to take up and off-load oxygen. It is manufactured from bovine haemoglobin by an extraction and purification process. This process has been validated for the removal of potential contaminants including plasma proteins, red blood cell stroma, endotoxin bacteria, viruses and the agents that are thought to cause transmissible spongiform encephalopathies (BSE). Hemopure is a sterile, pyrogen-free balanced salt solution containing glutaraldehyde cross-linked bovine haemoglobin polymers, which range in size from 130 to 500kD and have an average molecular weight of 250kD. The product's oxygen dissociation curve is right-shifted with a P50 of 38mmHg for human haemoglobin. In contrast to human haemoglobin whose oxygen affinity relies on adequate levels of 2,3-diphosphoglycerate, the affinity of bovine haemoglobin for oxygen is regulated by the concentration of chloride ions in the plasma. It has a dose dependent intravascular half-life of sixteen to twenty-four hours. When stored within a temperature range of 2 degrees to 30 degrees, it is stable for at least two years, can be infused directly without reconstitution, and does not require typing or cross matching. Studies in an artificial capillary model suggest that the free form in plasma facilitates the diffusion of oxygen into the pulmonary capillary blood more rapidly and more efficiently than normal diffusion across the alveolar-capillary barrier and the red blood cell membrane.

Long-term studies have demonstrated that transfusions of human blood products in breast and colon cancer patients have been implicated in diminished survival and disease free interval. An alternative to blood transfusion, which has no immune modulation effects, is conceptually attractive in the setting.

AIM:

To attempt to avoid red cell transfusions in patients undergoing mastectomy and immediate autologous tissue reconstruction with the secondary objective being to evaluate flap behaviour in the presence of improved tissue oxygenation despite a significant surgical anaemia.

MATERIAL AND METHODS:

20 Women undergoing mastectomy and immediate autologous tissue reconstruction of the breast received from one to four units (30g per unit) of Hemopure, avoiding any transfusion of human blood products at all.

Data on haemodynamics was collected before, during, and after infusion. Data of clinical outcome in terms of wound healing and recovery of haemoglobin levels was also collected.

RESULTS:

Hemopure administration resulted in prompt and effective relief of symptoms of anaemia after administration of half to one unit of product. Haemoglobin levels returned to normal within four weeks of surgery. All flaps and reconstructions healed primarily without complications.

CONCLUSION:

Administration of Hemopure permitted major cancer surgery to be undertaken safely without resorting to conventional red blood cell transfusion and any immune modulation, with could be expected from human blood transfusion. The absence of wound and flap complications despite significant declines in haemoglobin levels strongly support the concept of improved tissue oxygenation with the use of polymerized cell free haemoglobin solution.

IL– 1

Rejuvenation of Expired Blood for Blood Transfusion

Takashi Yonetani and Antonio Tsuneshige

Department of Biochemistry and Biophysics and the Johnson Research Foundation, University of Pennsylvania Medical center, PA, USA

The blood specimens collected for blood transfusion by Red Cross are stored at blood banks for limited periods as either “the whole blood” or “the packed red blood cells” after separation of serum plasma from the whole blood. After the expiration of the specified storage periods, large fractions of them, as much as 20% of the stored blood specimens, are discarded. The serum fraction of the whole blood can be used to extract useful components such as blood clotting factors and serum albumin. However, the red blood cell fraction is an industrial waste that can pollute the environment, though small portions of it have been used to extract hemin (iron protoporphyrin IX) for industrial uses.

During the blood storage, 2,3-diphosphoglycerate, an allosteric effector of hemoglobin in red blood cells, is gradually depleted and the oxygen affinity of intra-erythrocyte hemoglobin increases, rendering such stored red blood cells unsuitable for blood transfusion. We have recently developed a simple method (1,2,3) reducing the oxygen affinity of intra-erythrocyte hemoglobin. This is accomplished by treating the expired red blood cells with limited quantities of nitric oxide as a heme-specific allosteric effector (4) to convert the expired red blood cells having high oxygen affinity to those having low oxygen affinity. This method allows rejuvenating the expired blood for blood transfusion (1,2,3).

Our previous studies on the α-nitrosyl derivative of human adult hemoglobin, tetrameric hemoglobin in which only two α-subunits are complexed with nitric oxide (4) have shown that the α-nitrosyl hemoglobin is a cooperative, low-affinity oxygen-carrier. Our method allows converting all the intra-erythrocyte hemoglobin into α-nitrosyl hemoglobin (a-NO RBC), by incorporating nitric oxide into intact human erythrocytes to a 50% saturation of hemes and exclusively bound to α-subunits. Oxygen equilibrium measurements show that α-NO RBCs also show reduced oxygen affinity (P_{50}) and diminished Bohr effect (i.e., the pH-dependence of P_{50}). Although its oxygen-carrying capacity is reduced by 50%, because two α-subunits are already complexed with nitric oxide, and only two β-subunits are capable of oxygen binding, α-NO RBCs can efficiently deliver oxygen to tissues under normal physiological conditions with no apparent adverse effect during blood transfusion.

(1) Yonetani, T. and Tsuneshige, A. (2000) US Patent #6,087,087 (07/11/2000), (2) Tsunesgige, A. and Yonetani, T. (2001) Art. Cells, Blood Subs. And Immob. Biotech. 29, 347-357, (3) Tsuneshige, A. and Yonetani, T. (2002) Adv. Expl. Med. Biol. (eds S. Evans, J. Biaglow, and D. F. Wilson), Klewer Press, pp. 1-6, (4) Yonetani et al., (1998) J. Biol. Chem. 273, 20323-20333

IL–II Some Fundamentals about Perfluorocarbons and Perfluorocarbon Emulsions

Jean G. Riess

University of California at San Diego and Alliance Pharmaceutical Corp., San Diego, USA

This presentation is intended to provide basic understanding about the properties of perfluorocarbons (PFC) and their emulsions, and their relevance to in vivo oxygen delivery. Exceptionally strong intramolecular binding and weak intermolecular cohesiveness of liquid PFCs result in a unique combination of high O_2 and CO_2 dissolving capacities (on the order of 50 and 200 vol.%, respectively) and extreme chemical and biological inertness. Uniquely also, PFCs are both hydrophobic and lipophobic. They are not metabolized and are excreted with the exhaled air. The challenge was to identify a PFC that had a relatively low vapor pressure, yet was readily excreted, was capable of producing stable emulsions and was easy to manufacture. Perfluorooctyl bromide, a slightly lipophilic PFC, obeys these conditions.

Intravascular use supposes the preparation of a stable, sterile, ready-for-use submicron-size PFC-in-water emulsion. Phospholipids are privileged as emulsifiers as they effectively reduce the PFC/water interfacial tension and have a long history of use in pharmaceuticals. Particle growth in such emulsions is driven by molecular diffusion (Ostwald ripening). It can be slowed down by addition of a higher molecular weight PFC such as perfluorodecyl bromide. Other emulsion stabilization principles rely on the use of fluorocarbon-hydrocarbon diblocks that modify the interfacial film. Heat sterilized PFC emulsions ca. 0.15 (m in size can be prepared that are stable for over 2 years.

In vivo oxygen delivery by PFC emulsions has been established through preclinical experimentation and human clinical trials. Gaseous PFC-based microbubbles have been licensed as contrast agent for diagnostic ultrasound imaging.

J.G. Riess, Chem. Rev. 101, 2797-2919 (2001).

IL–III

Clinical Application of Perfluorocarbons for Organ Preservation

Shinichi Matsumoto MD PhD

Kyoto University Hospital Transplantation Unit, Kyoto, Japan

Perfluorocarbons (PFC), which store and release high levels of oxygen, have been examined as oxygen carriers. PFC was first used for organ preservation as a component of the two-layer method (TLM) (University of Wisconsin [UW] solution-PFC plus oxygen) of pancreas preservation. Pancreata preserved in the TLM are oxygenated through the PFC and substrates are supplied by the UW solution. This allows pancreata stored in the TLM to generate adenosine triphosphate during storage and prolong the preservation period. In the canine model, the TLM has been shown to repair warm ischemically injured pancreata during preservation, improve pancreas graft survival after transplantation, and improve islet yields after isolation.

Currently the TLM was used for pancreas preservation before clinical whole pancreas transplantation. In this first clinical trial of 10 cases, the TLM had no adverse effect on the recipient patients after transplantation. Furthermore, the morphologic quality of the human pancreas grafts after reperfusion was excellent compared with the pancreas stored in UW solution. In addition, there was no acute rejection episode of pancreata preserved by the TLM. Very recently the TLM was clinically used before whole pancreas transplantation in Japan for the first time with a promising result.

The TLM resuscitated cold ischemic injury caused by UW cold storage and resulted in increasing human islet yields and success rate of transplantation. Furthermore, human pancreas preservation by the TLM without UW cold storage resulted in the best isolation results and improved success rate of islet transplantation. In addition, the TLM enabled to use aged donor pancreas for islet transplantation.

Thus preservation of human pancreata by the TLM has become an important process for successful islet transplantation. Clarification of the mechanisms and optimization of the TLM before human islet isolation would further contribute to the islet transplantation.

On the other hand, the TLM can also prolong preservation of heart in rodent model and small bowel in rodent and canine models. There organs are notoriously difficult to be preserved using standard UW solution and PFC would play important roles to overcome the limitation of preservation time.

IL—IV Modified Hemoglobins Increase Eosinophil Migration into Intestinal Villus Lamina Propria

Ann L. Baldwin and J. Edward Valeski

University of Arizona, Tucson, Arizona, U.S.A.

Diaspirin cross-linked hemoglobin (DBBF-Hb) and polyethylene glycol-conjugated hemoglobin (PEG-Hb) have both been considered as potential "blood substitutes". However, previous studies have shown that a 5 ml bolus injection of either substance (10 mg/ml) into the circulation of anesthetized Sprague-Dawley rats produces intestinal epithelial disruption and mast cell degranulation within 15 min. Eosinophils were observed in electron micrographs of these tissues. Since eosinophils play an important role in the pathogenesis of inflammatory diseases, toluidine blue-stained, plastic embedded sections of the above intestinal tissue, that had previously been immersed in diaminobenzoate to stain for eosinophilic granules, were used to count the number of eosinophils per villus by means of light microscopy. Tissue from control animals (injected with buffered saline containing 2% bovine serum albumin), showed 4.0 $\pm$ 0.2 (SEM) (n=154 villi) eosinophils/villus. Tissues from animals injected with DBBF-Hb or PEG-Hb, showed 9.6 $\pm$ 0.5 (n=84) and 8.1 $\pm$ 0.4 (n=78) eosinophils/villus, respectively. Corresponding values for numbers of degranulated mast cells per villus were 0.7 $\pm$ 0.1, 1.4 $\pm$ 0.2 and 2.1 $\pm$ 0.2. In both cases these values were all significantly from each other ($p<0.05$). These results support the hypothesis that mucosal mast cell degranulation, induced by some modified hemoglobins, causes recruitment and activation of eosinophils that then augment and sustain gastrointestinal responses through the release of pro-inflammatory mediators. Supported by NIH HL53047.

IL－V

Approach to Clinical Trial of HbV (Liposome Encapsulated Hemoglobin Vesicle) Considering Medical Ethics and Study Efficacy

Masuhiko Takaori, M.D.

Horonable Superintendent of East Takarazuka Sato Hospital, Takarazuka, Japan

Recently developed artificial blood (HbV), liposome encapsulated hemoglobin vesicles suspended in the physiological saline, has been tested for its efficacy and safety in a number of animal experiments. The HbV seemed considerably acceptable as artificial blood and to be faced on clinical trials in the near future. The clinical study, however, must be regulated by human ethics and its design should be composed adequately to obtain scientific evidence. Three plans for phase Ⅱ study were designed, namely (1) autologous blood transfusion with normovolemic hemodilution with the HbV, (2) substitution of homologous blood transfusion for patients with uncommon blood type and (3) for substitution of homologous blood transfusion for patients with unexpected massive hemorrhage during surgery. In these studies, commercially available plasma substitute should be used compensating absence of colloid osmotic pressure in the HbV. As control for the HbV, the plasma substitute alone was used associated with infusion of the saline of same volume of HbV used. Those study are designed focusing on oxygen metabolism in tissue and hemodynamic stability with the HbV infusion compared with the plasma substitute. Among those plans, the autologous blood transfusion with normovolemic hemodilution with the HbV is proposed to be most preferable and practical and seems consistent to obtain significant results evaluating the efficacy of the HbV.

IL–VI

Design and Evaluation of Oxygen Therapeutics

A. Gerson Greenburg, MD PhD

Brown Univ.,School of Medicine, Providence, RI, USA

The evolution in nomenclature from blood substitutes to oxygen therapeutics for the class of agents that deliver oxygen reflects well the intended use of these solutions. Humans are obligate aerobic organisms; perfusion of tissues with oxygen is essential for survival. In clinical situations associated with decreased tissue perfusion improved delivery of oxygen to tissues is perceived as a need. Blood is a complex solution with at least five major functions including oxygen delivery, intra-vascular volume, oncotic pressure, and immune and coagulation functions. Replacing the totality of blood function would be an engineering challenge and designing a solution for that end would be difficult. Thus, in the “early years”, replacing the oxygen delivery aspect of blood was the goal.

How best to accomplish that goal became the question. Identification and solution of problems, e.g. coagulopathy, oxygen off-loading, persistence so that development could move forward was required. Chemical modification of hemoglobin to improve the P50 and persistence was an initial objective. Modification is predicated on the availability of “pure” hemoglobin solutions thus forcing an exploration of purification techniques. Options for hemoglobin modification that produce a stable, reproducible molecule using intra and intermolecular cross-linking techniques were evaluated; eventually a polymerized molecule evolved.

With a molecule of choice in hand, a multifaceted approach directed at scale-up production and pre-clinical testing was undertaken. Pre-clinical models are needed; they do not necessarily predict all clinical situation observations. Early clinical models test for safety and efficacy and require the use of concurrent reference controls. If a solution has multiple functional facets then controls addressing each aspect are necessary and the experimental complexity grows exponentially. The design of clinical trials, the pivotal studies on which licensure is granted, requires defined endpoints reflecting efficacy with an acceptable safety profile. Efficacy endpoints and the key elements of what constitutes safety are issues open for discussion.

Shepherding the concept of chemical modification of hemoglobin from the basic chemistry processes and laboratory evaluation into early clinical trials and onto full Phase III clinical evaluation affords a unique perspective on the field. Given our greater appreciation of the role of oxygen in tissue function, repair and recovery, these oxygen therapeutic solutions with unique properties, in part due to their acellularity, are on the cutting edge of modern therapeutics. They will garner greater attention as we appreciate more and more the importance of tissue perfusion in maintaining overall homeostasis.

S—I—1

Targeted O_2 delivery by Low-p50 Hemoglobin: A New Basis For Hemoglobin-Based Oxygen Carriers.

[1,2]Robert M. Winslow, [1]Amy G. Tsai, [2]Kim D. Vandegriff, and [1]Marcos Intaglietta

[1]Department of Bioengineering, University of California, San Diego, and [2]Sangart, Inc., San Diego, CA, USA

We measured O_2 delivery to capillaries of the hamster skinfold, comparing a new modified hemoglobin (MP4, P50 5.4 mmHg) to a first-generation polymerized bovine hemoglobin (PolyBvHb, P50 54.2 mmHg). Microscopic measurements of PO_2 and the oxygen equilibrium curves permitted analysis of O_2 delivery to capillaries by red cells and plasma hemoglobin separately. The total arteriolar-venular O_2 difference, $C(a\text{-}v)O_2$, was higher for MP4 animals (4.15 ml/dL) compared to the PolyBvHb animals (2.30 ml/dL), and arterial base excess was directly proportional to the $C(a\text{-}v)O_2$. Higher red cell capillary oxygen extraction in the MP4 animals (63%) compared to PolyBvHb (24%) animals represents a new mechanism of action of cell-free hemoglobin: plasma MP4 targets O_2 delivery from both red cells and MP4 to capillary networks. High oxygen affinity serves to preserve total O_2 as blood approaches capillaries, but permits release in low tissue PO_2 environments.

Disclaimers

Drs. Winslow and Vandegriff are employees of Sangart, Inc. Dr. Vandegriff holds stock options in the company. Dr. Winslow, is President, CEO and Board Chairman of Sangart, and is its principal shareholder. Dr. Intaglietta is a member of the Board of Directors of Sangart, Inc.

Acknowledgments

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S－Ⅰ－2 Hemopure®: Clinical Development and Experience

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Hemopure [HBOC-201, hemoglobin glutamer-250, (bovine)], approved for use in South Africa and under review by the U.S. FDA, was developed as an alternative to red blood cell (RBC) transfusion in patients undergoing surgery. Hemopure contains glutaraldehyde-crosslinked and -polymerized bovine-derived hemoglobin in a balanced electrolyte solution and is stable for 3 years when stored at temperatures of 2-30℃ C. Hemopure has a right-shifted oxygen equilibrium curve with a P_{50} of 43 torr and a dose- dependent half-life of approximately 19 hours. The clinical effects of Hemopure have been studied in more than 800 treated human subjects in 22 clinical trials including multicenter, randomized, controlled, parallel group studies in a number of clinical settings. Efficacy was determined by the avoidance of allogeneic RBC transfusion in a clinically significant number of patients when Hemopure was used as an oxygen bridge to maintain hemodynamic stability until regeneration of endogenous RBC. Since patients were randomized to Hemopure or RBC at the first perioperative allogeneic transfusion decision, only those destined to receive RBC were enrolled. In four major RBC-controlled studies, 32%, 27%, 43% and 59% of patients, respectively, who received Hemopure avoided transfusion of RBC. The mean number of RBC units administered in the Hemopure group was significantly less than in the RBC group in three of the studies ($p<0.001$). Hemopure was well tolerated across a range of doses and dosing regimens. In addition to stabilization of vital signs, common effects included blood volume expansion and a transient increases in blood pressure (10-20 mmHg), serum enzymes, gastrointestinal symptoms and skin discoloration. Hemopure appears to be a feasible alternative to RBC in surgery patients requiring transfusion.

