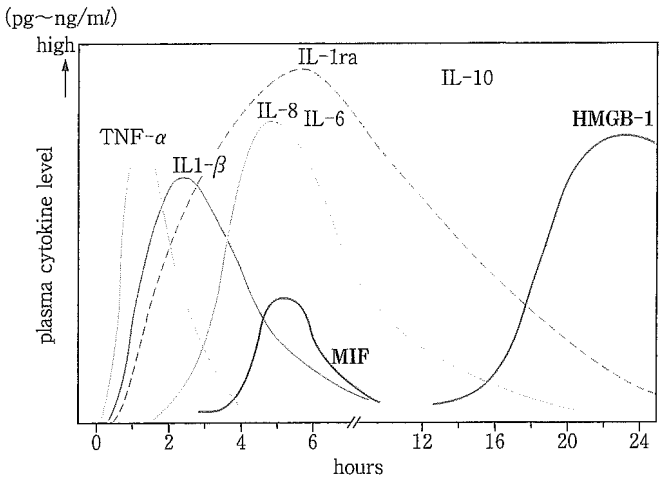

図 2 Cytokine time course

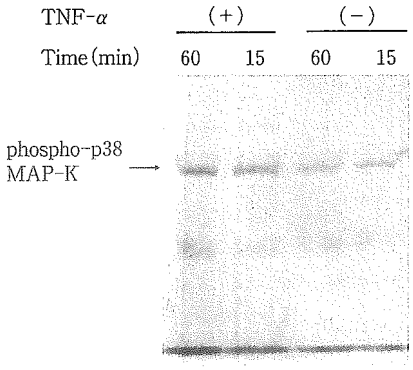

図 3 TNF-α 刺激後の phospho-p38 MAP-K 発現

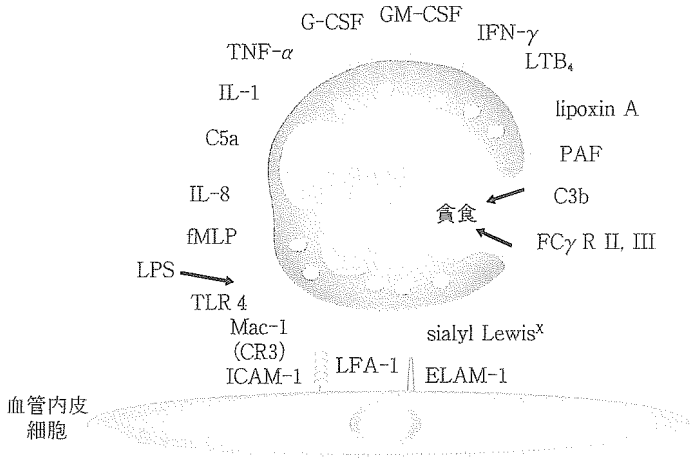

図 4 好中球上の各種受容体と接着分子(文献[3]より改変引用)

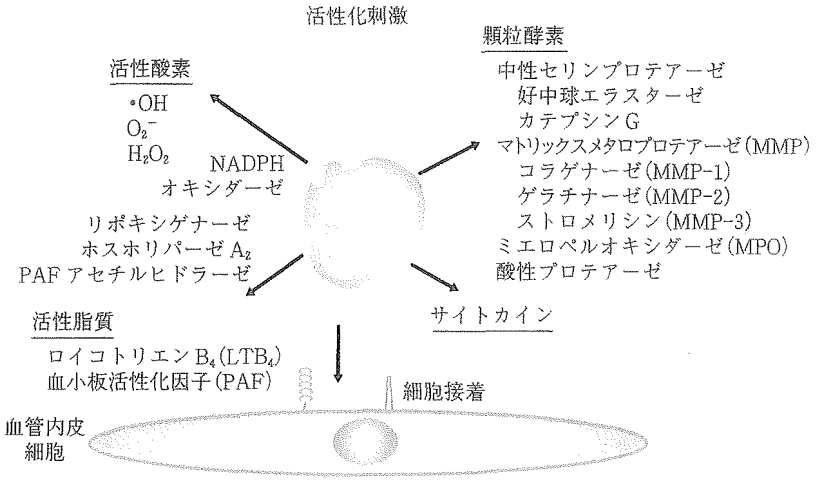

図 5　活性化好中球の産生/放出物質と接着能(文献[3]より改変引用)

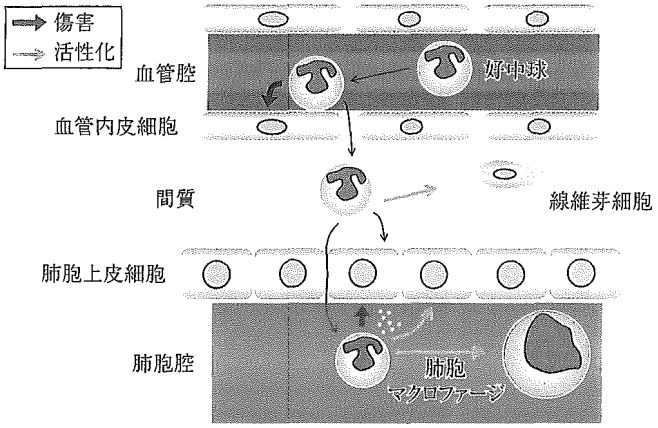

図 6　好中球による急性肺傷害の発症機序

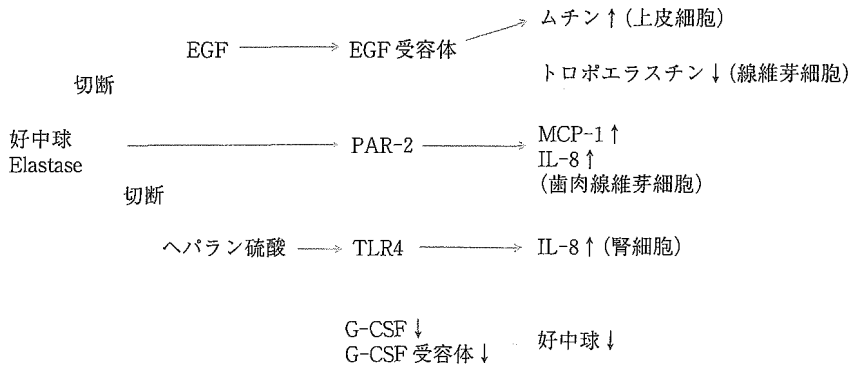

図 7　好中球エラスターゼの各種遠隔作用

熱傷，大手術などの一次刺激後に感染症などの二次刺激が加わると，一次刺激のみの場合に比し強い生体反応が惹起される(two hit phenomenon)．著者らは，その機序の一部に，炎症性サイトカインの過剰かつ遷延性の産生が関与していることを見いだした[5]．図8において，熱傷前負荷マウスでは，無処置マウスに比べて血液，肺組織中のTNF-α，MIP-2(ヒトIL-8相当)が二峰性に産生亢進している．これらのマウスで認められた急性肺傷害は，サイトカイン産生抑制剤であるJTE-607，methyl-PSL投与により，有意に抑制された(図9)．

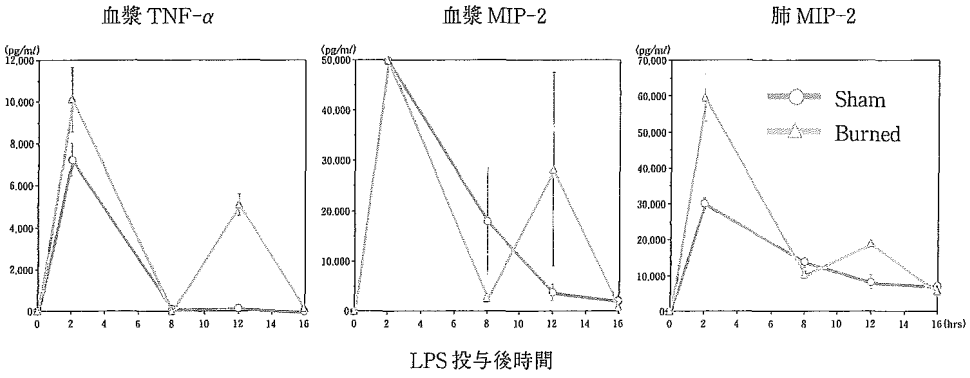

図8 対照および熱傷前負荷マウス血液，肺組織中TNF-α，MIP-2(文献[5]より改変引用)

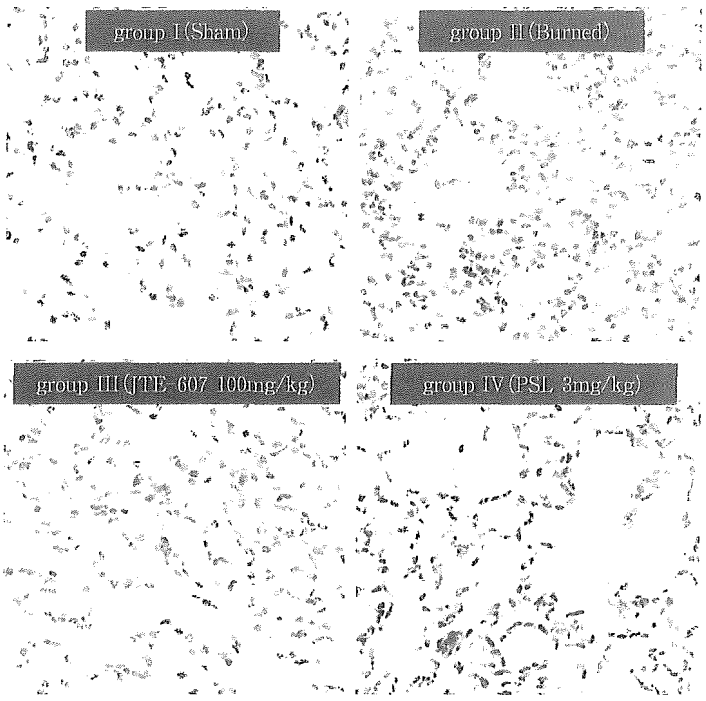

図9 肺組織所見(文献[5]より改変引用)

SIRS 患者血中で検出されるサイトカイン値は，患者間で大きなばらつきがある(**図 10**)．その一因として，サイトカイン遺伝子上に存在する遺伝子多型，特に一塩基変異多型(single nucleotide polymorphism：SNP)の関与が明らかとなっている．**図 11**，**図 12** に TNF-α, β遺伝子多型，**図 13** にシークエンスにより確認された IL-8 遺伝子上の SNP を示した．将来的には，これらの遺伝子多型を解析し，患者個々の特性に応じたオーダーメード治療を施すことが期待されている．

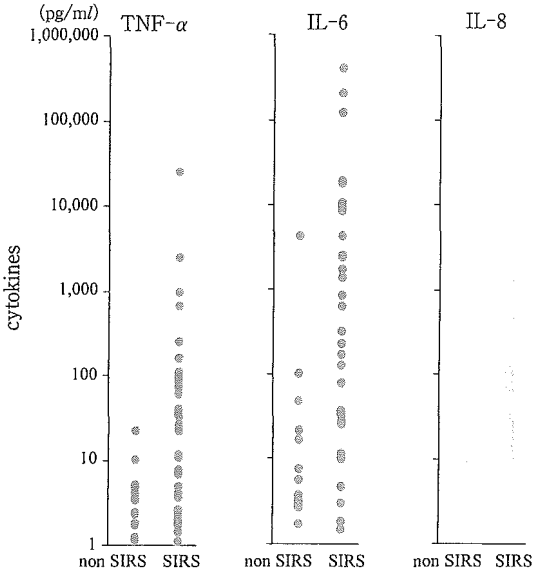

図 10　SIRS 病態下の血液中各種 cytokine 分布

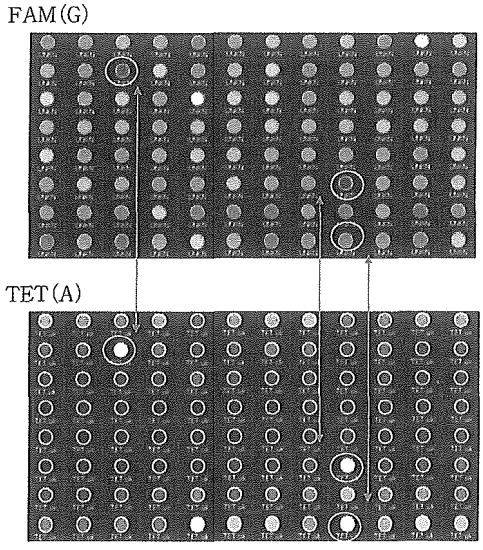

図 11　ABI Prism 7700 による TNF-α-308 SNP 解析

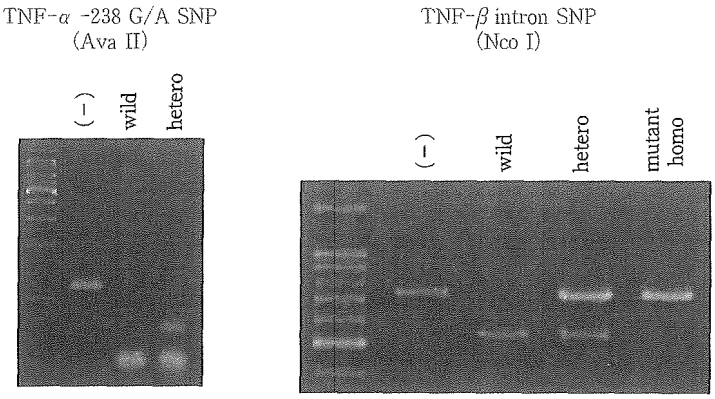

図 12 PCR-RFLP 法による TNF SNP 解析

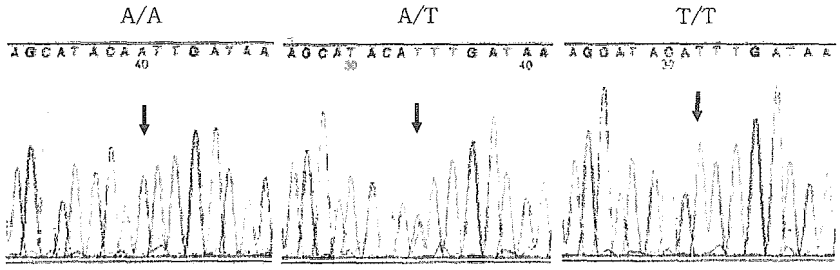

図 13 Direct sequense 法による IL-8 promoter 領域遺伝子多型の解析

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EMERGENCY MEDICAL SERVICES IN JAPAN: AN OPPORTUNITY FOR THE RATIONAL DEVELOPMENT OF PRE-HOSPITAL CARE AND RESEARCH

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☐ **Abstract—Japan is at a crossroads in the development of its Emergency Medical Services (EMS). At present, Japan has an essentially pure scoop-and-run, defibrillation system. However, there is a strong movement toward expanding the scope of paramedic practice to include more complex, Advanced Life Support (ALS) and trauma protocols to its nationally standardized pre-hospital protocols. The implications of introducing complex pre-hospital protocols guided by the use of existing scientific evidence to support such action is discussed in the context of Japan's unique opportunity to test many fundamental questions in pre-hospital medical care and the public's understanding and acceptance of these practices. Japan, a technologically advanced country that is not encumbered by entrenched "standards of care," has the opportunity to develop an efficient and rational EMS system.** © 2005 Elsevier Inc.

☐ **Keywords—EMS; pre-hospital; Japan; paramedic**

INTRODUCTION

Japan, a technologically advanced country of 130 million citizens, has a national Emergency Medical Services (EMS) system comprised of highly trained Emergency Life Support Technicians (ELST) whose complete protocols appear on six faces of a small, folded card containing the essence of their scoop-and-run-defibrillate pre-hospital philosophy. ELSTs in Hokkaido, Kyushu, or giant Tokyo—regions and cities differing in geography, transport distance, and epidemiology—are bound by the same protocols. A Japanese ELST can defibrillate ventricular tachycardia, perform CPR, and insert an oral-esophageal airway in a patient with no detectable vital signs. An ELST does not administer any drug, except for oxygen. On more than several occasions, we have heard and read that Japanese ELSTs and the Japanese public feel the pre-hospital scope of practice is severely limited compared with their counterparts in North America and other countries in the western hemisphere (1).