S–I–3 Clinical Development of a Perfluorochemical Emulsion (Oxygent™) as an Intravascular Oxygen Therapeutic

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Oxygent™ is a unique perfluorochemical (PFC) emulsion that belongs to a class of oxygen-carrying therapeutics currently in late-stage clinical development for use as temporary red cell substitutes. Oxygent is a 60% w/v PFC emulsion based on perflubron emulsified with lecithin, and has a median particle diameter of <0.2 μm. It provides several advantages over donor blood since it can be administered universally, is free of viruses and bacteria, can be easily manufactured in very large quantities from commercially available synthetic raw materials, and can be stored refrigerated for up to 2 years. In May 2000, Alliance and Baxter Healthcare Corporation formed a joint venture (PFC Therapeutics, LLC) to support the remaining clinical development and future commercialization of Oxygent. The oxygen transport efficacy of Oxygent has been documented in a variety of preclinical studies, and in several Phase 2 studies that have clearly demonstrated the ability of Oxygent to reverse physiological triggers and delay the need for blood transfusion in general surgery patients,[1] and also to decrease allogeneic blood transfusion in cardiac surgical patients.[2] In a recent multicenter European Phase 3 study, when used in conjunction with hemodilution (ANH) in major general surgery, Oxygent was able to significantly reduce and avoid red blood cell transfusion through hospital discharge in patients experiencing surgical blood loss in excess of 10 mL/kg.[3] In early 2001, a Phase 3 study in cardiac surgery was terminated early, due to a significant imbalance in cerebral adverse events (strokes). Full analysis of all clinical safety data revealed that these adverse events appear to have been linked to overly aggressive autologous blood harvesting just prior to cardiopulmonary bypass. Alliance and Baxter subsequently designed a new Phase 3 program in general surgery, in which Oxygent will be used to avoid blood transfusion without the need for ANH or autologous blood harvesting. Due to financial considerations, however, this new clinical program was placed on hold in 2002. Currently, efforts are underway to secure a new strategic and/or financial partner to help fund the final stage of development, to ensure that Oxygent becomes available to clinicians in the future.

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S—I—4

Clinical Results of Perftoran Application: Present and Future

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Perftoran (PF) was registered in Russia in 1996 as an oxygen carrying blood substitute manufactured by OJS SPC Perftoran. PF contains an emulsion of 10 vol.% of perfluorochemicals which are stabilised with Proxanol 268, with an average particle size of 0.07 mkm. More than 2000 patients were included in clinical studies of PF by the end of 2000 year. Distribution of patients treated by PF: 37 % - the treatment of severe anaemia, haemorrhagic and traumatic shock; 19 % - polytrauma and fat embolism; 13 % - ischemic brain edema at a craniocereberal trauma; 5 % - cardioplegia during heart reconstruction operations; 6 % - kidney transplantation; 15 % - acute ischemia of legs etc. We have shown that it is necessary to administer PF on the early stages of blood loss treatment: immediately after crystalloid solutions. In those cases PF diminished hypoxic and ischemic damages of tissues and improved blood rheology thanks, shortened the rheanimation period, improved central and especially peripheral haemodynamics and microcirculation, decreased edema and raised tissue pO_2. Some experimental and clinical experience give opportunity to use PF in the following additional fields in future: treatment of vessel damages in lower extremities; plastic surgery; treatment of ischemic stroke and myocardial infarction; activation of detoxification function of liver; inhibition of retrovirus infection; temporary and partly replacement of autoblood during large reconstruction operations.

S—I—5 HemoZyme® as Battlefield Hemorrhagic Trauma Protectant

Carleton Hsia and Li Ma

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HemoZyme®, a hemoglobin-based oxygen carrier with anti-inflammatory activities is being developed as a "small volume" hemorrhagic trauma protectant for battlefield-use. HemoZyme® works by providing adequate oxygenation in hypovolemic and hypotensive perfusion and by guarding against reperfusion injury from delayed red cell transfusion. As a result of 2002 ATACCC Oxygen Therapeutics (OT) Symposium in Florida, FDA may now consider a "Treatment Investigational New Drugs* " of a life-saving resuscitative fluid such as HemoZyme® for battlefield hemorrhagic trauma.

Battlefield experience in Afghanistan calls for an improved "golden-hour" extender as evacuation of combat hemorrhagic trauma patients to definitive field hospital care was delayed for up to 10 hours. Under this desperate life-threatening scenario, HemoZyme® as a hemorrhagic trauma protectant would work as a golden-hour extender. We have preliminary evidences of efficacy of HemoZyme® in animal models of lethal hemorrhagic shock. In these studies HemoZyme(out performed in terms of survival time against standard and experimental resuscitative fluids, including whole blood. Results will be also be presented to show that HemoZyme® has a well-understood pathophysiological mechanism, that it has been shown to be effective in multiple animal species with clear clinical endpoints (i.e. mortality) and that there is definitive pharmacokinetic and pharmacodynamic data to determine an effective dose in humans.

* Treatment Investigational New Drugs (Federal Register, May 22, 1987) are used to make promising new drugs available to desperately ill patients as early in the drug development process as possible. FDA will permit an investigational drug to be used under a treatment IND if there is preliminary evidence of drug efficacy and the drug is intended to treat a serious or life-threatening disease, or if there is no comparable alternative drug or therapy available to treat that stage of the disease in the intended patient population. In addition, these patients are not eligible to be in the definitive clinical trials, which must be well underway, if not almost finished.

S–I–6 The Development and Preclinical Testing of a Novel Second Generation Recombinant Hemoglobin Solution.

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First-generation hemoglobin therapeutics were designed more than 20 years ago, and subsequent preclinical and clinical testing has identified several issues with their use, including pulmonary and systemic vasoactivity, extravasation, serum enzyme increases, adverse effects on gastrointestinal motility, generation of myocardial lesions, and potential interactions between hemoglobin and endotoxin. In retrospect, these physiologic effects are not unexpected, since it is now known that an inherent property of all natural (wild-type) hemoglobins is the ability to interact strongly with nitric oxide (NO). This interaction, secondary to extravasation of the hemoglobin into parenchymal tissue, has been demonstrated to cause many of these observed effects.

To address these issues, Baxter modified the basic hemoglobin functionality using recombinant technology and site-directed mutagenesis in combination with various chemical modification strategies to produce a series of different hemoglobin variants with reduced NO reaction rates and an improved safety profiles. Substitution of selected amino acids into the heme pockets of α and β-subunits reduces the rate of reaction with NO by up to 30-fold relative to wild-type hemoglobin. The protein is made recombinantly in Escherichia coli and has no human or animal origin.

Extensive preclinical experiments conducted with a large number of these hemoglobin variants have demonstrated that the pulmonary and systemic hemodynamic responses can be dramatically reduced, the clearance of the product from the blood stream can be reduced and the resultant half-life of the final product can be extended, the effect on enzyme elevations seen following infusion of hemoglobin can be modified, the adverse effects on gastrointestinal motility seen with first generation hemoglobin solutions can be significantly attenuated, the generation of myocardial lesions can be significantly reduced, and depending on the particular recombinant hemoglobin variant examined, the interactions between hemoglobin and endotoxin can be altered.

Therefore, viable approaches exist for modifying the intrinsic biologic properties of hemoglobin to produce second generation hemoglobin products with improved pharmacologic properties relative to first generation products.

S—II—1

Genetation of Human Monoclonal Antibodies with a Human Myeloma Cell Line

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Continuous in vitro culture of a human myeloma cell line (Karpas-707) over twenty years reduced the doubling time from 70 to 35 hours. During this period the cells became also hypoxanthine-aminopterin-thymidine (HAT)-sensitive as well as ouabain resistant and less sensitive to the toxicity of polyethylene glycol (PEG). Following fusion with fresh tonsils and blood lymphocytes as well as with EBV-transformed B-cells it formed stable hybridomas that continuously secreted large quantities of human monoclonal antibodies. Molecular characterization by DNA sequencing of 30 of the hybridomas rearranged V-genes and analysis of germ-line diversity, somatic hyper mutation and heavy- and light-chain pairings revealed that the hybridoma-derived monoclonal antibodies are representative of antibodies from a wide population of human lymphocytes and at different stages in the maturation of the response which suggests that our myeloma cells can form stable hybridomas with any of the antibody producing B-cells that are formed in vivo. This could enable in due course to produce in vitro any of the in vivo generated human antibodies . Furthermore fusion between our myeloma cells to human CD4+ T-cells led to stable hybridoma that are CD4+. In addition it was also possible to generate stable hybridomas following fusion with the adherent human HepG2 cell line which was derived from hepatoma. Therefore it will not be unreasonable to expect that a fusion between our myeloma cells with other human cells that produce other humoral factors might lead to the in vitro production of other useful cytokines.

S—II—2

Isolation and Preparation of Therapeutic Antibodies with Neutralizing Activities Against Viruses, Toxins Secreted by Bacteria and Snake Venom

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We prepared a human antibody library, termed AIMS library, using a phage-display system. From this library, several kinds of antibodies specific against varicella-zoster virus (VZV) were isolated. While antibodies are initially isolated as phage antibodies, they can be easily converted to authentic human antibodies. They showed strong VZV-neutralizing activities. Therefore, they could be used as therapeutic drugs against VZV. In a similar way, many antibodies specific against various types of influenza viruses have been isolated. In the case of influenza viruses, however, they rapidly change their immunogenicity (a process termed antigen-drift). Since cultivation of influenza viruses under presence of virus-neutralizing antibodies, we could isolate mutant viruses that escaped from the antibodies by changing their sequence forming a neutralizing epitope. Using this system, we may prepare a new type of influenza vaccine that have already experienced the antigen-drift in vitro. From the AIMS library, we prepared several kinds of antibodies with neutralizing activities against diphtheria toxin (DT) and tetanus toxin (TT). The antibodies against DT have been shown to prevent the process of attachment of DT to the receptor expressed on the cells. In the case of habu snake venom, we could not isolate antibodies with neutralizing activities against the venom's toxic components from the AIMS library. However, using lymphocytes prepared from one volunteer who had five-time experiences to have been bitten by habu in Okinawa island, we prepared an antibody library and succeeded to isolate several antibodies with strong venom-neutralizing activities.

S—II—3

Production of Human Monoclonal and Polyclonal Antibodies by Use of TransChromo Animals

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We have developed TransChromo (TC) technology, which enables the introduction of megabase-sized segments of DNA into cells. We have used this approach to derive mice that carry megabases of human DNA by the use of a human chromosome fragment (HCF) as a vector. TC technology has been applied to the construction of the TC Mouse™, which incorporate entire human immunoglobulin (hIg) loci. Because of the instability of the Igk locus-bearing HCF2, the hybridoma production was less efficient than that observed in normal mice. Two approaches have been taken to solve this problem. One is simple cross-breeding the Kirin TC Mouse™ carrying the stable HCF14 with the Medarex YAC-transgenic mouse stably carrying 50% of the IgVκ gene segments. The resulting mouse, dubbed the KM MouseTM, performed as well as normal mice with regard to immune responsiveness and efficiency of hybridoma production. Another approach is construction of the human artificial chromosome (HAC) carrying both entire hIg heavy and light chain loci on a single chromosome fragment. The resulting mouse, dubbed the HAC Mouse™, also performed as well as normal mice. By using those mice several human monoclonal therapeutic antibody candidates targeting CD40, HLA-DR, TRAIL-R and BST2 have been produced.

Another application of TC technology is the production of polyclonal antibodies derived TC animals such as chickens and cows. To test the efficacy of human polyclonal antibodies derived from TC animals, feasibility studies were performed using antisera and purified IgG from TC mice immunized with several pathogens including Pseudomonas aeruginosa, methicillin-resistant Staphylococus (MRSA) and Japanese encephalitis virus (JEV). The TC mouse -derived antisera and IgG showed a much higher titer and efficacy in vitro and in vivo than either human serum or IgG prepared from human blood. The HAC was introduced into bovine primary fetal fibroblasts, which were used to produce cloned bovine TC calves. The HAC was retained well in calves and the hIg loci underwent rearrangement and expressed diversified transcripts. Human immunoglobulin proteins were detected in the blood of newborn calves.

S—II—4 Application of Baculovirus Membrane Display to Antibody Production

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Membrane proteins and nuclear receptors are major targets of drug discovery in the postgenome era. Monoclonal antibodies against membrane proteins are considered to be of great therapeutic value in the treatment of cancer, virus infection and many other diseases. However most membrane proteins have difficulties in functional expression and purification. We found that membrane proteins, such as HMG-CoA reductase and G protein coupled receptors, were functionally displayed on the surface of the budded baculovirus. We developed a system in which high affinity receptor was reconstituted on the viral membrane by co-infection of recombinant viruses harboring trimeric G protein subunits.

This baculovirus display system is considered to be useful for antibody production of membrane proteins. Also we are producing antibodies against nuclear receptors using gp64 fusion protein on the virus. N or C terminal part of each nuclear receptor is fused to gp64, and the recombinant viruses were immunized to mouse. This method is proven to be very useful to systemic production of monoclonal antibodies for multiple purposes such as western blotting, immunohistochemistry and gel-shift assay. So far over 30 antibodies have been produced out of 48 species of human nuclear receptor superfamily. These antibodies enabled us to examine distributional change and complex formation in transcriptional regulation. Information of localization of nuclear receptors will give new insights into drug action targeted to these nuclear receptors.

S—III—1 Development of Platelet Substitutes: Scientific and Regulatory Considerations.

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This presentation will review the status of attempts to develop platelet substitutes and discuss some of the products now under investigation. The term “platelet substitute” will include all modifications of liquid-stored platelets, as well as cellular and acellular products derived from cells and plasma.

The platelet is the most complex of all blood components and the normal platelet is capable of an array of activities. Many in vitro assays have been developed which measure the platelet's ability to adhere to foreign surfaces, release components of its granules and adhere to other platelets (aggregate). In vivo assays, in animals and in human subjects, include measurement of circulation time and correction of abnormal bleeding when platelets are deficient. Correlation of assay results with clinical utility has been most difficult and lies at the heart of the challenge to develop a "substitute" for platelets. This concept will be explored in the review of the scientific and regulatory aspects of this field.

Work has been reported on a wide array of potential products, including modifications of the liquid-stored product (platelets that have been stored in the cold, frozen or lyophilized), membranes or microparticles derived from platelets, use of other particles as hemostatic foci (liposomes, fibrinogen aggregates, red blood cells) and acellular agents that affect hemostasis in thrombocytopenic subjects (factor VIIa). These products and their status will be briefly reviewed.

The regulatory approach of the U.S. Food and Drug Administration (FDA) to platelet products has utilized in vitro assays to determine damage to the products during preparation, measurement of circulatory survival and demonstration of hemostatic utility. Modified platelets, or more extreme substitutes have been studied for altered thrombogenic potential. To date, no product has been approved by the FDA.

A clinically useful product may not display all functions of normal platelets. Such a product could not be used in all clinical situations, but would be valuable in selected applications. The application of this selective approach will be considered in light of potential platelet substitute products.

S—III—2 A Novel Approach to Development of Artificial Platelets in Japan

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In Japan, a research project has been launched since 1997 to develop artificial platelet / platelet substitutes with a support of the Health Science Research Grants (Artificial Platelets) from Ministry of Health, Labour and Welfare.

Requirements of ideal artificial platelets are 1) to possess one or two hemostatic functions of platelets, 2) to interact with residual platelets to accelerate plug formation, 3) not to inhibit normal platelet functions and production, 4) not to form thrombus in the circulation.

Our strategy is first to prepare artificial particles which have the ability to specifically adhere to the site of vascular injury upon infusion and to recruit the residual circulating platelet to form thrombus.

Based upon our understanding of molecular mechanisms of platelet adhesion and aggregation, hybrid-type plateletubstitutes are designed, that is, development of liposomes or albumin polymers, to which recombinant platelet membrane glycoproteins such as GPIb α (a receptor for von Willebrand factor) and/or GPIa/IIa (a receptor for collagen) and synthetic peptides of fibrinogen functional domains are immobilized. Among several candidates developed thus far, liposomes or albumin polymers carrying GPIb α and dodecapeptides c-terminal sequence of γ -chain of fibrinogen are the best to show the in vitro platelet functions to form thrombus under flow conditions. In vivo studies to demonstrate their hemostatic efficacy are being planned.

S—III—3 Observation of Microscopic Motion of Artificial Platelets in the Model Arteriole

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The development of artificial platelets is strongly required to overcome the largely increasing demand for the thrombocytopnia. Recently Takeoka et al. developed the albumin microspheres (AMS) conjugated recombinant glycoprotein Ibα, which is a promising candidate for artificial platelet. An important aspect of hemostasis is the biomechanical process to form the aggregates. Thus we studied the fluid mechanical behavior of AMS by observing the microscopic motion in the model arteriole.

In this study, we developed the experimental system to visualize the microscopic motion of artificial platelet toward ligand coating surface in the model arteriole by the High Speed Camera with Image Intensifier. Then concentration profiles along a radius and particle velocity vectors were measured.

At high shear rate, the concentration profiled of large rGPIbα-AMS showed large wall-near excess. This profile may accelerate the interaction between particles and wall. Once particles adhered to the surface, the trajectories flowing near the wall became fluctuating. To assess the fluctuation, we determined the drift angle ϕ, which is the angle of velocity vector from axial direction. The angle ϕ tends to be higher near the wall and this tendency is desirable to achieve the adhesion of particles on the wall.