This is not altogether surprising. By contrast to the Japanese ELST, a comparably certified paramedic in New York City, population 8 million, has at least 58 separate protocols (2). Fresno County, California, which has a mixed rural, suburban and urban environment, has a population of just 750,000 people. Paramedics serving this diverse area have over 36 protocols, which run the gamut of advanced cardiac life support to the treatment of burns, snakebite, and seizures (3). Paramedics in Fresno, New York City, San Francisco or Houston, Texas can administer any one of 30 or more medications (from aspirin, adenosine, amiodarone and morphine all the way to terbutaline and verapamil) based on protocols developed and elaborated by EMS programs starting in the late 1960s and early 1970s. A paramedic in Maine or Sacramento, California can perform naso-tracheal intubation, a cricothyrotomy or decompress a tension pneumothorax using a thoracostomy needle. All U.S. para-

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medics can instill intravenous fluids in the setting of hypotension and trauma, yet, the evidence for the benefits of pre-hospital management of trauma using intravenous fluids remains controversial (4). Similarly, no clinical trial has yet proven that epinephrine or amiodarone improves survival to discharge from the hospital and the most rigorous clinical trials to date only suggest that endotracheal intubation might have a beneficial effect on overall survival in the setting of cardiac arrest, despite well-known and common complications associated with the procedure (5,6). In some regions of the United States, many pre-hospital practices are being scrutinized, with some being withdrawn or curtailed as data on the inefficacy or danger of certain pre-hospital practices have emerged (7–10).

In this article, comparisons of health care economics, health care culture, epidemiology, and topics such as continuous quality improvement (CQI) are generally avoided, though useful tools for comparison do exist (11). Although our focus is the scientific basis for developing EMS in Japan, it is important to recognize that politics, the emotional and economic cost to society, corporate financial profits, and decisions about what pre-hospital system is right for Japan all enter the equation. We emphasize that in Japan, EMS is an entirely publicly funded service and patients do not individually pay the expense of their ambulance ride to the hospital. Thus, the economic significance of expanded practice, training costs, and utilization are not small.

The development of EMS in Japan has some temporal and philosophical parallels to that in North America. The Meiji Empress founded the Japanese Red Cross during the Reformation and Japanese physicians developed field treatments for the sick and wounded during their wars and natural disasters of the 19th and 20th centuries (12). The first ambulance services were started in pre-World War II days by the Tokyo Police Department and were intended for trauma, rather than medically ill victims. In 1935, there were six ambulances in the old Tokyo City. In 1961, the first designated hospitals providing 24-h emergency services were assigned (13,14). The impetus for increased emergency services was twofold. First, an increase in the economic power of Japan was represented by an increase in car ownership—and in fatal car accidents, as well as a successful bid to host the 1964 Olympics. Thus, the first great expansion of ambulance services and designated emergency facilities was organized with the 1964 Tokyo Olympics in sight (15). Through the 1960s and into the early 1970s, emergency medical care in Japan was dominated by surgeons (14). It was not until 1973 that the Japan Association of Acute Medicine (JAAM) was founded, largely by surgeons, ironically with little collaborative effort between surgeons, intensivists, internists and members of other specialties wishing to provide comprehensive emergency services in dedicated emergency departments (EDs). In 1991, members of JAAM, other dedicated physicians, citizens and politicians collaborated to implement the Emergency Life Saving Technicians (ELST) Act (13). That same year saw the introduction of the privately and publicly funded Foundation for Ambulance Development. Japanese EMS, in contrast to the U.S. EMS system, is a government-sponsored service managed through the auspices of the Fire Department. In 2001, there were approximately 5517 ambulances (16). In contrast to U.S. EMS systems, even those run by fire departments, patients calling 119, the national equivalent to the U.S. 911, are not charged for the service of transportation to the ED.

In 2002, approximately 207 ambulance units of the Tokyo Fire Department, alone, made 629,883 runs in response to "119" calls. Of these, more than 50% were made for patients over the age of 50 years (17). Thus, as the Japanese population ages, the nation faces an important issue: What is the agenda for the future of EMS in Japan?

Two recent events have pushed the EMS development agenda to the forefront of the Japanese public's consciousness. The first event was in Akita City, where the practice of endotracheal intubation was being performed illegally by pre-hospital ELST units for several years. When this was revealed, and the paramedics indicted, the citizens of Akita City regarded the paramedics as heroes, stirring debate about their professional fates and the "backwardness" of Japanese EMS. A second occurrence, in November 2002, was the tragic, sudden cardiac death of Prince Takamadonomiya at the Canadian embassy in Tokyo. As a result, there has been public outcry to expand the scope of practice in Japanese EMS from its basic life-support-based system.

In response to these events, and recent research in Osaka Prefecture and Tokyo, the Ministry of Public Management and Home Affairs, along with Japan Medical Association and JAAM, formed three committees to address fundamental issues of EMS practice in Japan (18,19). The three committees are for: 1) defibrillation by paramedics, 2) endotracheal intubation, and 3) the use of drugs. Each committee is composed of 9 to 10 individuals representing different specialties and sectors of society. For example, the defibrillation committee was composed of two physicians from Emergency Medicine, one pediatrician and one from the Japanese Medical Society. In addition, there was one legal advisor, two members from the fire department and two from related bureaucracies.

COMMENTARY

Until the death of the beloved cousin of the Emperor, ELSTs could not defibrillate without consent of a base

hospital physician. This event, and the abundant data supporting the use of rapid defibrillation to save lives in the setting of cardiac arrest, accelerated the policy of defibrillation without calling into the base hospital (20–22). Since April of 2003, Japanese ELSTs have been interpreting, with the aid of interpretive computers, cardiac monitor rhythm strips in the setting of pulseless ventricular tachycardias and fibrillation. However, Japanese paramedics are still highly restricted in their scope of practice. They are trained in the basics of advanced life support (ALS) and basic trauma life support (BTLS). Thus, they may maintain and protect the airway using the bag-valve-mask (BVM), laryngeal mask airway (LMA), or esophageal obdurator airway (EOA, similar to combitube), but only in the setting of cardiac arrest. Because they are now permitted to defibrillate without consulting a base-hospital physician, ELSTs may avoid life-threatening delays in the use of semi-automatic defibrillators. Consistent with other interventions in the Japanese EMS system, ELSTs may only start intravenous lines and administer lactated Ringer's solution in the setting of cardiopulmonary arrest. These restrictions in practice are in sharp contrast to the wide variety of interventions used in North America.

Our observation is that in Japan, there is building popular and political pressure to rapidly expand the scope of practice of ELSTs to be more comparable to that of North American paramedics and other western EMS systems. Although appealing, this is bound to be an expensive and controversial undertaking. By expensive, it is meant threefold: in economic, social and professional terms. By controversial, we mean that many of the general practices of pre-hospital ACLS and BTLS, although appealing and empirically useful, have not necessarily been proven effective by prospective studies and, at this time, the most rigorous data are lacking (5,6,23–31). Thus, in the United States and in North America in general, there are 30 to 50 protocols for any paramedic to choose from in any given situation—many of which probably do not require attention in the span of an ambulance's arrival and delivery of the patient to an ED, especially in an urban setting with short transport times. The most fundamental questions in pre-hospital care seem to have the same foci: how much to do at the scene of an accident, a cardiac arrest, an acute exacerbation of a chronic illness? For most prehospital interventions, there is little evidence of a positive effect on outcome (23,31). However, shorter prehospital time—inherent in scoop-and-run systems such as that in Japan, has been shown to be a critical factor for patients with cardiac arrest and trauma activation of prehospital systems (32,33).

Japanese ELSTs, as well as many medical doctors, are professionals who truly believe that expanding their scope of practice will result in lives saved. All medical professionals, doctors and emergency life-saving technicians alike, are sincerely dedicated to the proposition that our first duty is not to harm the patient. Many members of the medical profession in Japan have met proposed expansions of EMS services with skepticism and resistance. Because policies are set at a national level, all cities and prefectures are subject to the policies set by the Ministry of Health. Many involved in pre-hospital medical services in Japan may harbor resentment toward the medical profession, which can be perceived as holding back EMS development for reasons not entirely related to patient care. It is our opinion that in the long run, the best chance to help patients and not harm them is to test each proposed intervention in a randomized (and when possible, blinded), controlled trial (RCT). This type of testing is the gold standard of clinical inquiry and minimizes bias. For conditions such as cardiac arrest, meaningful endpoints such as "survival to discharge" would be used, rather than the dubious "return of spontaneous circulation (ROSC)" or "survival to admission" (5,23,24).

We know from Japan's centralized, Utstein-based EMS databases that 5517 ambulance units made 4,399,195 runs in response to "119" calls in 2001. The mean response time from call to arrival on scene for 4,399,195 ambulance runs was 6.2 min (35). In the year there were 88,058 out-of-hospital cardiac arrests (16). Japan's EMS databases and hospital record keeping, and essentially pure scoop-and-run/defibrillate system make it well situated to perform first-rate pre-hospital science.