S—III—4 In Vivo Evaluation of New Platelet Substitute Glycoprotein Ib-α Bound on Recombinant Albumin Polymer

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Platelet substitutes made of recombinant products are expected to be used clinically, because of their no risk of infection and possibly stable supply. A novel platelet substitute, glycoprotein Ib-α bound on recombinant albumin polymer (Gp Ibα-pol. Alb), we used in the present study, is known to associate with vWF in the presence of ristocetin. To evaluate how the platelet substitutes contribute to the in vivo hemostasis influenced by many factors, we employed the methods of photochemically induced thrombosis in rat mesenteric vein. After the injection of Gp Ibα-pol. Alb to thrombocytopenic rats made by gamma-ray irradiation, the occurrence of venous obstruction was monitored. Time required to induce vein-obstruction after the injection of the Gp Ibα-pol. Alb was measured and the results was expressed by the relative time ratios to the time by the injection of control pol. Alb. GP Ibα-pol. Alb required less than half of the time by saline injection to induce venous obstruction; the relative time ratio (Gp Ibα-pol. Alb / control pol. Alb) was 0.87 +/- 0.24 (mean +/- SD), indicating Gp Ibα-pol. Alb can accelerate the venous thrombosis formation on the injured endothelium. Rapid disappearance of the Gp Ibα-pol Alb from the circulation with 3.6 minutes of plasma half life and their accumulation to the liver and spleen were revealed by a study with 125-I-labeled Gp α-pol. Alb. Any thrombosis at intact vessels was revealed by immunohistopathological examinations; neither TAT nor PIC were elevated in the sera. We conclude that the novel platelet substitute, Gp Ibα-pol. Alb, is expected to function effectively also in vivo and is to be used safely to stop bleedings.

WS—I—1 Hemoglobin Encapsulation - Influence of Preparation and Wall Materials

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Monomethoxypoly (ethylene glycol)-b-poly-DL-lactide (PELA) microcapsules containing bovine hemoglobin (bHb) were prepared by four solvent removal methods based on W/O/W double emulsion technique. The optimum preparative condition for PELA microcapsules loaded with bovine hemoglobin was investigated. It was found that homogenization rate, type of organic solvent, and volume of the solidification solution influenced the activity of bovine hemoglobin encapsulated. By optimization of the preparation process, it was possible to obtain the oxygen binding affinity near to that of native hemoglobin. The effect of wall polymer had an important influence on the efficiency of the encapsulation. PELA copolymer containing MPEG 2000 block was found to produce a high entrapment efficiency. The results suggested that the multiple emulsion solvent-diffusion method using ethyl acetate as the organic solvent was an efficient process for preparation of the artificial red cells.

WS—I—2 Kinetics of Ligand Binding to MP4, a Polyethylene Glycol-conjugated hemoglobin

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Sangart is developing a polyethylene glycol (PEG)-conjugated hemoglobin, MP4, that is non-vasoactive and in early-phase clinical trials. MP4 is made by reaction of adult human hemoglobin (HbA) with a monofunctional maleimide-activated PEG. Physical properties of MP4, compared to HbA, are given in the Table. The kinetics of ligand binding are of particular interest due to the lack of vasoactivity and high O_2 affinity of MP4. Cell-free, modified hemoglobins have typically produced side effects attributed to vasoconstriction, generally assumed to result from NO scavenging. To explore this hypothesis, we first measured the kinetics of NO binding to deoxyMP4. The association rates were the same for MP4 and HbA at ~20 $\mu M^{-1}s^{-1}$. This result is consistent with our previous study showing that hypertension and NO binding do not correlate when molecular radius is increased and p50 is reduced (Rohlfs et al., J. Biol. Chem., 273: 12128, 1998). Second, to explore the molecular mechanism for the high O_2 affinity of MP4, we measured the kinetics of O_2 association and dissociation. Like NO, O_2 association rates were the same for both proteins and approach the limit of diffusion with values of ~40 $\mu M^{-1}s^{-1}$. For O_2 dissociation, we expected the rates for MP4 to be lower compared to HbA because of MP4's higher O_2 affinity. However, at a pO_2 of ~150 Torr, the O_2 off rates were approximately 2-fold higher for MP4 compared to HbA. Inspection of MP4's oxygen equilibrium curve shows that it is has low cooperativity and intersects the HbA curve. Therefore, the O_2 affinity of MP4 is higher only at pO_2 values lower than the intersection point. In conclusion, the rates of NO binding to HbA and MP4 are the same, which is consistent with theories that hemoglobin-induced vasoconstriction is mediated by mechanisms other than scavenging of NO in the vascular space. The unexpected O_2 kinetic findings show that, at the pO_2 in air-saturated buffer, the O_2 affinity of MP4 is actually lower than that of HbA.

	HbA	MP4
MW (kD)	64	95
Radius (nm)	3.2	9±2
p50 (Torr)	12	6
Hill number	2.8	1.3
Bohr effect (log p50/pH)	-0.5	-0.24
Viscosity (cP) (Hb = 4 g/dL)	1.0	2.5
COP (Torr) ([Hb] = 4 g/dL)	18	50

WS—I—3 Serum Albumin Including Synthetic Hemes as an Oxygen-Carrying Hemoprotein

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Serum albumin is the major plasma protein and serves two major roles: maintaining the colloid osmotic pressure, and transporting many helpful materials around the body. From a viewpoint of clinical application, it is of great interest to develop an O_2-carrying albumin, which could be of extremely medical importance.

We have recently found that synthetic hemes are successfully incorporated into recombinant human serum albumin (rHSA), providing a new class of hemoprotein [alubumin-heme (rHSA-heme)] which can bind and release O_2 reversibly under physiological conditions (in aqueous media, pH 7.3, 37 ℃). The physicochemical properties and O_2-coordination behavior of rHSA-heme satisfy the initial clinical requirements for red blood cell substitutes.

Administration of extracellular hemoglobin-based O_2 carriers often elicits an acute increase in blood pressure by vasoconstriction. This side effect is recognized to be due to the depletion of NO by the extravasuated hemoglobins. We have found that the administration of rHSA-heme does not induce such hypertensive action, because of its low permeability through the vascular endothelium.

Physiological responses to exchange transfusion with rHSA-heme solution into rats after hemodilution and hemorrhage (Hct: ca. 10%) have been evaluated. The declined mean arterial pressure and blood flow after a 70% exchange with rHSA and the further 40% bleeding of blood were significantly recovered up to about 90% of the baseline values by the injection of rHSA-heme. The renal cortical O_2-tensions and skeletal tissue O_2-tensions were also increased, indicating the in vivo O_2-transpoering efficacy of rHSA-heme.

WS—I—4 Highly Stable, Low-Sized Fluorocarbon Emulsions with Fluorocarbon / Hydrocarbon Diblocks as Interfacial Film Reinforcers

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Achieving fluorocarbon emulsions with small and narrowly-dispersed droplet sizes is important, as prolonged intravascular persistence, facilitated oxygen diffusion, reduced side effects, and longer room temperature storage are strongly related to the physicochemical characteristics of such emulsions.

We have prepared highly stable, small-sized perfluorooctyl bromide emulsions when using semifluorinated alkanes $C_nF_{2n+1}C_mH_{2m+1}$ (FnHm diblocks) as co-surfactants of phospholipids. After 6 months at 25℃, the average droplet diameter of an emulsion stabilized by such diblocks was only ~80 nm, as compared to ~180 nm for the reference emulsion stabilized by phospholipids alone. The diblocks are preferentially located within the phospholipid interfacial film. The stabilization effect and mechanism are conditioned by the adequacy between the Hm segment's length and that of the phospholipid' fatty chains.

Although research regarding the toxicity and pharmacokinetics of FnHm diblocks is still much more limited than that of fluorocarbons, preliminary studies indicate that diblocks display no cytotoxicity and have acceptable LD_{50} values and excretion rates, provided the lengths of Fn and Hm segments are appropriately chosen. The FnHm-stabilized emulsions do not induce any significant perturbation on the rheological behavior of red blood cells, even when a large dose is administrated.

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WS—I—5 Encapsulation Effects of Concentrated Hemoglobin with Phospholipid Membrane

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Hemoglobin(Hb)-vesicles encapsulate purified and concentrated Hb with thin phospholipids bilayer membrane with modulating their size and oxygen affinity. They have structural similarity with red blood cells, shielding the bioactivity of Hb and coencapsulating modulators such as allosteric effectors. The solution viscosity and colloid osmotic pressure could be controlled by adding colloid materials such as albumin and starch. The interaction of Hb-vesicles with blood components was evaluated with a microchannel array flow analyzer (MC-FAN), showing no interaction of Hb-vesicles with blood cell components. The excellent properties of Hb-vesicles, namely good processibility, long-term storage, blood compatibility, etc., are attributed to the surface-modification with polyoxyethylene (POE) chains and lipid components. Physicochemical points of POE modification are introduced as well as the design of novel POE-lipids. The Hb-vesicles are very safe against the generation and attack of active oxygen species such as H_2O_2 by encapsulating the concentrated Hb molecules with a saturated phospholipid bilayer membrane or co-encapsulating catalase etc.

WS—II—1 Effective Oxygen Transport in the Microcirculation After Exchange Transfusion with Colloidal Plasma Expanders and Hemoglobin Based Blood Substitutes

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Tissue oxygenation is determined by the availability of oxygen, the physical properties of the flowing blood, the partition of oxygen between the oxygen consuming compartments, and the self regulatory controls of the microcirculation. Substituting red blood cells with oxygen carrying and non carrying colloids usually lowers plasma and blood viscosity, an effect that is often detrimental to tissue survival due to the reduction of shear stress and consequent decrease in production of vasodilators by mechano transduction in the endothelium. Lowering plasma viscosity also enhances facilitated oxygen diffusion by molecular hemoglobin oxygen carriers in plasma, a process that over oxygenates the small artery and arteriolar wall leading to autoregulatory vasoconstriction, hypertension, reduced perfusion, reduced functional capillary density, decreased oxygen delivery to the tissue and an unfavorable acid base balance. Oxygen delivery to the tissue is also modulated by the manner in which oxygen is released by blood. A carrier with a high oxygen affinity releases oxygen when the blood/tissue gradient is large, and vice versa. This process can be exploited to minimize the amount of oxygen by the carrier while maintaining a uniform and adequate level of tissue oxygenation. This is possible because tissue oxygen regulation is set to insure an average tissue pO_2 of about 20 mmHg, while anaerobic metabolism starts when tissue pO_2lowers beyond about 1 mmHg oxygen partial pressure. This gap is a physiological mechanism that insures that no portion of the tissue falls below the limit for anaerobic metabolism, which can occur due to the semi random distribution of capillaries and the longitudinal capillary oxygen gradient. Therefore an oxygen carrier with high oxygen affinity, i.e., low p50, only delivers oxygen to those portions of the tissue that cannot be oxygenated by red blood cell that have already given up their oxygen in the districts with higher pO_2. The ensuing economy in oxygen transport, however, is only effective if the whole capillary network is functional, which requires the absence of vasoconstrictor stimuli. These considerations indicate that effective oxygen transport by molecular oxygen carriers used as blood substitutes requires high intrinsic viscosity and oxygen affinity. These properties lead to a blood substitute that is effective in small quantities, since it brings oxygen only to the portions of the tissue where it is lacking.

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WS—II—2 Roles of Platelet-Associated Adhesion Molecules in Regulation of Leukocyte Adhesion to Microvascular Endothelium

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Multistep mechanisms for leukocyte adhesion in vivo have been established by observation through an intravital microscope. However, roles of circulating platelets in regulation of leukocyte-endothelial cell interactions have not fully been investigated because of several technical difficulties to visualize their behavior in vivo. To overcome these difficulties, we have established a novel method to stain platelets with the fluorophore and to visualize their individual traffics with a resolution sufficient enough to determine site-specific velocity and density in a single vessel of organ microcirculatory system. High-speed intensified video microscopy allowed us to visualize fast-moving platelets at videorates of 300-1000 frames/sec, and normospeed replay of the video images at 30 frames/sec demonstrated that behavior of individual platelets in microvessels differ greatly between the cells flowing at centerline region and those flowing in the periendothelial space. Our results demonstrate that, under high shear rates greater than 600 sec^{-1}, platelets tend to form microaggregates and to flow along the periendothelial space, exhibiting transient skipping and rolling on the endothelium. They utilize GPIb-alpha on their surface for these shear-dependent interactions with the surface of the endothelium through its affinity to vWF. GPIb-alpha-mediated platelet adhesion appeared to help P-selectin-mediated leukocyte adhesion to microvascular endothelium at least in part, since its immunoneutralization caused a significant suppression of the stimulus-elicited microvascular adhesion of leukocytes. Although the roles of shear-dependent redistribution of platelets in regulation of the tissue delivery of inflammatory cells are largely unknown at present, these results suggest a possible role of platelets as a determinant of inflammatory impacts.

WS—II—3 From Microcirculation to Metabolism: New Methods to Assess Tissue Ischemia

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Artificial oxygen carriers, originally developed as blood substitutes supposed to reduce the need of blood transfusions, may have an enormous, yet barely explored potential in the treatment of diseases caused by acute vascular obstruction, e.g. myocardial, cerebral or mesenterial infarction. The survival of these tissues depends on a critical collateral circulation via neighboring vascular territories. It has been postulated that due to their physical and biochemical properties, artificial oxygen carriers may have a higher capability to deliver oxygen to ischemic, collateralized tissue than normal blood. Thus, experimental models are required on which this hypothesis can be tested. To this end, we developed a skin flap model in hamsters, which consists of an anatomically perfused part and an ischemic part nourished by a collateral perfusion. The preparation allows for monitoring the microhemodynamics with intravital microscopy, microcirculatory blood flow with laser Doppler flowmetry, partial tissue oxygen tension with Clark-type microprobes and tissue metabolism with microdialysis. Microcirculatory blood flow was significally reduced in the ischemic tissue to one fourth to one third of the anatomically perfused tissue. Partial tissue oxygen tension was 10.9 $\pm$ 3.8 mmHg in the ischemic tissue and 23.5 $\pm$ 5.3 mmHg in the anatomically perfused part. Lactate increased from 2.1 $\pm$ 0.7 mmol/l to 2.8 $\pm$ 0.7 mmol/l after obstruction of the vascular blood supply to the tissue subsequently rendered ischemic ($P < 0.05$), and the lactate/pyruvate coefficient was raised from 24 $\pm$ 8 to 50 $\pm$ 24 ($P < 0.01$). Furthermore, the tissue underwent histological examination 6 hours after onsetting of ischemia. The immunohistochemical staining revealed a significant increase in apoptosis as well as an up-regulation of hypoxia induced factor in the ischemic tissue. In conclusion, we were able to develop an experimental model that allows for investigations on the interplay between microhemodynamics, tissue oxygenation and metabolism, as well as the vitality of ischemic, collateralized tissue.

WS—II—4 Effect of Artificial Oxygen Carrier on Neutrophil Movement in the Microvasculature of Hamster Cheek Pouch

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In this study, we examined whether artificial oxygen carrier, liposome encapsulated hemoglobin vesicle (HbV), alters rolling, adhering, and migration of neutrophils before and after the administration of leukotriene B_4 (LTB_4) in the microvasculature of hamster cheek pouch. Male golden hamsters (110-160g) were anesthetized with urethan (1.2 g/kg body weight, intraperitoneally). After catheters were inserted into the femoral vein and the carotid artery, approximately 40% of the rat's blood was withdrawn and replaced by HbV or albumin within 5 min. Ten min after the replacement, LTB_4 (300 nM) with the volume of 50 μl was topically applied to the cheek pouch. The microvasculature of the cheek pouch (unit:50×100 μm2) was observed with transillumination under a microscope. Neutrophils moving more than 10 μm /sec along the inner wall of the venule, those remaining at the same site longer than one min, and those moving from the venular wall into the interstitial space were designated as "rolling", "adhering", and "migration", respectively. Data were statistically analyzed with ANOVA. The application of LTB_4 caused a transient increase and a subsequent decrease in rolling, an increase in the stage of adhering, and migration from the vessel wall. The exchange of the blood by HbV and albumin caused decreases in hematocrit approximately by 40% in both groups. The blood pressure decreased significantly in the albumin group, but did not significantly altered in the HbV group. The heart rate did not significantly change in both groups. There were no significant differences in the alterations in rolling in both groups. Adhesion and migration of neutrophils were significantly less in HbV group than albumin group. In conclusion, this study demonstrated that HbV could inhibit the neutrophil adhesion and migration elicited by LTB_4. The mechanisms of this effect of HbV on neutrophil movement remain unclear, but it is suggested that HbV can be beneficial in septic or hemorrhagic shock when leukotriene-induced tissue injuries may occur.

WS—II—5 Studies on safety of Hb-Vesicles in Brain

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Hb-vesicles have an excellent oxygen transporting capability equivalent to that of normal red blood cells, and, reverse vital functions and anaerobic metabolism produced during controlled hemorrhagic shock in rodent model. However, general concerns have been raised about the toxicities of the hemoglobin-based oxygen therapeutics on neural tissues. It is clinically known that intracerebral hemorrhage associated with stroke and head trauma produce brain inflammation and neural cell death. The delayed damage could result through a variety of mechanisms including local brain ischemia, release of toxins by blood breakdown, thrombin release or leukocyte infiltration. Evidences have been also shown that released hemoglobin from the red blood cell breakdown could cause brain cell injury. Potential breakdown of the blood brain barrier (BBB) during the shock is an additional ingress of peripheral blood into brain. Since hemorrhagic shocks frequently accompanied hemorrhagic conditions in brain/spinal cord, neurotoxicity of Hb-vesicles should be evaluated and be compared with that produced by the standard trauma resuscitation. The initial phase of Hb-vesicle neurotoxicity studies will be done *in vivo* utilizing rodent animal model system which simulates experimental condition of Hb-vesicle exchange transfusion studies.

Wister rat (male, ~300g) will be anesthetized with sodium pentobarbital, 50 mg/kg i.p., and placed on a stereotactic frame. A midline scalp incision will be made and a hole will be drilled in the skull (3 mm lateral to midline, 0.02 mm anterior to coronal suture). A 25-gauge needle with a syringe will be secured in the holder and the frame. The needle tip is advanced into the striatum 5.5 mm below the skull surface steriotactically and the testing fluid suspension, 50 μl each of the following compositions will be injected into striatum.

The testing fluid suspensions consist of (1) Hb-vesicle suspended in 5% albumin with Hb concentration of 10 g/dl and the final Hb-vesicle suspended compositions in autologous whole blood matching to the two conditions of Hb-vesicle (a) 50 and (b) 90%-exchange transfusion. (2) One hundred % Hb-vesicle suspension in 5% albumin will also be tested to compare the effect produced with the autologous whole blood. The controls consist of (a) 5% albumin in isotonic physiologic saline, and (b) autologous whole blood.

The testing suspension (total 50 μl) will be injected over 5 min, the needle will be left in place for 3 min, then removed slowly. The bone whole will be sealed with bone wax, the scalp wound will be sutured, and the animal will be placed individually in a cage with free access to food and water. The control for the process of injection, additional animals will receive injection of 50ul sterile 0.9% saline solution into the same site. One, 24, 48, 72 hours, 1 and 4 weeks after the injection, the animals will be reanesthetized with sodium pentobarbital and perfused through the heart with ice cold 4% paraformaldehyde in 0.1 mol/l phosphate-buffer saline (PBS). The animals with saline injection will be killed at 48 hours. The brain will be removed and cut coronally approximately 2 mm on either side of the needle entry site.

The brain inflammation and cell death will be evaluated pathohistologically. Terminal dUTP nick-end-labeling positive dying cells, neutrophil infiltration, CD8 a immunoreactive lymphocytes (possible natural killer cells), and microglial reaction will be evaluated on each of the tested animals and toxicity produced by Hb-vesicle will be evaluated against that produced by autologous whole blood. During the intracranial hemorrhage, the red blood cells slowly hemolyze. The oxyhemoglobin will be released in the cerebrospinal fluid and transformed into bilirubin by an enzyme-dependent process. Neurotoxicity of hemoglobin and its metabolites have been demonstrated in cortical cells and spinal cord *in vivo* and *in vivo*. Accordingly, production of CSF heme derivatives, oxyhemoglobin and methemoglobin, will be determined in the CSF samples collected prior to the sacrifice of the animals. If any noted difference is observed for Hb-vesicle tested animals, the rate of release of hemoglobin from Hb-vesicles and its heme metabolites will be evaluated in brain homogenate *in vivo*.

WS–III–1 Hemoglobin, Transcriptional Activator and Suppressor. How to Tip the Balance?

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In the 1990's, our group discovered that acellular hemoglobin (Hb) is a potent inducer of the nuclear factor kappa B (NF-κB) that is involved in the regulation of inflammatory genes (ACBSIB 25(1&2):193-225,1997; ASAIO J 44:M356-M367,1998). We found that the activation of NF-κB by Hb, might be dependent on Hb's pro-oxidant potential and the extent of Hb-mediated cellular oxidative stress that shifts GSH/GSSG into an oxidative equilibrium. In this study, however, the high GSSG that should have inhibited DNA binding activity of NF-κB, did not prevent the expression of inflammatory genes, cytokines and adhesion molecules. Our more recent study, revealed that Hb-activated NF-κB can not prevent apoptosis (ASAIO J 48(2):193,2002). Recently, it was suggested, that Hb may also induce other oxygen and redox-regulated transcription factors, hypoxia inducible factor-1α (HIF-1α) and activator protein-1 (AP-1) (ACBSIB 29(6):415-425,2001). HIF-1α can be of particular significance, since its target genes involve erythropoietin, transferrin, heme oxygenase, endothelin, vascular endothelial growth factor, and nitric oxide synthase. The present study, was undertaken to investigate the effects of unmodified ferrous- and ferric Hb, and ferrous Hb-adenosine-glutathione complex on the induction of NF-κB and HIF-αa in normal and GSH deficient human coronary artery and brain capillary endothelial cells. After incubation, cells were evaluated for the GSH/GSSG ratio, nitric oxide fate and the activation of NF-κB and HIF-1α. The measurement of NF-κB and HIF-1α was done in nuclear extracts by using the TransAM™ method (Active Motif, Carlsbad, CA). Results indicate, that NF-κB and HIF-1αare differentially potentiated by oxidative conditions. Hyperoxia and the oxidative equilibrium activates NF-κB, which is in agreement with our previous observation. On the contrary, HIF-αa showed to be relatively stable in this condition. However, the hypoxic environment induced HIF-1α. It seems that this different molecular response of Hb is linked to its physiological and physico-chemical characteristics. Therefore, it is possible that Hb solutions, which in vivo alter the cellular redox state and have a strong hyperoxic effect, might trigger NF-κB and suppress HIF-1α. On the other hand, Hb solutions that raise the peripheral vascular resistance and produce hypoxic/anoxic conditions, might accelerate the expression of HIF-1α regulated genes. Perhaps, the key strategy to control transcriptional responses to Hb-based red cell substitutes is in the type of Hb chemical modification procedure that addresses Hb's intrinsic toxicity, particularly pro-oxidative and vasoconstrictive reactions. The pharmacologic modification of Hb with adenosine and GSH has shown to be an effective strategy.

WS—III—2 Hemodynamic and Metaboloc Changes after Administration of PEG-Hb in Hemorrhagic Shock in Swine

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Thirty anesthetized, fully instrumented piglets (BM～20 kg) were bled 50 % of their individually calculated blood volume, followed by replacement of 70 % of the shed volume. Pentastarch (PS, 10 %) was used as control. Animals in the treatment group were given one of the following polyethylene-glycol-linked tetrameric hemoglobin (PEG-Hb) formulas: PEG-Hb 4 % in Ringer's lactate, PEG-Hb 2 % in 5 % PS or PEG-Hb 4 % in 5 % PS. Following resuscitation, animals were observed for 120 minutes. After euthanasia, tissue samples for histopathology were taken from the heart, lungs, skeletal muscle, small intestine, liver, kidney and spleen.

Hemorrhagic shock (HS) occurred to a similar extent in all animals. As for hemodynamic parameters, responses were comparable between the groups in heart rate, mean arterial pressure, mean pulmonary artery pressure and systemic as well as pulmonary vascular resistances. Arterial lactate increased after hemorrhage, but returned toward baseline after resuscitation. Arterial pH and base excess fell during HS but also returned toward baseline post infusion. Oxygen delivery was normalized after resuscitation. Oxygen consumption and oxygen extraction ratio increased in the treatment group compared to controls. There were no abnormal histology findings in any of the animals in either group.

No detrimental changes were found after administration of PEG-Hb in this porcine model of HS in any of the parameters studied.

WS–III–3 Nanometer-sized Oxygen Carrier Alleviates Myocardial Infarction in the Rat

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Background. We tested a hypothesis that a 200 nm liposome-encapsulated-hemoglobin (LEH) may deliver oxygen beyond blockage through collateral circulation and limit ischemic damages after acute myocardial infarction (AMI) and reperfusion.

Methods. Pressure-volume loops were analyzed 10 and 40 minutes after occlusion of the left anterior descending artery and 20 minutes after reperfusion in rats pretreated with LEH (LEH+) or saline (LEH-) of 1% body-weight. End-diastolic and end-systolic pressure-volume relationships were defined and left ventricular size and function were analyzed before and after AMI and reperfusion.

Results. Although end-diastolic (EDV) or end-systolic (ESV) left ventricular volumes were not different, stroke volume (SV), end-diastolic pressure (EDP) and ejection fraction (EF) tended to be higher in LEH+ rats after AMI (Table) and reperfusion.

Conclusion. Nanometer-sized LEH appeared to be effective in preserving stroke volume and ejection fraction immediately after AMI and reperfusion in the rat.

	EDV (mcL)	ESV (mcL)	SV (mcL)	EDP (mmHg)	EF (%)
LEH- (n=8)	270 ± 79	218 ± 77	62 ± 16	14.0 ± 4.3	24.3 ± 7.4
LEH+ (n=10)	274 ± 95	200 ± 93	88 ± 28	15.6 ± 5.8	34.2 ± 14.0
P (+ vs -)	0.921	0.668	0.333	0.517	0.090

WS—III—4

Daily Repeated Infusion of Hb-vesicles (HbV) into Wistar Rats for Two Weeks: A Preliminary Safety Study.

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One of the safety studies of a new drug in the preclinical stage should be the daily repeated infusion in rodent and nonrodent for at least 14 days at different three dosages. In this study we tested Hb-vesicles (HbV, [Hb] = 10 g/dL, [lipid] = 5.6 g/dL) for the daily repeated infusion into rats. Twelve male Wistar rats (140 g body weight) received infusion of HbV (10 mL/kg/day) or saline through a tail vein for 14 consecutive days. The total volume of infusion was estimated to be 2.5 times as much as total blood (56 mL/kg). Among 12 rats, 6 rats were sacrificed 1 day after the final infusion, and the others after 2 weeks of interval.

All the rats well-tolerated and survived and the body weight continuously increased until their sacrifice. There was no significant hematological change except that Hct tented to decrease slightly in the HbV group, probably due to the accumulated volume of HbV particles in blood. One day after the final infusion spleen and liver weights increased significantly. Histopathological observation indicated significant HbV accumulation in liver and spleen, however, there was no sign of organ damage. Serum clinical laboratory tests indicated significant increases in lipid components derived provably from HbV particles. After 2 weeks of interval, spleen and liver weight returned to the original levels, however, significant amount of hemosiderin was confirmed without serum iron increase. All the concentrations of the lipid components returned to the original levels. Judging from these results, there was no sign of significant toxicity of HbV at the level of dosage employed.

WS—III—5 Blood Substitute Resuscitation as a Treatment Modality for Moderate Hypovolemia

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Blood substitute resuscitation as a treatment modality for moderate hypovolemia (~40% blood loss) in a canine model has been evaluated using Oxyglobin™ (Biopure Glutamer-200/Bovine; a hemoglobin-based oxygen-carrier) and Hespan™ (6% hetastarch; a non-oxygen-carrier) as resuscitants. Autologous (shed) blood served as control. Nine dogs were studied - after splenectomy, each dog was hemorrhaged (32-36mL/kg) and randomly assigned to 3 resuscitation groups. Microvascular, systemic function and oxygenation characteristics were measured simultaneously in prehemorrhagic, posthemorrhagic and postresuscitation phases for correlation -- microvascular changes in the bulbar conjunctiva were non-invasively measured via computer-assisted intravital microscopy and systemic function and oxygenation changes were measured via standard operating room procedures. Prehemorrhagic microvascular characteristics were similar in all animals (venular diameter=41±12 μm, A:V ratio=~1:2, red-cell velocity=0.5±0.3mm/s). All animals showed similar significant ($P<0.01$) posthemorrhagic changes: ~19% decrease in diameter (34±7 μm), A:V ratio=variable, and ~80% increase in velocity (0.9±0.5mm/s). All animals also showed similar posthemorrhagic systemic function and oxygenation changes. Control blood resuscitation restored posthemorrhagic microvascular changes to prehemorrhagic values (diameter=39±6 μm, A:V ratio=~1:2, velocity=0.6±0.4mm/s). Oxyglobin™and Hespan™ restored microvascular changes in similar manner to prehemorrhagic values (Oxyglobin™: diameter=38±3 μm, A:V ratio=~1:2, velocity=0.6±0.4mm/s; Hespan™: diameter=38±7 μm, A:V ratio=~1:2, velocity=0.5±0.4mm/s). After resuscitation, control blood restored all systemic function and oxygenation changes to prehemorrhagic values. Both Oxyglobin™ and Hespan™ resuscitation restored systemic function changes, but not oxygenation changes, to prehemorrhagic values. This was an unexpected finding because of the oxygen-carrying capability of Oxyglobin™. This study suggests strongly that volume replenishment may play a significant role, instead of oxygen-carrying capability, in the treatment of moderate hypovolemia.

WS–III–6 Evaluation of Anionic Liposome-Encapsulated Hemoglobin in Rabbits.

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Liposome-encapsulated hemoglobin (LEH) is being investigated as an oxygen-carrying substitute. We manufactured LEH of lipid composition DSPC/Chol/DMPG/α-Tocopherol (46/42/10/2, molar ratio) and containing stroma-free human hemoglobin (Hb) by a combination of microfluidization and ultrafiltration. Oxygen affinity of Hb was modified by pyridoxal phosphate and radioactive Tc-99m labeling was enabled by co-encapsulating glutathione inside the liposomes. One half of this batch of anionic LEH was externally loaded with PEG-DSPE. Thus, two anionic preparations- one without PEG-DSPE and the other with PEG-DSPE were obtained. The average size of the LEHs was ~150 nm; the lipid concentration was 29.5 mg/ml; [Hb]/[Lipid] ratio was 1.53 and; p50 was 21.7 mm Hg. About 16 mg of lipid (Tc-99m-LEH, 2.3 mCi) was injected to investigate distribution and circulation kinetics in rabbits. After 24h, anionic LEH and PEG-anionic LEH accumulated in liver, to the extent of 35.3% and 11.5, respectively. Blood borne activity was 13.5 and 35.7%, respectively. The circulation $T_{1/2}$s of anionic LEH and PEG-anionic LEH were 9.6h and 16.5h, respectively. To evaluate the LEH-induced thrombocytopenia, small amount of LEH (4 mg lipid) was injected in rabbits that had been given autologous In-111-platelets 30 min prior to LEH injection. It was found that PEGylation significantly reduced the thrombocytopenia induced by LEH injection. Within 2 min of anionic LEH injection, the circulating In-111-platelets dropped to about 35% of baseline level; PEG-anionic LEH reduced the circulating activity to about 58% of the baseline value. There was also a significant drop in circulating white blood cells after LEH injections as evidenced from the complete blood count (CBC) data. The pattern of platelet counts obtained from CBC closely paralleled the profile of In-111-platelets after LEH injection.

WS–III–7 Effect of Pegylated Hemoglobin on Microvascular Oxygen Transport and Function in Acute Anemia after Isovolemic Hemodilution

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A new formulation of polyethylene glycol conjugated hemoglobin (MalPEG-Hb, 4 g/dl Hb, viscosity of 2.5 cP, COP 49 mmHg, p50 5.4 mmHg, Sangart Inc., San Diego) was used in a exchange transfusion protocol aimed at simulating a clinical scenario of blood loss replacement where the initial volume restitution is made with a colloidal plasma expander, and upon reaching the transfusion trigger either blood or MalPEG-Hb is provided. The hamster window chamber model was hemodiluted to 35% of the original hematocrit reaching a moderate hemodilution level. The exchange was continued with an additional exchange transfusion with either collected blood (fresh frozen plasma and packed RBCs) or MalPEG -Hb. The final hematocrit in the two groups were 18% and 11% respectively. Total hemoglobin concentrations were 5.9 and 4.7 g Hb/dl for the blood and MalPEG-Hb groups respectively. Combining microvascular flow and pO_2 data allowed to calculate oxygen consumption by the tissue which had the relative values of 0.55 and 0.53 ml O_2/min g Hb respectively. Oxygen delivery had relative values of 3.82 and 2.87 ml O_2/min which reflected the difference in hemoglobin concentration. These results show that MalPEG-Hb is equivalent to RBCs on a per gram of hemoglobin basis in transporting oxygen in the microcirculation.

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F—I—1

Comparison of Platelet Substitutes Made of PolyAlb and Vesicles

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Albumin-polymerized particles (polyAlb)[1,2] and phospholipid vesicles[3] bearing recognition proteins have been developed as a candidate for platelet substitutes. As recognition proteins, we used recombinant glycoproteins; rGPIbα and rGPIa/IIa, that recognize von Willebrand factor (vWf) and collagen, respectively, and fibrinogen.

Under flow conditions, the rGPIbα-vesicles rolled on the vWf surface in the direction of flow, like platelets. On the other hand, rGPIbα-polyAlb attached to the surface of the vWf-immobilized plate and accumulated. The rolling phenomena of the rGPIbα-particles under flow conditions would be attributed to the flexibilities of the particle to which rGPIbα was conjugated. Fibrinogen-polyAlb recognized to the GPIIb/IIIa on the surface of the activated platelet and accumulated to the platelet-immobilized surface. It was found that the remaining platelets in thrombocytopenic blood were involved in the aggregation of fibrinogen-polyAlb with platelets, rGPIa/IIa-polyAlb and rGPIa/IIa-vesicles were attached to the collagen surface and accumulated.

1) Biomacromolecules, 1:290-295, 2000. 2) ibid, 2:1192-1197, 2001. 3) Biochem. Biophys. Res. Commun., 296;765-770, 2002.

F—I—2

Real-Time Visualization of Artificial Platelet Interacting with Immobilized Matrix and Native Platelet Bound on the Matrix Surface under Controlled Blood Flow Conditions

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Platelets are known to play crucial roles in hemostasis and thrombus formation, especially in the presence of blood flow. Platelet surface adhesive protein of glycoprotein (GP) Ibα and some of collagen receptor including GP Ia/IIa play particular important roles not only in initial adhesion of platelet at site of vascular injury but also in platelet cohesion. We have developed real-time imaging system enabling to detect shape changes of native platelets as well as artificial platelet composed of albumin polymer and adhesive proteins of GP Ibα and GP Ia/IIIa on their surface under blood flow condition by using ultra-fast laser confocal microscopy with video-enhancement. Outer surface of native platelet were visualized by fluorescination with FITC-conjugated specific monoclonal antibody against GP IIb/IIIa, while artificial platelet composed from albumin polymer was visualized by direct fluorescination of albumin by FITC. On the surface of collagen, GP Ia/IIa conjugated albumin polymer directly and firmly adhered on the surface with multiple bond formation, resulting relatively round shape of the artificial platelet, while on the immobilized von Willebrand factor surface, GP Ibα conjugated albumin polymer less firmly adhered on the surface with elongated shape due to small number of bond formation. Artificial platelets interacting with native platelet bound on the collagen surface shows the shapes similar to those bound on the VWF surface, suggesting the importance of the presence of GP Ibα to make artificial platelet enabling to interact with native platelets.