Thus, Japan has an opportunity to test many fundamental hypotheses important to the practice of pre-hospital patient care. Though there are legal barriers (e.g., nationally uniform pre-hospital practice laws that prevent local and regional clinical trials), and perhaps cultural ones as well, there is no standard of care to interfere with the ethical performance of randomized controlled clinical trials of interventions such as endotracheal intubation vs. "simple" hyperventilation by BVM, LMA or combitube. Similarly, there is no technical barrier to a trial of epinephrine vs. placebo. Even landmark studies such as the OPALS series from Canada have had to use retrospective controls (whose results are subject to the Hawthorne Effect type biases) (6,21,22,32). In Japan, methodological shortcuts can and should be assiduously avoided when possible and validated tools, such as the Utstein template and true randomization, should be standard and, fortunately, are already the basis for record keeping by Japanese ESLTs and their base hospitals (36).

The public, anywhere in the world, expects and deserves the protection that effective government oversight provides. Many arguments have been put forth suggesting that endotracheal intubation, vasopressors and antidysrhythmics, applied in certain pre-hospital settings,

may save lives (36,37). These interventions deserve the most rigorous scrutiny and testing (25).

Other interventions, such as pre-hospital administration of anti-seizure medications, oral dextrose for hypoglycemia, and morphine for the pain of a fractured long-bone indeed may be convenient and warrant less scrutiny, while broadening the repertoire of Japanese ELSTs in the field. The tenets of evidence-based medicine suggest that evidence from research is only one component to be considered in clinical decision-making, with individual clinical circumstances, patient and citizenry preferences, and clinician's expertise determining therapeutic action or restraint. When rigorous scientific evidence is lacking, yet years of clinical experience and acumen suggest an intervention is effective and safe, it is reasonable to try that intervention until there are data to suggest otherwise.

In the broader picture, basic questions are: whose judgment and under whose control will protocols be driven, based on what quality of evidence? How much money will be dedicated to research and how much time is needed for that research? Who will be accountable when patients come to harm through ELST error and how will policy disagreements be handled at a national level? (10,38) New interventions such as endotracheal intubation and the administration of potentially dangerous medications increase the complexity of the ELST's or paramedic's curricula and will likely add significant expense to the publicly funded pre-hospital system. In the United States (even with rapid sequence intubation), misplaced endotracheal tubes and multiple intubation attempts in the field are a common and dangerous occurrence (39–42). The limited *clinical* experience of technicians, lack of evidence for positive effects on outcome, and the prolongation of scene times the more interventions are introduced should be primary considerations before the scope of EMS practice is broadened. Furthermore, skills requiring the most technical knowledge—and judgment—deteriorate fastest, thus increasing danger to patients before they have ever arrived in the hospital where larger teams can work together with the ELSTs to clarify the patients' needs (43). Everyone needs to consider, also, the cost of expanded protocols in terms of drugs, equipment acquisition and maintenance, as well as extra training and re-certification costs. In Japan, if ESLTs are to start giving medications, their legal status as healthcare providers certified to do so will need to be revisited.

The "Chain of Survival" concept that is generally embraced should be based on rigorous evidence that the chain, in fact, improves survival and minimizes harm (44). In North America, we have a system that is based at least as much on tradition as on evidence. Invasive field procedures and risky medications may be overused due to the so-called "technical imperative." The technical imperative has been expressed as, "if a procedure can be taught, it will be used with a frequency greater than its indication" (45). Any system introducing new practices should consider this pitfall with the greatest of attention not only to the risks and benefits of such practices but to the adversarial relationship that may be created among physicians, nurses and paramedics (45).

Fortunately, there has been a recent surge in high quality pre-hospital research that might clarify long-standing controversies in EMS practice (7,21,22,32,44). Some research, which has been performed with rigor, could still introduce new practices prematurely due to overenthusiastic endorsement or over-interpretation about its applicability (46–48). Thus, individual studies showing promise for a particular therapy should be repeated before being put into general practice and sub-group analyses showing significant effects should be viewed with caution, though the finding may be excellent for hypothesis generation (48).

Opportunities for international collaboration to further the development of EMS in Japan, for its benefit and that of the rest of the world, abound. The results of systematic and rigorous scientific inquiry will surely benefit all Japanese citizens and help enlighten the practice of EMS worldwide. With good public education and a rational approach to EMS development, there is good reason for optimism that victims of cardiac arrest and other critically ill patients can be given optimal treatment or therapeutic restraint to optimize survival in the out-of-hospital setting (6,8,49). However, before the people of Japan commit vast amounts of time, money and emotion to an expanded EMS system, we urge caution and scientific rigor.

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Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model

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Abstract

Phospholipid vesicles encapsulating Hb (Hb-vesicles: HbV) have been developed for use as artificial O_2 carriers (250 nm ϕ). As one of the safety evaluations, we analyzed the influence of HbV on the organ functions by laboratory tests of plasma on a total of 29 analytes. The HbV suspension ([Hb] = 10 g/dl) was intravenously infused into male Wistar rats (20 ml/kg; whole blood = 56 ml/kg). The blood was withdrawn at 8 h, and 1, 2, 3, and 7 days after infusion, and the plasma was ultracentrifuged to remove HbV in order to avoid its interference effect on the analytes. Enzyme concentrations, AST, ALT, ALP, and LAP showed significant, but minor changes, and did not show a sign of a deteriorative damage to the liver that was one of the main organs for the HbV entrapment and the succeeding metabolism. The amylase and lipase activities showed reversible changes, however, there was no morphological changes in pancreas. Plasma bilirubin and iron did not increase in spite of the fact that a large amount of Hb was metabolized in the macrophages. Cholesterols, phospholipids, and β-lipoprotein transiently increased showing the maximum at 1 or 2 days, and returned to the control level at 7 days. They should be derived from the membrane components of HbV that are liberated from macrophages entrapping HbV. Together with the previous report of the prompt metabolism of HbV in the reticuloendothelial system by histopathological examination, it can be concluded that HbV infusion transiently modified the values of the analytes without any irreversible damage to the corresponding organs at the bolus infusion rate of 20 ml/kg.

Keywords: Biomimetic material; Blood; Drug delivery; In vivo test; Liposome; Nanoparticle

1. Introduction

Liposomes or phospholipid vesicles have been extensively studied for the application of drug delivery system, and some are now approved for a clinical use as antifungal or anticancer therapies [1]. Another promising application is to use vesicles for encapsulating a concentrated human Hb. The resulting Hb-vesicle (HbV) can serve as an O_2 carrier with ability comparable to red blood cells (RBC) [2–4]. The advantages of the Hb-based O_2 carriers (HBOCs) are the absence of blood-type antigens and transmission of known and unknown blood-borne disease, the possibility to improve the rheological properties of blood flow according to the needs of patients, and stability for long-term storage. These characteristics will make it possible to use the HBOCs both in elective and emergency situations [5,6]. In this sense, the infusion of HBOCs becomes superior to the conventional blood transfusion that still has the potential of mismatching, infection such as HIV and hepatitis virus, and the problems of only 2–3 week preservation period. The acellular Hb modifications including polymerized Hb and polymer-conjugated Hb are now undergoing the final stages of clinical trials [7,8]. However, the cellular structure of HbV (particle diameter, ca. 250 nm) most closely mimics the characteristics of natural RBC such as the cell membrane function of physically preventing direct contact of Hb

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with the components of blood and vasculature during circulation [9]. In comparison with some acellular Hb modifications, the Hb encapsulation in vesicles suppresses hypertension induced by vasoconstriction, a theory that is suggested to be due to the high affinity of Hb with nitric oxide and carbon monoxide as vasorelaxation factors [10,11]. Moreover, the surface modification of HbV with polyethylene glycol (PEG) chains not only prolongs the circulation half-life [12] but also prevents the intervesicular aggregation and guarantees the homogeneous dispersion in the plasma phase that provides a prompt blood flow in the microcirculation and the resulting sufficient tissue oxygenation [13,14].