F—I—3

High Level Expression of Recombinant Human Prethrombin-2 in Mammalian Cells

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We have developed a high-level expression system for human prethrombin-2 in mammalian cells. We have improved an expression plasmid containing the chicken β-actin promoter, well known as a powerful promoter. Structural gene coding prethrombin-2 was placed under the control of the promoter in the plasmid. Chinese hamster ovary cells and mouse myeloma cells were transfected with the plasmid. The cells were subjected to gene amplification and cloning. The expression levels of recombinant prethrombin-2 were over 100 μg/mL in both cell lines. The recombinant prethrombin-2 was activated to α-thrombin by recombinant ecarin, the prothrombin activator derived from snake venom. And we have been able to develop and optimize a large-scale production using over 1000 L-scale fermenters.

We then developed a purification method for recombinant a-thrombin. Recombinant a-thrombin was purified from the conditioned medium of the prethrombin-2 producing myeloma cell line.

Highly purified activated α-thrombin (sp. act.; ca. 2500 NIH Units/mg) was obtained and the final yield was estimated to be about 40 %.

Functional properties of the purified recombinant a-thrombin, such as the conversion activity of human fibrinogen to fibrin and hydrolytic activity of thrombin-specific chromogenic substrate S-2238, were similar to plasma-derived human α-thrombin. This system is suitable for large-scale production of pathogen-free recombinant a-thrombin, which can be used in the place of thrombin derived from human or bovine blood.

F—I—4 Hemorrhagic Shock in Air Breathing Pigs Treated with Bubble-Forming Intravenous Dodecafluoropentane Emulsion.

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In pigs, artificially ventilated with oxygen enriched (up to 26% O_2) air, potentially lethal hemorrhagic shock can be successfully treated by intravenous infusion of 0.3 ml/kg of a 2% dodecafluoropentane (DDFP) emulsion (DDFPe) forming oxygen transporting intravascular microbubbles (1). However in order to simulate a more field-realistic trauma situation the present study was undertaken on spontaneously air breathing pigs.

Pentobarbital anesthetized pigs were bled 36 ± 1 (SE) ml/kg (about 50% of the blood volume) over a 60 min period. The post-hemorrhage systolic blood pressure was 76 ± 2 mm Hg. In the course of 30 min, the control animals (Cs) (n = 6) then received preparation blank at 0.3 ml/kg i.v. Treatment animals (Ts) (n = 5) received 0.3 - 0.5 ml DDFPe/kg i.v. of a 2% DDFPe (i.e. 0.006 - 0.01 ml DDFP/kg). After the sham treatment, blood pressure and muscle Po_2 levels decreased in Cs and they died in 61 ± 8 min. Following DDFPe treatment, the muscle Po_2 in Ts stabilized at pre-hemorrhage levels, urine production continued and they retained systolic blood pressures above 100 mm Hg until euthanasia 5.5 hrs post hemorrhage.

Arterial blood DDFP concentrations of 0.45 - 1.0 μg/ml declined to trace amounts in about 1.5 hrs. The short lasting presence of DDFP suggests a corresponding bubble life span. This poses an interesting aspect on the pathophysiology in this shock model namely, while the direct effect on vital oxygen supply was relatively short, the treatment gave the organism time to activate effective innate defense mechanisms. Alternatively, the bubbles may have become volume stabilized by shells of organic blood-derived molecules.

It is noteworthy that this therapeutic effect was achieved with extremely small doses of DDFPe in combination with air breathing thus contrasting with the obligatory oxygen breathing when erythrocyte substitutes based on liquid fluorocarbons are used. Moreover, part of the beneficial effect of the DDFP emulsion can probably be ascribed to the volume replacement effect achieved when, at body temperature, the liquid DDFP (boiling point 29°C) undergoes phase transition and expands.

We conclude that the treatment modality described above holds considerable promise as a first line intervention in hemorrhagic shock.

1. Tyssebotn, I.M., Bergoe, G.W., Lundgren, C.E.G., Abstract, p. 41, Syllabus of Fourth International Symposium on Current Issues in Blood Substitute Research, June 5-8, 2002, Stockholm, Sweden.

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F—I—5

Hemoglobin Mediated Contraction of Isolated Blood Vessels: Why Is Precontraction Necessary?

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A putative mechanism for hemoglobin (Hb) mediated vascular contraction is ferrous heme-iron scavenging of endothelium derived basal nitric oxide (NO). In the isolated rat aorta, however, Hb mediated contraction occurs only in vessels with prior myogenic tone enhancement (precontraction) with an agonist. To investigate mechanism, we tested a hypothesis that, in the isolated rat aorta, endothelial NO synthase is minimally active in the basal state but upregulated upon treatment with a contractile agonist. Rat thoracic aortic rings prepared with or without the endothelium were bathed in an oxygenated Krebs buffer at 37C. Isometric tension responses to Hb were monitored in the vessel rings precontracted with several distinct types of contractile agonists. In endothelium intact vessel rings precontracted with 25 nM NE, 40 mM KCl, 1.5 μM AVP, 3.4 μM PGF2a, or 0.2 mM 5HT, 2 μM Hb elicited a significant additional contraction (Mean±SD, N=5-8/group, $P<0.05$, t-test): 36.8±1.6%, 28.6±1.4%, 18.8±1.3%, 20.0±1.0%, 16.7±1.1%, respectively. While pretreatment with calmidazolium (calmodulin antagonist), staurosporine and 2,5-dihydromethlcinnamate (protein kinase inhibitors) had no effect, pretreatment with 15 mM 2,3-butanedione monoxime (intercellular gap junction inhibitor) prevented Hb mediated additional contraction. We conclude that, in the isolated rat thoracic aorta and perhaps other vessels, agonist induced contraction appears to upregulate endothelial NO release. Subsequent Hb treatment should then cause a reduction in NO diffusion to the smooth muscle leading to further contraction. This contraction coupled NO release appears to be mediated through a Ca^{++}-calmodulin independent mechanism.

F—I—6

Research on the Purification of Hemoglobin for RBC Substitute

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For the large- scale manufacture of hemoglobin-based red blood cell substitutes, to get highly pure Hb at a high recovery is the first problem to solve. In our method, we deoxygenate hemoglobin by passing nitrogen gas through it and adding non-toxic reduct- ant (vitamine C, tea-polyphenol, etc.) Then we add low molecular weight saccharides (glucose, HES, etc) to it as protectants and adjust it's PH to 6.0. After this we heat the solution at 60℃ for ten hours. We centrifuge it at 5000rpm at 4℃ and ultrafiltrate and filter it . The resultant is a highly purified hemoglobin solution. The content of non-hemoglobin proteins has been markedly reduced to less than 1% as indicated by HPLC. The SDS?PAGE pattern shows that before the purification, there are more than three bands of non-hemoglobin. After heating, there are only one band remained. The oxygen-binding capacity remains unchanged after heating. Maintained in phosphate buffer (15imOsm) for 4 hours under stirring, the recovery of Hb can reach 94%, which was shown to be related to the blood-store time in vitro. At present, we are studying the efficacy of virus-inactivation with this method in accordance with the regulations of the Chinese government.

F—II—1 Pharmacodynamic Study of Polyethylene Glycol Conjugated Bovine Hemoglobin (PEG-bHb) on Animals

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In this pharmacodynamics study, Sprague-Dawley(S-D) rats and Beagle dogs were used. In hemorrhage shock models of rats and dogs, blood pressure rose in 6% PEG-bHb treated animals significantly than dextran 70, even than whole blood (iso-volume of hemorrhage) transfusion. 6% PEG-bHb could ameliorate animals? micro-circulation markedly, not only reduces the viscosity but also may improve the blood flow, autogenous blood only recovered 50% blood flow, and there is no effect on bloods flow when used isovolumic dextran 70. Tissue oxygenations of rats were evaluated by the oxygen dependent quenching of phosphorescence using an Oxyspot phosphorimeter, and the results showed that capability of oxygen-carrying of PEG-bHb was near to native blood and superior to dextran 70. Based on these effects, the survival rates of animals treated with PEG-bHb were close to that of the whole blood transfusion. Similar results were achieved in part exchange transfusion models of rats and dogs. And data suggested that half of hemorrhage transfusion is the reasonable therapeutic dosage. Moreover, the survival time of rabbits of 95% exchange transfusion by PEG-bHb was above 25 hours. In conclusion, PEG-bHb is effective blood substitute with powerful tissue oxygenation and blood volume expansion.

F—II—2

Hemoglobin-Vesicles as Oxygen Carriers: Influence on Phagocytic Activity and Histopathological Changes in Reticuloendothelial System.

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Hb-vesicles (HbV) have been developed for use as artificial O_2 carriers (particle diameter, 250 nm) in which a purified Hb solution is encapsulated with a phospholipid bilayer membrane. The influence of HbV on the reticuloendothelial system was studied by carbon clearance measurements and histopathological examination (Am. J. Pathol. 159, 1079-1088, 2001). The HbV suspension ([Hb] = 10 g/dL) was intravenously infused in male Wistar rats at dose rates of 10 and 20 ml/kg, and the phagocytic activity was measured by monitoring the rate of carbon clearance at 8 hrs, and at 1, 3, 7 and 14 days after infusion. The phagocytic activity transiently decreased one day after infusion by about 40%, but it recovered and was enhanced at 3 days, showing a maximum of about twice the quiescent level at 7 days, and then returned to the normal value at 14 days. The initial transient decreased activity indicates a partly, but not completely, suppressed defensive function of the body. The succeeding increased phagocytic activity corresponds to the increased metabolism of HbV. The histopathological examination with anti-human Hb antibody, hematoxylin/eosin, and oil red O stainings showed that HbV was metabolized within 7 days. Hemosiderin was very slightly confirmed with Berlin blue staining at 3 and 7 days in liver and spleen, though they completely disappeared at 14 days, indicating that the heme metabolism, excretion or recycling of iron proceeded smoothly and iron deposition was minimal. Electron microscopic examination of the spleen and liver tissues clearly demonstrated the particles of HbV with a diameter of about 1/40 of red blood cells in capillaries, and in phagosomes as entrapped in the spleen macrophages and Kupffer cells one day after infusion. The vesicular structure could not be observed at 7 days. Serum clinical laboratory tests indicated no abnormal values except lipid components such as cholesterol and phospholipid that transently increased but returned to the original level. Even though the infusion of HbV modified the phagocytic activity for 2 weeks, it does not seem to cause any irreversible damage to the phagocytic organs. These results offer important information for evaluating the safety issues of HbV for clinical use.

F—II—3

The Effect of Liposome-Encapsuled Hemoglobin on Tissue Oxygen Metabolism of Small Intestine Following Hemorrhagic Shock in Rats

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Introduction: Intestine is known as a vulnerable tissue to hypoxia. Animal studies have demonstrated that liposome-encapsuled hemoglobin (LEH) is an effective resuscitative fluid for hemorrhagic shock (HS). The purpose of this study was to examine if LEH improves oxygen metabolism in rat small intestine after HS.

Methods: HS was initiated by withdrawing blood and mean arterial pressure (MAP) was maintained at 40 mmHg for 30 minutes. Rats were then resuscitated for 120 min with shed blood + 2 x normal saline (NS) or LEH (equivalent volume to shed blood) + 2 x NS. The small intestines were harvested at four different time points; before HS, before resuscitation, at the after resuscitation start 40 min and at the end of resuscitation with shed blood or LEH. The tissue levels (umol/g) of lactate and alanine were measured by ^{1}H magnetic resonance spectroscopy (^{1}H-MRS). Data were expressed as mean ± SD and compared using one way ANOVA followed by Tukey HSD (N= 5 in group).

Results: For MAP, there was statistically no difference between shed blood group and D. Resuscitation with LEH equally to shed blood, significantly reduced intestinal contents of lactate and alanine compared to those levels at the end of HS (* $p < 0.05$).

Conclusion: LEH appears to have a comparable oxygen carrying capacity to blood and may serve as a useful blood substitute.

F—II—4

Multiangle Laser Light-Scattering Method for the Study of Bovine Hemoglobin Dissociation

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Hemoglobin (Hb) is an oligomeric protein, composed of four monomeric subunits. Hb molecule may undergo dissociation from a single native tetramer to two dimmers which is called hemoglobin dissociation. In this article the dissociation of bovine Hb is studied by measurment of the average MW of the samples using the multiangle laser light-scattering method.Advanced multiangle laser light-scattering technique is a powerful method to determine the absolute molecular weights of the protein in solution. Two different methods, microbatch multiangle light-scattering (MALS) and on-line size-exclusion high-performance liquid chromatography light scattering with refractive index detector, are used to measure the average molecular weight of bovine Hb in different concentration respectively. The results of the two methods are agreed well. From the results, it can be concluded that the average molecular weigh of bovine Hb will be about 54 kDa when the bovine Hb concentration is more than 0.15 mg/ml, and will be about 36 kDa when the concentration is less than 0.03 mg/ml. The other conclusion which can be derived from these results is that the dissociation of bovine Hb is related to the pH and the tetramer shows more stable in the pH range of 6-9.

F—II—5

Protective Effects of a Novel Perfluorocarbon Emulsion Administered during Cardiopulmonary Bypass

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Purpose: To evaluate the clinical usefulness of perfluorooctylbromide (PFOB) emulsion during cardiopulmonary bypass (CPB) under a condition of excess hemodilution which can occur during heart operation. Methods: Female beagles (10-12 kg, n=15) were performed CPB for two hours under a mile hypothermic condition. Beagles were divided into three groups: Control(C), Hemodilution (HD: phlebotomy of 900 ml + Albumin 60 ml + Ringer's solution 900 ml)and PFOB (phlebotomy of 900 ml + Albumin 60 ml + PFOB 600 ml + Ringer's solution 300 ml). Hemodynamic parameters (heart rate: HR, mean arterial pressure: MAP, central venous pressure: CVP) were monitored and blood cell counts (WBC, RBC, Plt and Ht), parameters of liver and renal function (GOT, GPT, Cr and BUN) and lactate were measured periodically during CPB. In addition, lung water content was measured after CPB. Results: 1) CVP was markedly decreased in PFOB ($p<0.05$). HR was markedly elevated in HD and PFOB compared with C ($p<0.05$). MAP was significantly elevated in PFOB compared with HD ($p<0.05$), but markedly decreased compared with ($p<0.05$). 2) WBC and Plt did not differ significantly among three groups, although RBC and Ht were markedly low in HD and PFOB ($p<0.05$). 3) Peak values of GPT, BUN and Cr were significantly elevated only in HD group. PFOB significantly inhibited the elevation of lactate observed in HD ($P<0.05$). 4) An increase in lung water content detected in HD was prevented in PFOB ($p<0.05$). Conclusion: It was suggested that PFOB is clinically useful because PFOB prevented the deterioration of hemodynamics, metabolism and organ functions induced by an excess hemodilution during CPB.

F—II—6

Engineering Greater Temperature Dependence for O_2 Binding to Hemoglobins and Myoglobins

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The effects of distal pocket amino acid substitutions on the temperature dependence of O_2 affinity have been investigated using sperm whale cobalt myoglobin mutant as a model system that is resistant to oxidation. Single and double mutations at the Leu(B10), His(E7), Val(E11), Ile(FG5), and Ile(G8) positions in cobalt myoglobin were chosen because similar replacements have been used to reduce the rate of NO scavenging in recombinant Hb-based blood substitutes. Rate constants and P_{50} values for O_2 binding were measured at temperatures ranging from 5 to 37℃. The standard reaction enthalpies, ΔH^0O_2, ranged from -20 to -55 kJ/mol. Stabilizing bound oxygen by electrostatic interaction increases the temperature dependence of P_{50}.and is the strongest when His(E7), Gln(E7) and Phe(B10) are present. Loss of hydrogen bonding interactions decreased the temperature dependence of P_{50}, as seen for Val(E7), Leu(E7), and Phe(E7) mutants. Replacement of Val(E11) with either Ile(E11) or Leu(E11) caused an increase in ΔH^0O_2, from -40 to -50 kJ/mol, and the proximal Ile(FG5) to Phe mutant also has a significantly increased ΔH^0O_2. These results have been used to design multiple mutants with abnormally large temperature dependencies for O_2 binding. The new myoglobins are being used to construct an artificial gill and a filter-based O_2 separator. We are also designing hemoglobins with abnormally large P_{50} temperature dependencies. The goal is to construct a Hb-based blood substitute that can be refrigerated with a low P_{50} and enhanced resistance to autooxidation and heme loss, but still have a high P50 for efficient O_2 transport at 37℃.

F—II—7

Effect of NRC on the Oxygen Metabolism in Acute Massive Hemorrhaged Rats

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Objective

NRC (Neo Red cell) is the liposome encapsulated hemoglobin which has been developed as an artificial oxygen carrier to substitute the blood transfusion during massive hemorrhage in elective surgery. The purpose of this study is to examine the effect of NRC on systemic and tissue oxygen metabolism in acute massive hemorrhaged rats.

Method

SD rats (300 - 400 g) were anesthetized with 1.5% isofluran, immobilized with pancronium bromide and were mechanically ventilated on 45%oxygen. After isovolemic hemodilution (15mL/kg) using hespander, 20 mL/kg was bled and then 20 mL/kg of NRC was administered. Blood pressure and plasma lactate level, as index of systemic oxygen metabolism, were measured. The adenine nucleotide contents in brain and liver were measured as index of tissue oxygen metabolism. Saline and human red blood cell suspension (RBCs) were administered as control to compare with NRC.