According to the clinical conditions HbVs are supposed to be applied for, the organism is faced with the metabolism of a large amount of both Hb and lipids, because the dose rate of HbV is significantly large. The HbV particles, as well as phospholipid vesicles, infused in the blood stream are finally captured by phagocytes in the reticuloendothelial system (RES, or mononuclear phagocytic system, MPS) [4,15]. In a previous report, we clarified by the histopathological studies of rats receiving 20 ml/kg of HbV infusion that the HbV particles were captured and metabolized within 7 days in RES mainly in the spleen and liver [16]. Transmission electron microscopy provided a clear image of the HbV particles in the phagosomes 1 day after infusion, but they disappeared within 7 days. Staining with the anti-human Hb antibody, Berlin blue, and hematoxylin/eosin showed prompt metabolism of Hb molecules with no morphological changes in the liver and spleen. The phagocytic activity decreased and then transiently increased, but tended to return to the original level. From these studies, we did not see any irreversible damage to the organs.

Serum laboratory tests are the most common diagnostic tools to monitor organ functions clinically. However, both the PEG-modified HbV particles and the chemically modified Hb solutions remained in the plasma even after usual centrifugation to remove RBC, showing significant interference effects due to the light absorption by Hb and light scattering by the particles. These interference effects hindered the accurate evaluation of plasma laboratory tests and have been regarded as a serious issue for the development of HBOCs [17,18]. However, quite recently we have clarified by an in vitro experiment that the simple removal of PEG-modified HbV as a precipitate by ultracentrifugation (50,000 *g*, 20 min) or by conventional centrifugation in the presence of a high-molecular-weight dextran diminished most of the interference effects [19]. Using this simple procedure, we aimed to evaluate the safety of HbV by the laboratory tests of plasma after bolus intravenous infusion of HbV at a rate of 20 ml/kg, the same experimental model as in the previous study [16].

2. Materials and methods

2.1. Preparation of PEG-modified HbV

The PEG-modified HbV was prepared in a sterile condition as previously reported in the literature [10, 20–22]. Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Society of Red Cross, Japan (Tokyo, Japan). The encapsulated purified Hb (38 g/dl) contained 14.7 mM of pyridoxal 5′-phosphate (PLP, Sigma) as an allosteric effector at a molar ratio of PLP/Hb = 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5/5/1 (Nippon Fine Chem. Co., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (NOF Co., Tokyo, Japan, 0.3 mol% of the total lipid). The HbCO solution and the lipid powder were mixed and stirred for 12 h at 4°C. The resulting multilamellar vesicles were extruded through membrane filters with a final filter pore size of 0.22 μm. Thus prepared PEG-modified HbV was suspended in saline at the Hb concentration of 10 g/dl, and filtrated (pore size: 0.45 μm). The physicochemical parameters of the HbV are as follows: particle diameter, 251 ± 80 nm; [Hb], 10 g/dl; [metHb], <3%; [HbCO], <2%; phospholipids, 4.0 g/dl; cholesterol, 1.7 g/dl; and oxygen affinity (P_{50}), 30 Torr. The endotoxin content was precisely measured by modified *Limulus* Amebocyte lysate gel-clotting analysis that has been developed by our group recently, and confirmed that the endotoxin content was less than 0.1 endotoxin unit/ml [23].

2.2. HbV infusion and procedure for the plasma laboratory tests

All animal studies were approved by the Animal Subject Committee of Keio University School of Medicine and performed according to NIH guidelines for the care and use of laboratory animals (NIH publication #85-23 Rev. 1985). The experiments were carried out using 40 male Wistar rats (200–210 g, Saitama Experimental Animals, Kawagoe, Japan). They were anesthetized with diethylether inhalation, and the HbV suspension was infused into the tail vein at a dose rate of 20 ml/kg ($n = 5$ for every time point). Ten rats were used to obtain the control values. All the rats were housed in cages and provided with food and water *ad libitum* in a temperature controlled room on a 12 h dark/light cycle.

After 8 h, and 1, 2, 3, and 7 days, the rats were anesthetized with 1.5% sevoflurene inhalation (Maruishi Pharm. Co., Osaka, Japan) using a vaporizer (Model

TK-4 Biomachinery, Kimura Med., Tokyo). Polyethylene tubes (PE-50, Natsume Co., Tokyo) were implanted in the carotid artery for withdrawing blood into heparinized syringes for the Hct, HbV concentration, and plasma laboratory tests. The animals were finally laparotomized and sacrificed with acute bleeding from the abdominal aorta and the liver and spleen were obtained for weight measurements. The control rats received the same procedure for the measurements.

A part of the withdrawn blood (6 ml) was centrifuged to obtain plasma which was turbid and red/brown colored due to the presence of PEG-modified HbV particles especially in the samples taken at 8 h, 1 and 2 days after infusion. The plasma was ultracentrifuged (50,000 *g*, 20 min) to remove the HbV particles. The obtained transparent plasma specimens were stored at −80°C until the laboratory tests at BML, Inc. (Kawagoe, Japan). The selected analytes were total protein, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ -glutamyltransferase (γ-GTP) alkaline phosphatase (ALP), cholinesterase (ChE), leucin amino peptidase (LAP), creatine phosphokinase (CPK), amylase, lipase, total cholesterol (Total-Chol.), cholesteryl ester (Chol.Ester), free cholesterol (Free-Chol.), HDL-cholesterol (HDL-Chol.), β-lipoprotein, triglyceride (TG), free fatty acid (FFA), phospholipids, total lipids, uric acid (UA), blood urea nitrogen (BUN), creatinine (CRE), K^+, Ca^{2+}, inorganic phosphate (IP), and Fe^{3+}. In our previous study, it was confirmed that the concentrations of the plasma components in terms of the above analytes did not change after the ultracentrifugation at 50,000 *g* for 20 min [19]. Since rat albumin is slightly insensitive to the bromcresol green method, the values were corrected according to Takano et al. [24].

2.3. Histopathological examination of pancreas

After sacrificing the animals by acute bleeding from the abdominal aorta, the pancreas was resected for a histopathological study. The organs were fixed in a 10% formalin neutral buffer solution (Wako Chem. Co., Tokyo) immediately after the resection, and the paraffin sections were stained with hematoxylin/eosin.

2.4. Data analysis

Differences between the control and a treatment group were analyzed using a one-way ANOVA followed by Fisher's protected least-significant difference (PLSD) test. The changes were considered statistically significant if $p<0.05$.

3. Results

All the rats receiving the bolus infusion of HbV at a dose rate of 20 ml/kg tolerated the infusion and survived until intentional sacrifice. There was no noticeable change in appearance such as piloerection.

3.1. Hct and circulation persistence of HbV

The control Hct was 42±1%, and it decreased slightly to 40±1% at 1 day after HbV infusion. The estimated Hb concentration of HbV in plasma just after infusion was about 6 g/dl, and it gradually decreased to 4.4±0.3 g/dl at 8 h, 1.9±0.2 g/dl at 1 day, 1.3±0.1 g/dl at 2 days, and 0.8±0.01 g/dl at 3 days (Fig. 1). At 7 days, HbV was not detected at all in the plasma phase.

3.2. Spleen and liver weights

The changes in the spleen and liver weights were expressed as percents of the body weight (Fig. 1). The liver weight ratio (control, 4.81±0.17%) showed a significant increase 1 day after the infusion (5.29±0.27%, $p<0.01$), and then it returned to the original level at 2 days. Spleen weight ratio significantly increased from 0.32±0.05% to 0.66±0.06% 3 days after the infusion ($p<0.01$), however, it was reduced to 0.41±0.02% at 7 days.

3.3. Plasma laboratory tests

The plasma fraction after centrifugation of the blood sample for 3 days after the HbV infusion was turbid due to the presence of PEG-modified HbV. However, ultracentrifugation of the plasma produced transparent and light-yellow plasma phase and PEG-modified HbV was precipitated at the bottom in a tube. There was no sign of the presence of Hb in the supernatant, indicating that there was no hemolysis of both RBC and HbV.