Results

NRC dose-dependently improved blood pressure, systemic oxygen metabolism, and oxygen metabolism in the liver. The efficacy of NRC on systemic oxygen metabolism was about 1.5 times superior to that of RBCs.

Conclusion

These results suggest that NRC is useful in improving both systemic and organ oxygen metabolism after acute massive hemorrhage, and that its lower oxygen affinity compared to human erythrocytes is favorable on systemic oxygen metabolism under some amount of oxygen administration.

F—II—8

Increased Survival Time during Second Hemorrhage When First Hemorrhage is Resuscitated with Hemospan™ Compared to Blood or Colloid

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The major obstacle faced in the development of hemoglobin-based oxygen carrying solutions is the offset of oxygen delivery by vasoconstriction and bradycardia. The vascular endothelium is supposed to initiate this limitation, as it senses the oxygenated plasma. A new product, polyethylene-glycol-linked hemoglobin (PEG-Hb), designed to reduce plasma oxygen availability, was used in three different formulations. PEG-Hb possesses low oxygen diffusive capacity, high oxygen affinity and high viscosity. Juvenile 20-kg pigs were resuscitated to normovolemia after a 40 % hemorrhage, by either of four solutions: PEG-Hb 4 % (MP4), PEG-Hb 4 % in Pentastarch 5 % (HS4), Pentastarch 10 % (PS) or the animal's own shed blood served as controls. After a 30 minutes equlibration time, a second hemorrhage was initiated and stepwise continued until death. The survival time was 128 and 80 minutes after resuscitation with HS4 vs. blood, and was in the order: HS4>MP4>PS>blood. PEG-Hb significantly increases the tolerance to hemorrhage in this surgical double hemorrhage model, which indicates that PEG-Hb might be advantageous when control of hemorrhage during surgery is compromised.

F—III—1

Cardiac Output in Hamsters During Progressive Exchange Transfusion with Oxygen and Non-Oxygen Carrying Blood Replacement Fluids

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A modified thermodilution method was developed to measure cardiac output (CO) in awake animals with limited blood volume weighing < 100 g. This technique allows correlating systemic flow changes to those measured in the microcirculation of window preparations. The animal is instrumented with a jugular vein catheter placed proximal to the subclavian vein and a thermocouple positioned at the aortic arch. Two days after implantation, room temperature saline is injected (150 μ l) into the jugular catheter and the temperature change recorded. Cardiac index at baseline conditions (CO/weight) was 197.0 ± 18.8 (ml/min)/Kg (n=16). Stepwise exchange transfusion was performed in another animal group using dextran 70 until hematocrit was reduced by 60% after two exchange transfusions of 40% and 35% of the blood volume. Results show CO increased by 34% and 54% from baseline after each exchange step. Findings suggest that during moderate hemodilution, lowering of blood viscosity and increase in cardiac output may be mechanisms which allow for maintenance of baseline tissue oxygenation and capillary perfusion previously observed in the microcirculation of the window chamber model. Animals were further exchange transfused with either dextran 70 or a Hb-based oxygen carrier (Biopure, veterinarian product, 13 g/dl) until the final hematocrit was reduced by 75%. CO decreased by approximately 10% but the difference between groups was not statistically significant. Results indicate extreme hemodilution leads to significant vasoconstriction as evidenced by both groups showing a 63% decrease in functional capillary density from baseline.
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F—III—2

Microvascular Flow and Tissue Oxygenation after Hemorrhage and Resuscitation with MalPEG-Hb and Polymerized Bovine Hemoglobin in Awake Hamsters

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The effects of two oxygen-carrying plasma expanders were compared in a model of hemorrhagic shock and resuscitation in hamsters implemented with the dorsal skinfold window chamber. Animals were hemorrhaged by 50% of the estimated blood volume and resuscitated after one hour of hypovolemia with 50% of the previously withdrawn volume of either MalPEG-Hb (4 g/dl Hb, viscosity of 2.5 cP, COP 49 mmHg, p50 5.4 mmHg, Sangart Inc., San Diego) or a polymerized bovine hemoglobin (PBH) (Oxyglobin®, 13.1 g/dl Hb, viscosity of 2.0 cP, COP 45 mmHg, p50 54.2 mmHg, Biopure Inc., Boston).

Microvascular diameter, velocity and functional capillary density (FCD), tissue pO_2, base excess and mean arterial pressure were assessed after one hour of resuscitation.
MalPEG incrased FCD 64% vs. 52% for PBH. Microvascular flow increased 16% for MalPEG-Hb relative to baseline and remained decreased by 55%. Total hemoglobin concentrations were 7.5 g/dl (MalPEG-Hb) and 8.8 g/dl (PBH) and tissue pO_2 8 mmHg and 13 mmHg, respectively. Base excess changes were similar with +11.4 mmol/l and +11.6 mmol/l. The presence of MalPEG-Hb improves microvascular blood flow whereas tissue oxygenation is higher with PBH.
Research supported by NIH R24-HL64395, R01-HL 62318 and R01-HL62354.

F—III—3

Effect of the Synthetic Amino-Lipids Formulating Hemoglobin-Vesicles (HbV) on the Circulation Level of the Blood Cells

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The four kinds of dialkyl-aminolipid, which were delivered from glutamic acid were synthesized as vesicular components. The cationic aminolipid (1) was synthesized by esterification of the carboxyl groups of the glutamic acid. The twitter-ionic dialkyl-aminolipid (2) as a main component of the bilayer membrane, the anionic- (3) and PEG-lipids (4) for stabilizing the aminolipid vesicles were delivered from 1. The circulation level of the platelets was significant decreased with injection of the vesicles including anionic phospholipids (DPPG), however no change for the vesicles including 3. The incorporation of anionic 3 into phospholipids vesicles successfully increased the encapsulation efficiency of the hemoglobin. The aminolipid 2 formed bilayer vesicles in buffer solution (PBS, pH 7.4). The dispersion stability of the aminolipid vesicles was successfully increased with incorporation of the 3 and 4. The encapsulation efficiency of hemoglobin into the aminolipid vesicles was equal with that of the phospholipid vesicles. The leakage of the hemoglobin was not observed over one week at physiological conditions (pH 7.4, 37 ℃).

F—III—4

$(PEG_{5K})_6$-Hb: A Non-Hypertensive Hemoglobin Molecule Generated by Conservative PEGylation

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In an attempt to simplify the PEGylation of hemoglobin (Hb) for generating non-hypertensive Hb molecules, a new conservative PEGylation technology has been developed. It combines the high reactivity of maleimides towards thiols with the propensity of iminothiolane to derivatize the ε-NH_2 groups into PEG-maleimide reactive thiol groups. The method has the added built-in benefits of conserving the positive charge of the (-amino groups in the PEGylated molecule, high flexibility and oxy conformational specificity. Several PEGylated Hbs have been generated using this new technology. One product, [$(PEG_{5K})_6$-HbA] that carries six copies of PEG-5000 chains per Hb was non-hypertensive when analyzed using hamster top loads and rat 50% exchange transfusion models. This PEGylated Hb has (i) a hydrodynamic volume corresponding to that of a globular protein of 256 kDa molecular mass; (ii) a molecular radius of 6.8 nm; (iii) high O_2-affinity; (iv) increased viscosity and oncotic pressure. These properties are consistent with the emerging new paradigms for the design of Hb-based blood substitutes. Another conservatively PEGylated Hb, $(PEG_{20K})_2$-HbA, with two PEG-20K chains [one each on Cys-93(β)], exhibiting most of the properties of $(PEG_{5K})_6$-HbA, remains vasoactive. One possible explanation for this difference in vasoactivity arises from a higher degree of surface shielding occurring for the $(PEG_{5K})_6$-HbA. The simplicity, flexibility and the high efficiency of the PEGylation reaction intrinsic to this new PEGylation technology makes the large scale production of non-hypertensive Hb molecule cost-efficient and should facilitate the development of new classes of Hb-based therapeutics.

F—III—5

The Influence of Hemodilution and Oxygen Affinity of Hemoglobin Vesicles on the Oxygenation in Ischemic Hamster Flap Tissue

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In previous experiments, we have shown that the oxygenation in ischemic and hypoxic, collateralized hamster flap tissue could be improved by normovolemic hemodilution with lipsosome encapsulated hemoglobin vesicles (HbV) dissolved in 6% dextran 70 (HbV-Dx70). The aim of this study was to investigate the effect of HbV at various degrees of hemodilution and the influence of oxygen affinity of the HbV. To this end, hamsters were exposed to a gradual normovolemic hemodilution of 20%, 40% and 60% blood exchange with Dx70 and HbV-Dx70. The P50 was set at either 15 mmHg (HbV15) or 30 mmHg (HbV30). The Hb concentration of the final solutions was 7.5 g/dl. Microvascular blood flow (measured by intravital microscopy) continuously increased to approximately 150% of baseline after the 60% hemodilution in all animals ($p < 0.01$ vs. control and baseline, ns between hemodilution groups). Tissue oxygenation (Clark-type microprobes) was transiently raised to 117 $\pm$ 15% of the baseline value during hemodilution with Dx70 ($p < 0.05$), whereas it was gradually increased after each step of hemodilution with the HbV solutions, reaching 163 $\pm$ 36% ($p < 0.01$) and 217 $\pm$ 67% ($p < 0.01$, $p < 0.05$ vs. HbV30-Dx70) after the 60% hemodilution with HbV30-Dx70 and HbV15-Dx70, respectively. From our results we conclude that ischemic tissue may benefit from normovolemic hemodilution with HbV solutions even at a high degree of blood exchange. Our results suggest that HbV provides better oxygen delivery to that tissue than red blood cells. Furthermore, better oxygenation was achieved by using the HbV with the higher oxygen affinity.

F—III—6

Ability of Polymorphonuclear Neutrophil to Respond to Infection in Presence of Cell-Free Hemoglobin. An in vitro Study.

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Cell free hemoglobin was reported to be able to potentate infection. Since some hemoglobin-based oxygen carriers were developed for clinical use in infectious environment such as septic shock or in trauma patients, it appears important to check the possible alteration of antibacterial functions of leukocyte after infusion of hemoglobin solution. However, few studies have investigated the preservation of leukocyte functions in presence of large quantities of cell-free hemoglobin. We have previously demonstrated the absence of activation of these cells by infusion of HBOCs but the ability of PNN to respond to infection was not yet investigated. In this study, performed in vitro, human polymorphonuclear neutrophils were incubated for 30 minutes at 37°C under gentle stirring with purified cell free hemoglobin or modified hemoglobin solutions at two different concentrations: 0.16 g/L to simulate hemolysis conditions or 16 g/L, a clinically relevant concentration that may be found in the plasma after infusion of HBOCs. Afterwards, phagocytic function of these cells was analyzed by flow cytometry, oxygen-derived free radicals release was investigated by nitroblue tetrazolinium reduction and bactericidal activity against Escherichia Coli and Staphyloccocus aureus was measured. The preliminary results seem to show that in presence of cell-free hemoglobin at the concentration of 16 g/L, the number of bacteria ingested by the neutrophil decreased whereas the percentage of active cells was not modified as compared to the control. However, the oxygen-derived free radicals release did not appear to be altered by the presence of cell free hemoglobin whatever the concentration tested.

P—1

Combining Hemoglobin with Adenosine and Reduced Glutathione Attenuates Its Direct and Indirect Neurotoxic Potential

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With the current problems in blood availability and safety, the potential role of red cell substitutes has become critical. Current products under testing, however, have met with limited success. The problems revolve around blood vessel constriction and the pro-oxidant and pro-inflammatory properties of hemoglobin (Hb). Toward a resolution of these problems, we have developed a novel Hb modification procedure to formulate a more effective and non-toxic product. This red cell substitute is composed of purified Hb, crosslinked intramolecularly with o-ATP and intermolecularly with o-adenosine, and conjugated with reduced glutathione (GSH). In a recent study, we investigated the direct neurotoxic potential of our red cell substitute using cultures of human brain neurons and astrocytes (ASAIO J 46(2):231,2000). While the obtained data demonstrated a lack of neurotoxicity, this research failed to answer the question about the potential indirect neurotoxic effect of this product via the activation of brain capillary endothelial cells (EC). This question was important, since during a 1998 clinical trial, one Hb-based blood substitute (HemAssist, Baxter) was found to cause adverse effects in patients with acute ischemic stroke (Stroke 30:993-6,1999). Therefore, this study compared the effects of our novel red cell substitute and those of unmodified (U) Hb, using plasma as a control, on normal and glutathione depleted human brain capillary EC. Particularly, we focused on GSH depleted cells to mimic the condition of ischemic patients with a diminished ability to control oxidative reactions of Hb. Confluent EC grown on 0.4 μm porous cell culture devices, cover slips and cell culture plates, were incubated overnight with 0.4 mM Hb solutions or plasma. After treatment, the cells grown on porous devices were tested for permeability by determining the diffusion rate of 125I-albumin across the monolayer. EC grown on coverslips were evaluated for early and advanced apoptosis using Annexin V-FITC and propidium iodide fluorescence probes, respectively, and for the expression of adhesion molecules. The pro-oxidant effect of Hb on EC grown in cell culture plates was examined by the measurement of intercellular GSH, lipid peroxidation and 8-isoprostanes. Results indicate, that UHb increases cell permeability and shrinkage, initiates oxidative stress and apoptotic events, and inflammatory responses. These effects are aggravated in GSH depleted cells ($p<0.01$). Contrarily, our red cell substitute did not appear to induce oxidative stress nor to increase inflammatory reactions of normal and GSH depleted EC. The diffusion rate of 125I-albumin was similar to that of the control in both tested groups. Obtained results suggest a lack of indirect neurotoxic potential (via activation of brain capillary endothelium) of the Hb-adenosine-GSH complex. The effects of this red cell substitute can be linked with the type of modification procedure that lowers Hb pro-oxidant potential and the anti-inflammatory and cytoprotective properties of adenosine.

P—2 Duration of Efficacy of NRC Administered in Divided Doses

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In blood substitute, it is considered preferable to give a necessary amount in multiple divided doses under controlling the hemoglobin concentration and monitoring the patient status from the viewpoint of using an appropriate amount of drug. In this experiment, NRC was administered to a rat hemodilution model in divided doses to investigate whether it is possible to maintain the efficiency in improving oxygen metabolism by keeping hemoglobin concentration over the Critical DO2 level.

Using male CD (SD) IGS rats, 75% of the blood was exchanged with homologous plasma. Then, NRC was administered as a single dose of 20 mL/kg or as two divided doses of 10 mg/kg at an interval of 3 hours. The NRC-crit (NRC volume as percentage of blood) and methemoglobin rate of NRC were determined and hemodynamics, blood lactate and blood gases were measured. The rats were observed for 6 hours after administration.

When NRC was administered as a single dose of 20 mL/kg, “the half-life of efficacy” calculated from the hematocrit value and methemoglobin rate as an index of actual oxygen transport efficiency was estimated to be approximately 6 hours after administration. And it is indicated that efficiency in improving oxygen metabolism was maintained. In addition, when NRC was administered as two divided doses of 10 mL/kg at an interval of 3 hours, the similar tendency was observed, and the efficacy of NRC was verified on maintaining the hemoglobin concentration exceeding the level of Critical DO_2.

P—3 The Oxygen Delivery of Artificial Oxygen Carrier with High Oxygen Affinity for Ischemic Tissues

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The artificial oxygen carrier (Neo Red Cell, NRC) has been shown to be effective in the treatment of ischemic diseases such as cerebral infarction. And now, the NRC which has been controled with high oxygen affinity(HA-NRC) can also deliver oxygen more efficiently to restricted tissues with low oxygen content due to ischemia. In this report, the dynamics of oxygen supply for ischemic tissues with HA-NRC and low oxygen affinity NRC(N-NRC) was evaluated the oxygen release using a spinner flask in vitro, and the isolated liver of rats for an ex vivo as the ischemia-reperfusion model.

In the in vitro model, HA-NRC released substantial oxygen under the low dissolved oxygen condition compare with N-NRC. In the ex vivo model, isolated liver in an ischemic state was perfused with both artificial oxygen carrier with different oxygen affinity, and the oxygen consumption, lactate level of perfusate and the energy charge (EC) in the center or at the peripheral tissue in isolated liver were measured.

When the isolated liver was perfused with HA-NRC, the oxygen consumption of the liver was larger and the lactate level of perfusate was lower than that of perfused with N-NRC. Whereas the ATP level in isolated liver perfused with N-NRC was extend all around the isolated liver, when the isolated liver perfused with HA-NRC, EC in the center of liver was higher than peripheral. This result suggested that the oxygen can be released to the restricted area of isolated liver which was perfused with HA-NRC.

P—4

Pretreatment of Blood Serum Containing Hb-Vesicles for Accurate Clinical Laboratory Tests.*

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Hb-vesicles (HbV, diameter: 251 ± 81 nm) are artificial oxygen (O_2) carriers encapsulating concentrated hemoglobin solution with phospholipid bilayer membrane, and their O_2 transporting ability in vivo has been extensively studied. It is important to clarify the interference of the HbV suspension on serum clinical laboratory tests, and to establish a pretreatment method to avoid such an interference. The HbV suspension, acellular Hb solution ([Hb] = 10 g/dL), or saline was mixed with a pooled human serum at various mixing ratios up to 50 vol% ([Hb] = 5 g/dL), and the magnitude of the interference effect of HbV and Hb on 30 analytes was studied. The mixture of the HbV suspension and serum was ultracentrifuged (50,000g, 20 min) to remove the HbV particles as a precipitate and the supernatant was analyzed to compare with the saline control group. The HbV particles were also removed by centrifugation (2,700g, 30 min) in the presence of dextran (Mw. 200 kDa). The HbV suspension showed considerable interference effects in most analytes, and most of which were more serious than those of the acellular Hb solution. These findings are thought to be due to the light absorption of Hb in HbV and/or the light scattering derived from the suspension that interfere with the colorimetric and turbidimetric measurements. The components of HbV may also interfere with the chemical reaction of the assays. However, removal of the HbV from the supernatant diminished the interference with the most of the analytes. This would be an advantage of HbV in comparison with acellular chemically-modified Hb solutions.