As for the analytes that reflect the liver function, the total protein (control, 5.2±0.1 g/dl) and albumin (2.46±0.06 g/dl) slightly decreased to, e.g., 4.9±0.2 and 2.11±0.10 g/dl, respectively, with statistically significant differences ($p<0.01$) for 3 days after the HbV infusion (Fig. 2). They tended to return to its original level at 7 days ($p<0.05$). AST (control, 60±7 U/l) decreased to 46±3 U/l ($p<0.05$) and returned to the original level at 7 days. ALT (control, 32±5 U/l) only slightly increased to 40±8 U/l 1 day after the HbV infusion ($p<0.01$), but it returned to its original level 2 days after the infusion. LDH (control, 150±60 U/l) did not change significantly. ALP (control, 1265±231 U/l) decreased at 2 days (812±149 U/l) and 3 days (872±98 U/l) ($p<0.01$), but it returned to the control level at 7 days. γ-GPT (control, 1.6 U/l) and LAP (31±1 U/l) showed significant but minimal reductions

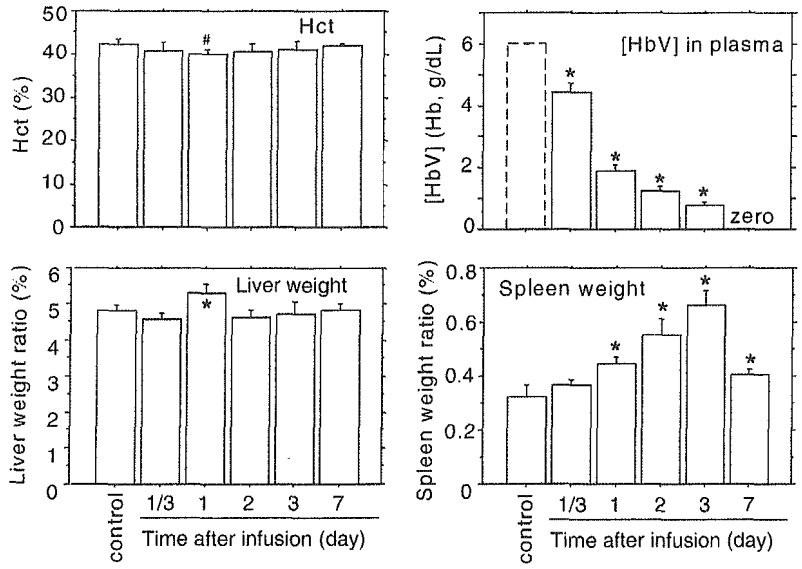

Fig. 1. Changes in hematocrit, concentration of HbV in plasma, and spleen and liver weights after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. The control value of [HbV] is the estimated concentration of HbV immediately after the infusion and expressed as with a dashed line. The spleen and liver weights are expressed as the ratio to the body weight (%).

($p<0.05$). ChE (control, 76 ± 18 U/l) did not show a noticeable change. Plasma total bilirubin (≤0.1 mg/dl) and Fe^{3+} showed some reductions but were maintained at a low level for 7 days in spite of the metabolism of a large amount of Hb.

CRE (control, 0.3 mg/dl) was maintained at a low level for 7 days. BUN (control, 16 ± 3 mg/dl) showed a slight increase at 7 days (21 ± 3 mg/dl) (Fig. 3). UA (control, 0.47 ± 0.19 mg/dl) increased to 0.70 ± 0.16 mg/dl at 3 days, however, it returned to a non significant level at 7 days. Amylase (control, 1613 ± 74 U/l) significantly decreased for 3 days after the infusion ($p<0.01$), but returned to its original level at 7 days. Lipase (control, 9 ± 1 U/l) showed significant increases ($p<0.01$) after the HbV infusion, and it tended to decrease after 3 days, and was reduced to a non-significant level at 7 days. CPK (control, 304 ± 116 U/l) decreased at 7 days ($p<0.05$), but did not show a noticeable increase during the experiment. As for the electrolyte concentrations, K^{+}, Ca^{2+}, and IP did not show any significant changes.

The most consistent changes were seen in the lipid components (Fig. 4). Total-Chol. (control, 73 ± 7 mg/dl), Free-Chol. (18 ± 2 mg/dl), Chol.Ester (59 ± 8 mg/dl), and HDL-Chol. (32 ± 4 mg/dl) showed significant increases and maximum values at 2 days ($p<0.01$). Free-Chol. increased to 39 ± 4 mg/dl, about twice the control value. However, it tended to decrease at 3 days, and returned to its control level at 7 days. β-Lipoprotein (control, 110 ± 42 mg/dl) slightly increased at 1 day (160 ± 33 mg/dl), but returned to its original level at 3 days. TG (control, 64.4 mg/dl) significantly decreased to 12.4 mg/dl at 2 days ($p<0.01$), but tended to increase to its original level at 7 days. Phospholipid (control, 132 ± 8 mg/dl) significantly increased to 150 ± 9 mg/dl at 1 day ($p<0.01$), and then returned to the original level at 3 days.

3.4. Histopathological examination of pancreas

The histology of pancreatic tissue 2 days after the infusion of HbV is shown in Fig. 5. There was no significant morphological change in spite of the increment of the pancreatic lipase activity.

4. Discussion

The clinical indications for the use of the HbV suspension as an artificial O_2 carrying fluid are estimated to be mainly preoperative or perioperative hemodilution, or resuscitation from hemorrhagic shock in emergency situations [25], both of which result in exchanging more than 20% of the original blood with the HbV suspension. Thus, the dose amount is extremely greater than that of stealth liposomes for drug delivery systems. HbV particles in the blood stream are finally captured by RES in the same manner as the conventional phospholipid vesicles [15]. In a previous study, we confirmed by the histopathological examination in a rat model that HbV particles were captured in the phagosomes of liver Kupffer cells and spleen macrophages without tissue damage, and they had completely disappeared within 7 days [16]. The transient splenomegaly and hepatomegaly in Fig. 1 seemed associated with the entrapment of HbV. The total weight change of

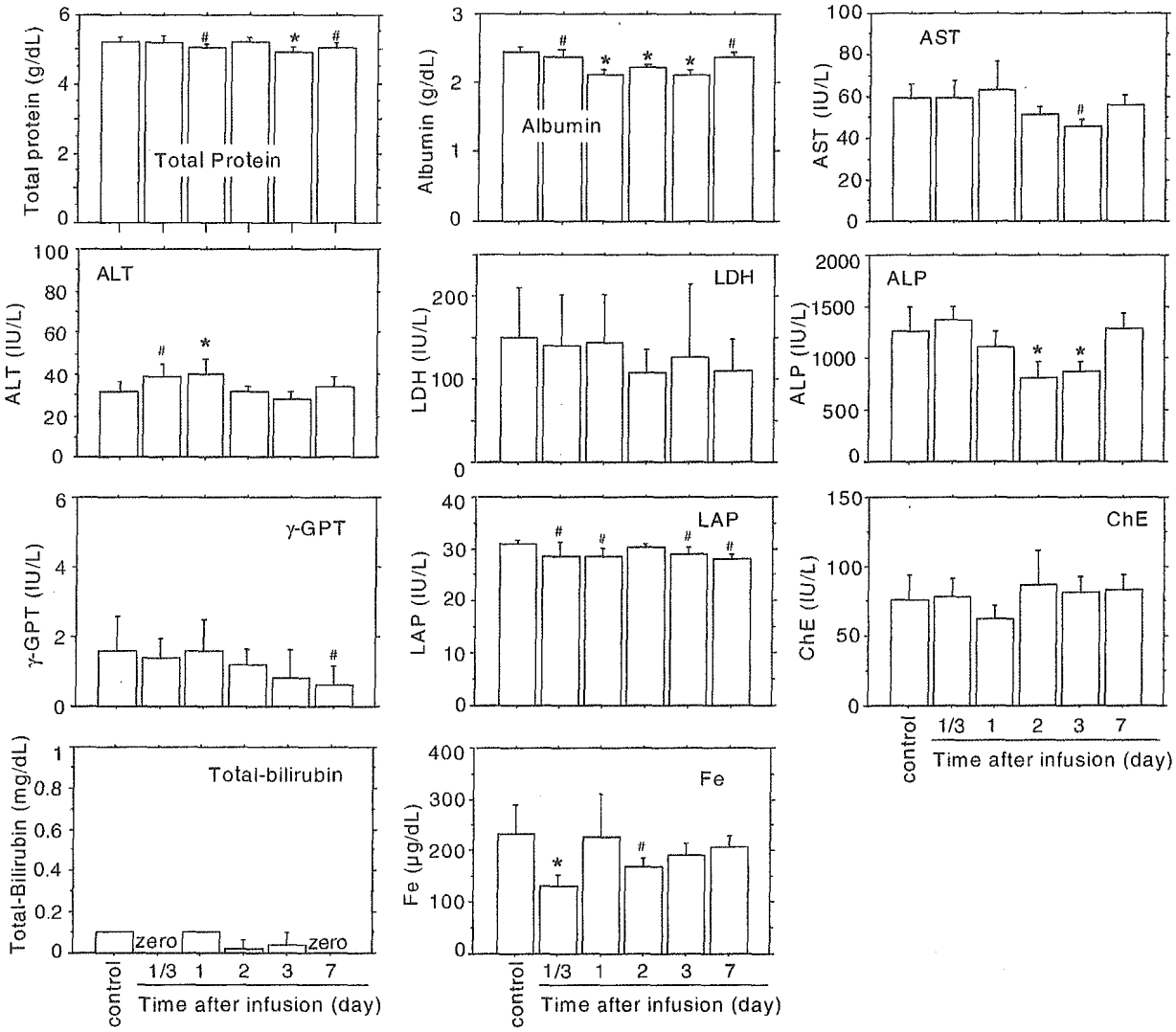

Fig. 2. Plasma laboratory tests representing the liver function and metabolism of Hb after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ-glutamyltransferase (γ-GTP) alkaline phosphatase (ALP), leucin amino peptidase (LAP), cholinesterase (ChE).