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P—5

Reduction of MetHb via Electron Transfer from Photoreduced Flavin and Restoration of O_2-Binding Ability of Hb-Vesicles as an O_2 Carriers

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Ferric methemoglobin is reduced to its ferrous form by photoirradiation either by direct photoexcitation of heme portion to induce electron transfer from the surrounding media (Sakai at al., Biochemistry 39, 14595-14602 (2000)) or by indirect electron transfer from a photochemically reduced electron mediator such as flavin. In this research we studied the mechanism and optimal condition that facilitate photoreduction of flavin mononucreotide (FMN) to $FMNH_2$ by irradiation of a visible light, and the succeeding reduction of metHb to restore the O_2 binding ability. Irradiation of visible light (450 nm) to a metHb solution containing FMN and an electron donor such as methionine and EDTA showed significantly fast reduction to ferrous Hb with a quantum yield (ϕ) of 0.12, that is higher than the method of direct photoexcitation of heme (ϕ = 0.006). The rate constant for the reduction of metHb with $FMNH_2$, measured with a laser flash photolysis system, should be larger than 10^{12} $M^{-1}s^{-1}$ which is 6 orders larger than the reported value. Native-PAGE and IEF electrophoresis indicated no chemical modification of the surface of the reduced Hb. Encapsulation of conc. Hb and the FMN/EDTA system with phospholipid bilayer membrane to form Hb-vesicels (HbV, diameter: 250 nm) as an artificial O_2 carrier also showed prompt photoreduction of metHb even in an aerobic condition. The reduced HbV showed reversible O_2 binding property. A conc. HbV suspension ([Hb] = 10 g/dL) was sandwitched with two cover glasses to form a liquid membrane with the thickness of about 10 μ m (close to capillary diameter in tissue, 5 μ m) and irradiation of visible light (221 mW/cm^2) completed metHb photoreduction within 10 sec. This fast photoreduction system thus demonstrates a possiblility for the in vivo use to restore O_2transporting ability of infused HbV when oxidized.

P—6 Compatibility of Albumin-Heme with Blood Cell Components

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Recombinant human serum albumin including 2-[8-{N-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-o-pivaloyamino)phenylporphinatoiron(II) (albumin-heme; rHSA-FeP) is a synthetic hemoprotein which has sufficient capability to reversibly bind and release O_2 under physiological conditions (pH 7.3, 37℃) similar to hemoglobin and myoglobin. In order to use this albumin-based O_2-carrier as a new class of red blood cell substitutes, its compatibility with whole blood was carefully investigated in vitro.

The interaction of rHSA-FeP with plasma was first evaluated from the thermodynamic behavior in rapid mixing of rHSA-FeP solution with rHSA by micro-calorimetry measurements. The transference of FeP to the free albumin molecules (large amount of excess) obviously took place, however, this transference of the water-insoluble FeP could only happen when rHSA-FeP directly contact with other albumin molecules.

After the addition of the rHSA-FeP solution into whole blood at 10, 20, and 44 vol%, the FeP concentration in the plasma phase remained constant for 6 hr at 37 oC in each group, and no significant time dependence was observed in the numbers of red blood cells, white blood cells, and platelets. The microscopic observations clearly showed that the shapes of the red blood cells have not been deformed during the measurement period. With respect to the blood coagulation parameters (prothrombin time and activated partial thromboplastin time), the coexistence of rHSA-FeP had only a negligibly small influence. Furthermore, the blood compatibility under dynamic flow conditions was also evaluated using a microchannel array flow analyzer. All these results suggested that the albumin-heme has no effect on the solution properties as well as the functions of the blood cells in vitro.

P—7

Exchange Transfusion of Albumin-Heme as an Artificial Oxygen Carrier into Anesthetized Rats: Physiological Responses and Oxygen Delivery

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Recombinant human serum albumin (rHSA) incorporating synthetic hemes, 2-[8-{N-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-o-pivaloylamino) phenylporphinatoiron(II) (FepivP) and 2-[8-{N-(2-methylimidazolyl)}octa- noyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-o-1-methylcyclohexanoylamino)phenylporphinatoiron(II) (FecycP), are artificial hemoproteins [albumin-heme (rHSA-FepivP, rHSA-FecycP)] which are able to reversibly bind and release dioxygen under physiological conditions (in aqueous media, pH 7.4, 37℃) like hemoglobin and myoglobin.

Physiological responses to exchange transfusion with albumin-heme solutions (rHSA-FepivP and rHSA-FecycP, [rHSA]: 5 g/dL, heme/rHSA: 4 (mol/mol)) into anesthetized rats after hemodilution and hemorrhage (Hct: ca. 10%) have been carefully evaluated. The declined mean arterial pressure (MAP) and blood flow after a 70% exchange with rHSA and the further 30% bleeding of blood, were significantly recovered up to about 90% of the baseline values by the injection of albumin-heme.

The mixed venous partial O_2-pressure (PvO_2) is one of the significant parameters for the O_2-supply. The decreased PvO_2 generally indicates a low O_2-saturation of Hb and implies declined O_2-transport. In the albumin-heme groups, the reduced PvO2 after 30% bleeding rapidly increased after the injection. Furthermore, the renal cortical O_2-tensions and skeletal tissue O_2-tensions also elevated, indicating the in vivo O_2-delivery of albumin-hemes.

P—8

Human Serum Albumin Hybrids Including Iron Complex of Protoporphyrin IX with a Proximal Base and their Dioxygenation

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Recombinant human serum albumin (rHSA) incorporating tetraphenylporphyrinatoiron derivative bearing a proximal base (imidazolyl group) can bind and release O_2 reversibly under physiological conditions like hemoglobin and myoglobin. We report herein the synthesis of novel iron(II) protoporphyrin IX derivatives containing two types of proximal base propionates (FePPs) and the dioxygen binding abilities of their rHSA hybrids (rHSA-FePP).

FePPs were synthesized from protoporphyrin IX via two steps. First, the proximal base (imidazolyl or histidiyl group) and ethyl group were introduced simultaneously into each propionic acid residue of protoporphyrin IX using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) in dimethyl formamide (DMF). Second, iron insersion was carried out using $FeCl_2$ in DMF to afford the corresponding compounds.

FePPs can bind and release dioxygen reversibly in DMF solution. rHSA-FePP was prepared by mixing the aqueous rHSA solution and ethanolic CO-coordinated FePP. The isoelectric point and circular dichloism sepecta of rHSA-FePP were as same as those of rHSA (pI 4.8). These results indicated that the surface charge distribution and second-order structure of rHSA host did not change after the FePP binding.

Under O_2 atomsphere, the UV-Vis. spectra of rHSA-FePPs showed the formation of the O_2-coordinated porphyrinatoiron. The O_2-binding properties of rHSA-FePPs are discussed detail.

P—9

Size-Exclusion High Performance Liquid Chromatography (SE-HPLC) with UV Absorbance, Light-Scattering and Refractive Index Detectors to Determine the Molecular Weight and Distribution of Molecular Weight of the PEGylated Bovine Hemoglobin(PEG-bHb)

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The method to determine the average molecular weight and distribution of molecular weight of the PEGylated hemoglobin(PEG-bHb) and their molecular weight of polypeptides in PEG-bHb with UV absorbance, light- scattering and refractive index detectors is described in this paper. The results indicate that when molar ratio of PEG to hemoglobin is 7:1, the average molecular weight of PEG-bHb(M_{CP}) is 95kDa, the average molecular weigh of protein moiety in PEG-bHb(M_p) is 68kDa, and their distribution are from 48-157kDa and 44-121kDa respectively; when the molar ratio is 10:1, the MCP is 106kDa, the MP is 70 kDa, and their distribution are from 56-216kDa, 49-135kDa respectively; when the molar ratio is 13:1, the MCP is 131kDa, the MP was 73kDa, and their distribution are from 81-272kDa, 41-144kDa respectively. It has been proved that this method is a simple and reliable way for the determination of average molecular weight and distribution of molecular weight of PEGlyted hemoglobin. This experiment also suggests that there exists not only aggregation in the frontal of elution peak of PEG-bHb, but dissociation of tretramer of hemoglobin into dimmer in the trail of elution peak, and the degree of aggregation is depended on the molar ratio of PEG to hemoglobin due to the present of doil PEG, the dissociation is dependant on the concentration of hemoglobin.

Key words: SE-HPLC, light-scattering, molecular weight, distribution of molecular weight, PEG-bHb

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P—10 Reduction of Methemoglobin Vesicles by Using Membrane Permeation of Reductants

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Oxyhemoglobin(oxyHb) of the Hb-based oxygen carriers is gradually autoxidized to the ferric state(metHb) and loses its oxygen binding ability. We have developed Hb vesicles which encapsulate concentrated Hb with phospholipid bilayer membrane and kinetically analyzed the reduction system of metHb in the vesicle by addition of reductants(cysteine(Cys), homocysteine(Hcy), ascorbic acid(AsA)) to the Hb vesicles dispersion. In the nitrogen atmosphere, metHb in the vesicle was reduced by Cys or Hcy due to the permeation through the phospholipid membrane, whereas not reduced by AsA. When the oxygen partial pressure was 40 torr, metHb in the vesicle was not reduced by Cys or Hcy. It was assumed that hydrogen peroxide (H_2O_2) generated during the autoxdation of the reductants was able to permeate through the membrane and oxidized Hb. On the basis of the assumption, we prepared the catalase-coencapsulated Hb vesicles and confirmed that metHb in the vesicle was effectively reduced by the reductants, showing the possibility of the reduction system of catalase-coencapsulated Hb vesicles by using membrane permeation of the reductants.

P—11

Increased Oncotic Pressure vs. Plasma Viscosity as Determinants of Functional Capillary Density Following Sequential Administration of Dextran 70 kDa and Hetastarch in Extreme Hemodilution

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Extreme hemodilution with 6% dextran 70 kDa, a low viscosity plasma expander, reduces functional capillary density (FCD) to pathological levels in the awake hamster chamber window model. However, if dextran 500 kDa, is administered in the final step, plasma viscosity increases and FCD is maintained. We hypothesized that the same effect could be obtained with 20% hydroxyethyl starch (HES) 200 kDa, a high viscosity (14.5 cp) and high oncotic pressure (COP > 200 mmHg) fluid. To determine whether the effect was due to increased viscosity and/or COP, we also used 10% HES (COP 90 mmHg, 4.3 cp). Two isovolemic hemodilution exchanges were performed with 6% dextran 70 until hematocrit (HTC) was reduced by 65%. This was followed by 35% hemodilution of blood volume with either 10% (H10) or 20% (H20) HES suspended in isotonic saline. Blood pressure was reduced to 65% of baseline in both groups. Heart rate reduced by 13% in the H20 and was unchanged in the H10 group. FCD was reduced by 27% and 22% in the H10 and the H20 group and the difference was not statistically significant. Final plasma viscosity was 1.2 cp in both groups and COP was 11.8 mmHg and 16.9 mmHg in the H10 and the H20 group. These results show that using a hyperoncotic plasma expander in extreme hemodilution maintains FCD, an effects that appears to be related to increased blood volume due to the elevated oncotic pressure.

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P—12 Systemic and Microvascular Responses to Hemorrhagic Shock and Resuscitation with Hb-vesicles.*

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The ability of Hb-vesicles to restore from hemorrhagic shock was evaluated in conscious hamsters with dorsal skinfold window preparation. HbV was suspended in 8% HSA at Hb concentrations of 3.8 g/dl (HbV(3.8)/HSA) and 7.6 g/dl (HbV(7.6)/HSA). Shock was induced by 50% blood withdrawal and mean arterial pressure (MAP) at 40 mmHg was maintained for 1 hr by the additional blood withdrawal. The hamsters receiving either HbV(3.8)/HSA or HbV(7.6)/HSA suspensions restored MAP to 93 ± 14 and 93 ± 10 mmHg, respectively, similar with those receiving the shed blood (98 ± 13 mmHg), which were significantly higher by comparison with resuscitation with HSA alone (62 ± 12 mmHg). Only the HSA group tended to maintain hyperventilation and negative base excess after the resuscitation. Subcutaneous microvascular blood flow reduced to about 10 - 20% of baseline during shock, and re-infusion of shed blood restored blood flow to about 60 - 80%, of baseline, an effect primarily due to the sustained constriction of small arteries A0 (diameter 143 ± 29 μ m). The HbV(3.8)/HSA group had significantly better microvascular blood flow recovery and non-significantly better tissue oxygenation than the HSA group. The recovery of base excess and improved tissue oxygenation appears to be primarily due to the increased oxygen carrying capacity of HbV fluid resuscitation.

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P—13

Studies on the Immunogenicity of PEGylated Bovine Hemoglobin (PEG-bHb)

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Methoxypolyethylene glycols of 5000 daltons(mPEG-5000) were covalently attached to the lysine residues of bovine hemoglobin . In order to conjecture the immunological responses after infusion PEG-bHb into humans, we did the next experiments on animals. First, using the type I hypersensitivity model to confirm which the degree of modification of PEG-bHb is safety. Guinea-pigs were sensitized 3 times interval days by the intramuscular injection of 0.5ml of the different modification of PEG-bHb (14%, 18%, 26%). After 14 days or 21days of the last sensitization, each animal received 2ml the same antigen solution i.v. at challenge. The results showed that the average modificational degree, which is attachment of PEG-5000 to the lysine residues, reached 18% is to be safety. Second, the effect of PEG-bHb (18%) on the humoral immunogenicity was ascertained by measuring the amount of antigen-specific antibodies present in the sera of immunized animals. The first immunization in a dose of 0.5mg of sample/rat/immunization (or 0.2mg of sample/mouse/immunization) was given intraperitoneally. The same dose was given repeatedly every two weeks and after 12 days of the last immunization the animals were exsanguinated. Serum levels of bHb-specific IgG or PEG-bHb-specific IgG were measured by ELISA method. The results shows that the intravenous antiserum did not yield detectable antibodies against PEG-bHb (18%). Inference can be drawn that there would unlikely emerge accidents in humans after infusion of PEG-bHb. Of course, it needs to be proven further.

P—14

Evaluation of Secondly Hemostasis for Oligopeptide-Conjugated Latex Beads Enhanced Effect as Platelet Substitutes

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We have studied prototypes of platelet substitutes and focused on the oligopeptides having a part of the amino acid sequence of fibrinogen as a recognition site to GPIIb/IIIa. The oligopeptides were conjugated to latex beads (1 μmφ) with N-succinimidyl 3-(2-pyridyldithio) propionate. The oligopeptide-conjugated latex beads (oligopeptide-LB, $1.0x10^5$/μL) were evaluated with thrombocytopenic blood ([platelet]=$2.0x10^4$/μL) under flow conditions (shear rate; $1600s^{-1}$). When thrombocytopenic blood adding the oligopeptide-LB was flowed on the collagen-immobilized plate (collagen-plate), the surface coverage (SC) of the $DiOC_6$-labeled platelets was gradually increased to 5.6±4.1% in comparison with the control experiment (bare LB, 3.1± 0.4%). The same experiment was carried out with FITC-labeled oligopeptide-LB, instead of platelets. The binding rate was initially low, and then steeply increased. The SC for the oligopeptide-LB was 13±2.6% (bare LB, 1.8±0.4%), and the overlap of the oligopeptide-LB was also confirmed. The above results suggest that the adhesion of the oligopeptide-LB was induced by the platelets which had already adhered on the collagen-plate, and the oligopeptide-LB was bound on the platelets, recruiting the flowing platelets in the aggregates.

P—15

Inflammatory Reactions Induced by an Application of Perfluorooctylbromide Emulsion During Cardiopulmonary Bypass with Moderate Hemodilution

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Purpose: To evaluate inflammatory reactions induced by application of perfluorooctylbromide (PFOB) emulsion during cardiopulmonary bypass (CPB) under a condition of excess hemodilution which can occur during heart operation. Methods: Female Beagles weighting 10-12kg (n=15) were performed CPB for two hours under a mild hypothermic condition. Beagles were divided into three groups: Control(C), Hemodilution (HD: phlebotomy 900 ml + Albumin 60 ml + Ringer's solution 900 ml) and PFOB (iphlebotomy 900 ml + Albumin 60 ml + PFOB 600 ml + Ringer's solution 300 ml)(n=5 each). During the experiment, we measured WBC, serum complement titer (CH50) and plasma vasoactive substances including bradykinin, histamine and calcitonin-gene-related peptide (CGRP). Results: ÇP) There was no difference C among three group. 2) In HD CH50 was markedly elevated compared to C and PFOB ($p<0.05$). 3) Bradykinin and histamine were significantly elevated in HD ($p<0.05$), but there was no difference among three groups in CGRP. Conclusion: A novel artificial red blood cell substitute, PFOB emulsion, may be useful during CPB, because it prevented the complement activation and liberation of bradykinin and histamine induced by hemodilution.

P—16 Effect of Surfactant and Perfluorocarbon Type on Droplet Size Stability of Oxygen-Carrying Microemulsions.

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Perfluorocarbon (PFC) microemulsions are very useful as intravenous oxygen carriers. A surfactant, typically egg-yolk lecithin is required to attain stability. The objective of this study is to evaluate the effect of three different types of lecithin and two PFCs on microemulsion droplet size stability.

Microemulsions were prepared using a microfluidizer. Six different formulations were prepared combining one of two PFCs with one of three aqueous lecithin concentrates. All formulations contained 40 % w/v PFC, and 9 % w/v lecithin. The PFCs tested were perfluorooctyl bromide and perfluorodecalin. Two egg-yolk lecithins, Epikuron 200 and Ovothin 160, and one soybean lecithin, Epikuron 170 (Lucas Meyer GmbH, Germany), were used. Droplet size was measured using a particle size analyzer on accelerated aging stability tests, which assessed droplet size stability up to 24 months.