these organs is 0.8% of the body weight (1600 mg for 200 g body weight), which should correspond to not only the accumulated HbV (635 mg for 20 ml/kg) but also to the increased amount of phagocytic or parenchymal cells and/or RBC. The organ weight ratios tended to return to their original levels as HbV disappeared from the blood stream, and there was no deteriorative sign of morphological change in the main organs such as the liver, spleen, lung, kidney, and heart. To confirm the safety more in detail, we analyzed for the first time, the plasma laboratory tests on 29 analytes without any interference effect of the PEG-modified HbV simply by removing it from plasma by ultracentrifugation [19].

Our results indicated no irreversible sign of organ damage after the bolus infusion of HbV at a dose rate of 20 ml/kg (cf. whole blood = 56 ml/kg). Especially, liver is one of the main organs of the trapping and metabolism of HbV. However, we did not see an increase in the physiological meaning of the parameters representing the liver function. As for the parameters representing the renal function, there were slight changes in CRE, BUN, and UA without any physiological meanings. CPK did not significantly change, indicating that the intactness of the cardiac function and skeletal muscular function should be preserved.

Amylase and lipase that represent pancreatic function showed slight changes. The amylase activity slightly decreased while the lipase activity significantly increased from 9±1 IU/l at control to 30±9 IU/l at 2 days. The lipase activity was measured by an enzymatic method that was specific for pancreatic lipase. Therefore, the increment should not be attributed to the hepatic or

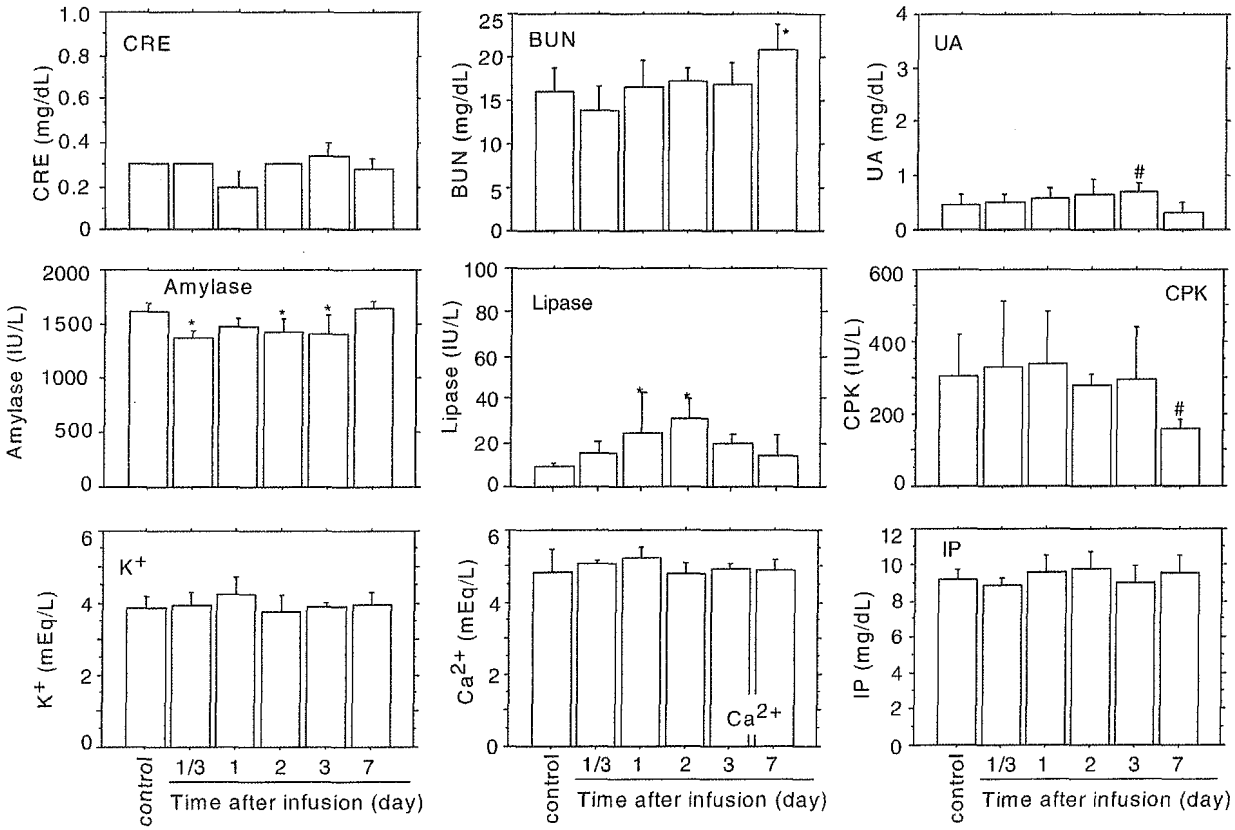

Fig. 3. Plasma laboratory tests representing renal, pancreatic and myocardial function, and electrolytes after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: creatinine (CRE), blood urea nitrogen (BUN), uric acid (UA), creatine phosphokinase (CPK), inorganic phosphate (IP).

lipoprotein lipase. However, this level of increment was significantly smaller than the reported value for the Wistar rats of pancreatitis. Hofbauer et al. [26] reported that acute necrotising pancreatitis increased lipase activity from 10 to 475–5430 IU/l. It was reported that the injection of liposome amphotericin B raised the serum lipase activity, and one possible reason was speculated to be the enzyme induction in the pancreas by the presence of a large amount of lipids from the liposomes [27], because pancreatic lipase hydrolyze not only TG but also phosphatidylcholine [28]. This speculation was also supported by our results that the profiles of the transient increases in the lipid components coincided with that of lipase, but not with amylase. The cause of this modification is not clear at the present time. Histopathological analysis showed no significant pathological change in the pancreas. However, the pancreatic function should carefully be monitored in the ongoing safety studies.

Significant and consistent increases were seen in the lipid components with maximum at 1 or 2 days. They should be derived from the HbV particles because they contain a large amount of cholesterol (ca. 1200 mg/dl) and DPPC (1840 mg/dl) in the infused suspension ([Hb] = 10 g/dl). The gradual increases in cholesterols by 2 days after infusion and no Hb release from HbV in the plasma indicate that they should be liberated from RES after HbV are captured by RES and destroyed in the phagosomes. This is also supported by the fact that the maximum concentrations were seen at 2 days when the HbV in the plasma had mostly disappeared from the blood. It has been reported that the infused lipid components of the phospholipid vesicles are trapped in the Kupffer cells, and diacylphosphatidylcholine is metabolized and reused as a component of the cell membrane, or excreted in the bile and in the exhaled air [29–31]. Cholesterol is finally catabolized as bile acids in the parenchymal hepatocytes. There should be no direct contact of HbV and the hepatocytes because HbV is so large that it cannot diffuse across the fenestrated endothelium into the space of Disse [11]. Cholesterol from HbV should reappear in the blood mainly as lipoprotein cholesterol after entrapment in the Kupffer cells [32], and then excreted in the bile after entrapment of the corresponding lipoprotein by the hepatocytes [33]. We speculate that the main components of the lipid bilayer membrane of HbV, the phospholipids and cholesterol, would gradually be redistributed