Droplet size varied between 16 and 52 nm. Droplet size was not influenced by accelerated time. The type of lecithin highly affected particle size, being lower using Ovothin 160, followed by Epikuron 200. Significant sedimentation was noticed in Epikuron 200 microemulsions, occurring immediately after preparation.

In conclusion, stable PFC microemulsions are being prepared. All formulations present long-term droplet-size stability on accelerated aging tests. These results are being corroborated by normal on-shelf stability. Droplet size appears to be affected mainly by the physical characteristics of the lecithin concentrate. The aqueous lecithin concentrate used and the presence of cholesterol and fatty acids in Ovothin 160 may be involved in its better performance as a surfactant.

P—17 Resuscitation from Hemorrhagic Shock with Hemoglobin-Vesicles Suspended in Recombinant Human Serum Albumin.

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A phospholipid vesicle encapsulating a concentrated hemoglobin solution (Hb-vesicle, HbV) has been developed to provide O_2 carrying capacity to plasma expanders. Its ability to restore the systemic condition after hemorrhagic shock was evaluated in anesthetized Wistar rats for 6 hrs after resuscitation. The HbV was suspended in 5% recombinant human serum albumin (HbV/rHSA) at an Hb concentration of 8.6 g/dl. Shed autologous blood (SAB), washed homologous RBC suspended in HSA (wRBC/rHSA, [Hb] = 8.6 g/dL), and HSA were used as controls. Shock was induced by 50% blood withdrawal. The rats showed reduction of mean arterial pressure (MAP) to 32 ± 10 mmHg, and significant metabolic acidosis and hyperventilation. After 15 min, the shocked rats were received either of HbV/rHSA, SAB, wRBC/rHSA, or rHSA alone (n = 8). The rats in the HbV/rHSA group restored MAP to 93 ± 8 mmHg at 1 hr, similar with those receiving SAB (92 ± 9 mmHg), which were significantly higher in comparison with resuscitation with rHSA alone (74 ± 9 mmHg), and wRBC/rHSA (79 ± 8 mmHg). There was no significant difference in blood gas parameters between the groups, however, 2 of 8 rats receiving rHSA alone died before 6 hrs after resuscitation, while in the other groups all rats survived. Histological examination of the HbV/rHSA group demonstrated no morphological abnormalities in lung, liver, and kidney. Spleen in the HbV/rHSA group showed accumulation of HbV particles in macrophages as a normal clearance pathway for HbV. All the groups showed a slight ischemic damage in myocardium indicating that ischemic damage did not heal after 6 hrs. Blood serum tests indicated slight increment of alanine aminotransferase / aspartate aminotransferase serum activities for all the groups resuscitated with the fluids containing HbV or RBC but not with the rHSA group. As a conclusion, HbV suspended in rHSA is effective for the restoration from hemorrhagic shock that is comparable with shed autologous blood.

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Oxygen Release from Hb-vesicles: Comparison with Red Blood Cells and Acellular Hemoglobin Solution Using an Artificial Oxygen Permeable Narrow Tube with 28 μm Inner Diameter.

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The O_2 releasing behavior of Hb-vesicles (HbV, diameter, 250 nm) was examined using O_2 permeable fluorinated ethylenepropylene copolymer tubes (inner diameter, 28 μm; outer diameter, 100 μm) exposed to nitrogen-saturated deoxygenated saline containing 10 mM sodium dithionite. Measurements were performed using an apparatus built on an inverted microscope that contained a scanning-grating spectrophotometer with a photon count detector connected to two photomultipliers and an image processor through a video camera. The rate of O_2 release from a sample solution flowing in the narrow tube (centerline flow velocity: 1 mm/s) was determined based on the visible absorption spectrum (500 - 600 nm). Both HbV and fresh human RBC were suspended in 5% human serum albumin solution at [Hb] = 10 g/dL, and they were mixed at various mixing volume ratios. The mixtures of acellular Hb solution and RBC were also tested.

Since HbV was homogeneously dispersed in albumin solution, increasing the HbV content resulted in thicker marginal RBC-free layer. Irrespective of the mixing ratio, the O_2 releasing rate was similar with that of RBC alone. On the other hand, the addition of acellular Hb solution (10 g/dL) to RBC by 50 vol% significantly enhanced deoxygenation. This outstanding discrepancies of O_2 releasing rate between HbV and acellular Hb solution should be due to the differences in molecular size (250 nn vs. 8 nm) and viscosity of the marginal RBC-free layer of the mixture.

P—19 Effects of Hemoglobin Vesicles on Blood Cells and Complement in Rat

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Hemoglobin vesicles (HbV) are human hemoglobin encapsulated into lipid bilayer with polyethylene glycol surface modification and have been developed as an artificial oxygen carrier. Evaluation studies of the biocompatibility in vitro using human blood have revealed that the function of platelets and neutrophils was little affected and that coagulation activity was not changed by HbV. In the latest study, we have assessed the effects of HbV in vivo regarding on blood cells and complement.

The HbV suspension (3.3 mL, final 20%) was injected from a tail vein of WKAH rats under ether anesthesia. Either empty liposome or saline was also injected into rats as a control. Peripheral blood was sampled at 6 hours, 1, 3 and 7 days after the injection. Total numbers of white blood cells, red blood cells and platelets were unchanged in the HbV, empty liposome and saline groups during the observation period. The rise of granulocyte ratio, concomitantly with the fall of lymphocyte ratio, was observed at 6 h after the injection both in HbV and empty liposome group. The changes were more prominent in the HbV group. The ratio of leukocytes returned to the level of pre-injection at 1 day after the injection and subsequently unchanged up to 1 week. Although the ratio of lymphocytes was decreased at 6 h post-injection, little change was observed in the distribution of lymphocyte subset such as T cells, B cells, $CD4^+$ cells and $CD8^+$ cells. Complement activity (CH50) of sera was decreased by 10% in the HbV group at day 3, but returned to the level of the saline group at 2 weeks after the injection.

The HbV administration affected leukocytes and complement in the peripheral blood of rat, but the changes were transient and small. These results suggest that host defense may be sufficiently maintained after the HbV administration.

P—20 Prolongation of the Oxygen-Carrying Ability of Catalase-Encapsulated Hb-Vesicles in vivo

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Hemoglobin (Hb) binds oxygen reversibly in the ferrous state and is gradually oxidized to metHb (Fe^{III}). In the use of the Hb-based oxygen carriers, the oxidation of ferrous Hb to nonfunctional metHb is an important concern. In the Hb vesicles (particle diameter 250nm), in which a purified Hb solution is encapsulated with a phospholipid bilayer membrane, the reduction of metHb by ascorbic acid and glutathione does not occur because of the low membrane permeability of these reductants. And reactive oxygen species (H_2O_2, O_2^{-}*) are also other causes of the metHb formation.

We co-encapsulated catalase with Hb to suppress the metHb formation of the Hb vesicles and measured the rate of the metHb formation in vivo (Hb vesicles; 20mL/kg, Wister rats; 200g, n=4). It was clarified that H_2O_2 was the major factor for metHb formation, because H_2O_2 permeated through the bilayer membrane but not O_2^{-}*. The in vivo study indicated that the metHb formation of the catalase-coencapsulated Hb vesicles ([catalase]=6.0×10^4 unit/mL,) was suppressed, and the half-time of the metHb formation was 30 h. We found that the metHb formation in the Hb vesicles in vivo was largely caused by H_2O_2, and was suppressed in the presence of the catalase in the vesicle.

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Detection of Lipopolysaccharide in Hemoglobin-Vesicles by Limulus Amebocyte Lysate Test with Pretreatment of Surfactant.

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A suspension of Hb-vesicles (HbV; diameter = 251 ± 80 nm) is an artificial O_2 carrying fluid developed for the substitution of the red blood cell function. The method to quantitatively measure the bacterial endotoxin (lipopolysaccharide, LPS) in the HbV suspension has to be established because the conventional methods with Limulus amebocyte lysate (LAL) does not allow the accurate measurement due to the presence of large amount of lipids that interact hydrophobically with LPS, and shield the activity of LSP to clot the coagulant of LAL. This interference was evident from the isothermal titration calorimetry that clearly demonstrated the insertion of LPS molecule into the phospholipid bilayer membrane. In this report we tested solubilization of HbV with deca(ethylene glycol) dodecyl ether ($C_{12}E_{10}$) to release the entrapped LPS in vesicles as a pretreatment for the succeeding LAL assay of the kinetic-turbidimetric gel clotting analysis (detecting wavelength, 660 nm). The surfactant $C_{12}E_{10}$ interferes with the gel clotting in the concentration dependent manner, and the optimal condition for the measurement was considered in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration, and we found out the condition that allows the measurement of LPS higher than 0.1 EU/mL in the HbV suspension. Moreover, utilization of histidine-immobized agarose gel was effective to concentrate the trace amount of LPS from the $C_{12}E_{10}$-solubilized HbV solution and to wash out all the inhibitory elements. The LAL assay with the LPS-adsorbed gel resulted in the detection limit of 0.0025 EU/mL. The pretreatment with $C_{12}E_{10}$ would be applicable to not only HbV but also other drug delivery systems using phospholipid vesicles encapsulating or incorporating functional materials.

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Pharmacokinetics of the Hemoglobin-Vesicles (HbV) in Rats

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The phospholipid vesicles encapsulated hemoglobin solution (40 g/dL) (HbV) are useful materials as a red blood cells (RBC) substitute. We report on the circulation persistence and biodistribution of the HbV labeled with 99m-technetium (^{99m}Tc). The ^{99m}Tc-HbV was injected into rats from tail veins at 15% and 25% to blood volume. The circulation half-life (t1/2) of ^{99m}Tc-HbV was determined to be 15 hrs and 24 hrs for 15% and 25% groups, respectively. While, the empty vesicles (control) was eliminated significantly faster (t1/2=6 hrs) from blood circulation than HbV. The biodistribution data showed the major organs to eliminate the 99mTc-HbV and control vesicles from the blood circulation were liver, bone marrow, and spleen. The longer circulation life with increasing the injection dose was caused by decreasing the distribution ratio to liver. While, the remarkable difference of the circulation life between control vesicles and HbV was caused by the distribution into spleen. The distribution ratio of the control vesicles showed four times higher values than that of HbV. The large injection dose and encapsulation of Hb prolonged the circulation life of the HbV.

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40% Blood Exchange-Transfusion with Hb-vesicles in Rats and Observation of Hematological and Serum Clinical Laboratory Tests for 2 Weeks.

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In a clinical setting, volume of blood transfusion usually does not exceed 20 vol% of total blood volume. As a safety study 40 vol% of blood volume in a Wistar rat was exchange-transfused with a suspension of Hb-vesicles (HbV) with recombinant human serum albumin (rHSA). The safety of HbV/rHSA was evaluated by monitoring blood pressure, blood gas parameters, and the recovery of hematological, histopathological and serum clinical laboratory tests for two weeks.

Rats were anesthetized with Sevoflurene inhalation, and blood was continuously withdrawn from right common carotid artery and either of HbV/rHSA ([Hb] = 10 g/dL) (n = 6) or rHSA (n = 6) alone was infused through the right jugular vein to reach to 40% blood exchange. Both groups showed stable systemic parameters for 6 hrs including blood pressure and blood gas parameters and there were no significant differences between the groups. At this level of hemodilution the rHSA group can effectively compensate for the reduced oxygen transport probably by increased cardiac output.

For the longer period of observation, rats were anesthetized with i.p. pentobarbital and exchange-transfused with either of HbV/rHSA (n = 20) or rHSA alone (n = 20) through right common carotid artery, sutured, and housed for two weeks in cages provided with food and water ad libitum. All the rats in the both groups survived for 14 days. Hct returned to the original level 7 days after blood exchange. In the serum clinical laboratory tests, there was a transient increase in lipid components for the HbV/rHSA group, however, no sign of organ damage was confirmed. The accumulated HbV particles in liver and spleen was completely disappeared within 14 days. These results indicate that HbV can be used for hemodilution without irreversible damage to the organs.

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Tumor Oxygenation Using the Hemoprotein (rHSA-FeP) and the Hemoglobin-Vesicle (HbV) in a Rat LY80 Tumor Model.

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It is supported that oxygenation of tumor cell may raise the efficacy of chemotherapy and radiotherapy by number of ex vivo studies. The purpose is to investigate the effect of oxygen carriers and oxygen on tumor tissue PO_2 (TPO_2) in a rat tumor model.

Human serum albumin (HSA) iscorporating synthetic hemes, the tetrakis (o-pivalamido) phenylporphinatoiron (II) derivative, is an artificial hemoprotein (rHSA-FeP) which is able to bind and release dioxygen under physiological conditions. The hemoglobin vesicle (HbV) is a red cell substitute encapsulating purified concentrated Hb in a phospholipid vesicle. Above-mentioned rHSA-FeP and HbV were used as oxygen carriers (kind gifts from Waseda University). The LY80 tumor cells were injected into the right thigh of Donryu rats 1 week before experiment. At the experiment, rats were maintained by mechanical ventilation with 1% halocen (FiO_2 = 0.99). TPO_2 was estimated with Oxyspot™ at twenty spots of the tumor before administration of oxygen carriers. After four minutes of administration of each oxygen carrier and 5% rHSA (as control) at the rate of 2.5 ml/min/kg, the average of each 20 spots was calculated every 30 seconds. The measurement of TPO_2 was continued fifteen minutes. The increase ratio (TPO_2 @after administration / TPO_2 before administration) of rHSA-FeP, HbV and 5% rHSA was maximum 2.5 (P = 0.014), 1.8 (P = 0.048) and 1.0, respectively. We conclude that rHSA-FeP and HbV was potent useful agents in oxygenating tumor tissue. Tumor oxygenation, which may have synergistic effect with other cytotoxic therapy, merits further investigation.

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Nano-sized Oxygen Transporter Alleviates Cerebral Infarction in the Rat

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Background. Treatment of cerebral infarction (CI) has been limited to indirect therapies aiming at reduction of edema or oxygen demand. We tested a hypothesis that an ultra-small (200 nm) liposome-encapsulated-hemoglobin (LEH) may ameliorate tissue hypoxia and limit ischemic damages in a rodent CI model.

Methods. LEH was intravenously infused 1% of body weight 5 minutes before (PR) and 5 minutes after (PT) occlusion of the right middle cerebral artery in SD rats. Severity of edema was compared with CI rats without treatment (CT) 24-hour later on T2 weighed relative signal strength obtained with MRI system at cortex, striatum, hippocampus and pyriform lobe (Table).

Results. LEH was effective in reducing brain edema in all areas except in pyriform lobe in PR and PT than in CT. Edema reduction was more prominent when NRC was infused before CI, but NRC after CI was still effective in reducing edema without significant difference between administration before and after CI. Monoclonal antibody revealed human hemoglobin (=LEH) in capillaries throughout the CI tissues.

Conclusion. Nano-sized LEH was effective in reducing brain edema before as well as after CI in the rat.

P—26 NRC as a Blood Substitute: Duration of Efficacy in Various Species

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Introduction: NRC as a liposome encapsulated hemoglobin has a long circulation time in blood achieved by modification of the liposomal surface with polyethyleneglycol. Due to generation of methemoglobin, hemoglobin of blood substitutes loses its oxygen transport efficiency. We have thus reported that duration of efficacy of blood substitutes should be discussed correctly based on its half-life of efficacy, as the time to reduction by half of actual oxygen transport efficiency, in consideration of methemoglobin generation. We therefore studied circulation time of NRC as liposomes in blood and the rate of methemoglobin generation in blood in various species.
Methods: Normal rats, rabbits, dogs and cynomolgus monkeys were intravenously administered NRC at a dose of 20ml/kg. NRC-crit value and ratio of methemoglobin were determined by centrifugation method with glass capillary for measurement of hematocrit value and cyanomethemoglobin method, respectively, in blood withdrawn at designated times.
Conclusion: We confirmed that there are clear differences among various species in circulation time of NRC as liposomes in blood. Further, we found differences in rate of methemoglobin generation among various species. Rate of methemoglobin generation tended to be slower in large animals. Based on these results, we discuss expectations for duration of efficacy in human.

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Effect of Pegylation on Stability, Toxicity, and Pharmacokinetics of Perfluorocarbon Emulsion as Blood Substitutes

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Perfluorocarbon(PFC) emulsion has been developed as a candidate for oxygen carrying artificial blood substitutes and some of them have been clinically applied. The short half-lives of PFC emulsion in blood, however, limit the clinical application and the effort to prolong the half-lives is still required. We have been developed a new technique for preparing PFC emulsion using a new type high pressure homogenizer, and also examined new materials for improving the stability, toxicity and pharmacokinetics of PFC emulsion. FC43, perfluorodecalin(PFD), and perfluorooctylbromide(PFOB) were used as PFC. Highly purified Egg yolk lecithin, or hydrogenated lecithin were used as surfactant and perfluoroalchol esters with fatty acid were used as cosurfactant. Polyethylene glycol derivatives of distearyl phosphatidyl ethanolamine (PEG-DSPE) were used for modifying surface of PFC emulsion. The cosurfactant and PEG-DSPE remarkably improved physical stability of PFC emulsion. Under 100% O2 atmosphere, emulsion containing 50%(W/V)PFC were iv injected to rats at the dose of 30% volume of whole blood, and whole blood were periodically collected using hematocrit tube. After centrifuge, fluorocrit (the volume ratio of PFC to whole volume) were measured. The use of PEG-DSPE remarkably prolonged the half-life of PFC emulsion in blood for any PFC emulsion of FC43, PFD, and PFOB. Toxicity was also improved by PEG-DSPE. These results suggest the usefulness of pegylation for PFC emulsion

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