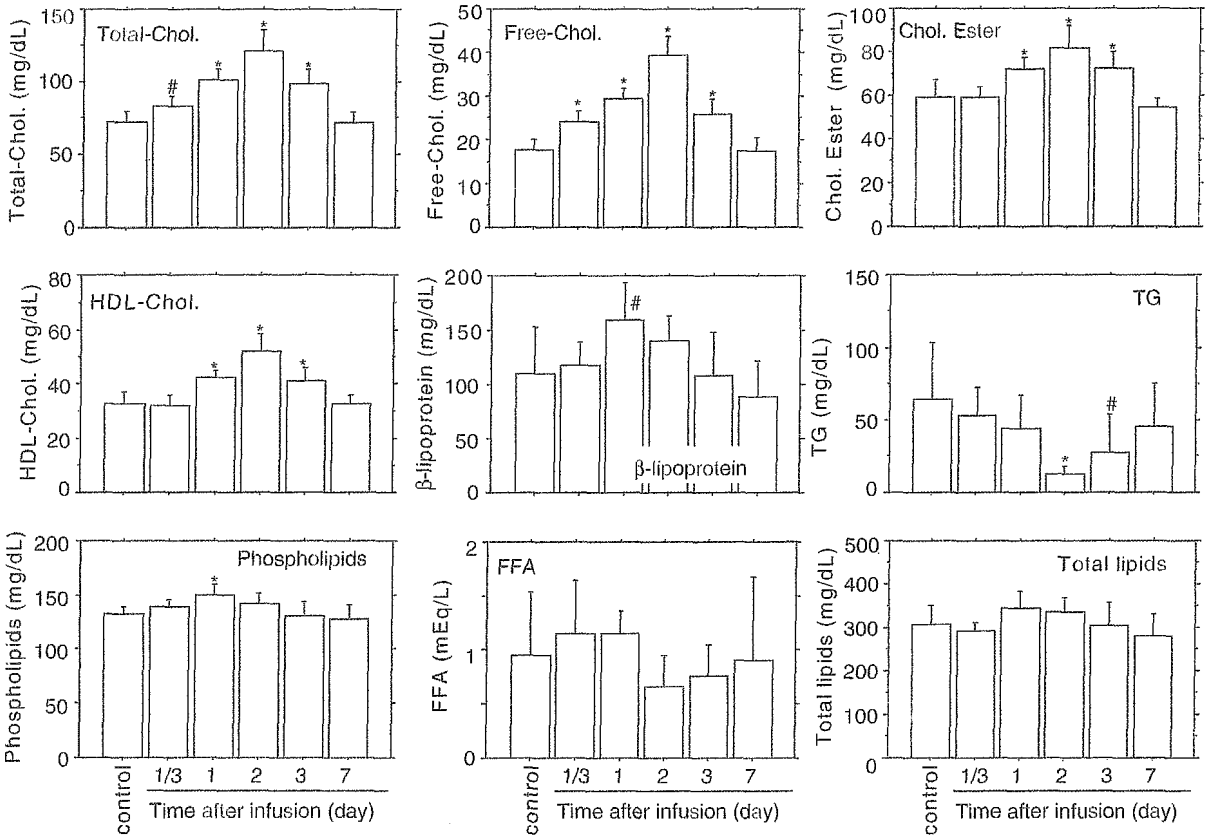

Fig. 4. Plasma laboratory tests representing lipid metabolism after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: total cholesterol (Total-Chol.), free cholesterol (Free-Chol.), cholesteryl ester (Chol.Ester), HDL-cholesterol (HDL-Chol.), triglyceride (TG), free fatty acid (FFA).

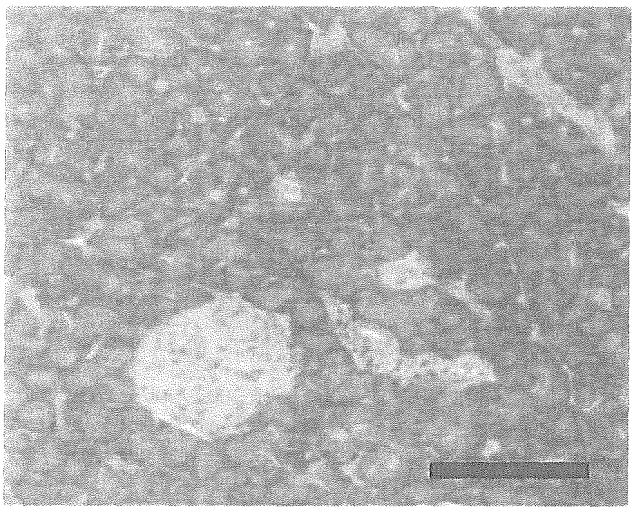

Fig. 5. Histology of pancreas 2 days after the infusion of HbV (20 ml/kg). Bar indicates 100 μm (HE stain).

or metabolized in the same manner. However, a precise pharmacokinetic study is necessary using radiolabeled materials to demonstrate the metabolic and excretory routes of the lipids. Transient, but significant increases in the lipid components raise the necessity of a further study to clarify the influence of a large dose of HbV especially on a lipemic model.

During the metabolism of Hb, there should be a release of bilirubin and iron. However, they did not increase for 7 days. In a previous study, the anti-human Hb antibody staining was effective for detecting the special and temporal distribution of human Hb of HbV both in the spleen and liver [16], and we made it clear that human Hb disappeared within 7 days. The released heme from Hb in HbV may probably be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells in the liver and in the spleen [11,34]. Bilirubin should be excreted in the bile as a normal pathway, and there should be no obstruction or stasis of bile in the biliary tree. Berlin blue staining revealed the presence of hemosiderin 3 and 7 days after HbV infusion, and it disappeared after 14 days [16]. A similar observation was reported for a polymerized Hb that was captured by the Kupffer cells while showing subsequent hemosiderin formation [35]. Normally, iron from a heme is stored in the ferritin molecule [36]. Ferritin in the lysosomal membrane may form paracrystalline structures and

eventually aggregate in mass with an iron content as high as 50%. These are hemosiderins composed of degraded protein and coalesced iron. Both ferritin and hemosiderin release iron molecules, and they are anticipated to induce hydroxyl radical production and succeeding lipid peroxidation [37,38]. However, iron release from hemosiderin is substantially less than that from ferritin, thus iron molecules in hemosiderin are relatively inert [39]. Plasma iron, mostly bound to transferrin, remained constant after HbV infusion. The iron concentration should be coordinately regulated through the "iron regulatory proteins" that sense the levels of iron for hematopoiesis and metabolic needs [40], and the excess amount of iron should be stored in an insoluble and less toxic form as hemosiderin. Together with the time course of the histopathological changes, the results of the plasma laboratory tests indicate that the metabolism of heme and the recycling or excretion of iron molecule is within the physiological capacity and suggested to be on the physiological pathway that has been well characterized for the metabolism of senescent RBC [41].

5. Conclusion

In this study, the plasma laboratory tests after the infusion of HbV (20 ml/kg) did not demonstrate an irreversible sign for a deteriorative damage to the organs. Plasma bilirubin and iron, which were considered to be released during the metabolism of the Hb molecule, did not increase during the observation period. This may be due to the moderate rate of Hb metabolism in RES after the entrapment of HbV with a moderate length of circulation time. The lipid components significantly increased at 2 or 3 days after infusion. These may be derived from the membrane component of HbV entrapped in RES. The complete normalization of the lipid components indicates that they are metabolized in a normal metabolic and/or recycling pathway. The precise biodistribution and fate of the components should be confirmed by a radioisotope technique. Our results have demonstrated the safety of HbV using only healthy rats, while rats in hemorrhagic shock, septic shock, or lipemia have to be tested in the ongoing safety studies. It should also be emphasized that the data cannot be extrapolated to large animals or humans, which may react differently to such a large dose of HbV.

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Hemorrhagic Shock Resuscitation With an Artificial Oxygen Carrier, Hemoglobin Vesicle, Maintains Intestinal Perfusion and Suppresses the Increase in Plasma Tumor Necrosis Factor-α

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It is known that damage to the intestinal mucosa followed by systemic inflammatory response is one of the leading causes of shock related morbidity and mortality. In this study, we examined the ability of an artificial oxygen carrier hemoglobin vesicle (HbV) to sustain systemic and intestinal perfusion during hemorrhagic shock. In rabbits, hemorrhagic shock (40% of the estimated blood volume) was resuscitated with 5% albumin (alb group), HbV suspended in 5% albumin (HbValb group), or washed red blood cells suspended in 5% albumin (RBCalb group). Plasma tumor necrosis factor (TNF)-α level was measured in rats under the same experimental protocol. No significant intergroup differences were seen in systemic hemodynamics. In contrast, parameters of intestinal perfusion significantly deteriorated in the alb group but were equally well sustained in the HbValb and RBCalb groups. Also, a significant increase in plasma TNF-α level was seen in the alb group but not in the RBCalb or HbValb groups. These results indicate the proficient oxygen transporting capability of HbV and its potential efficacy in shock resuscitation. *ASAIO Journal* 2004; 50:458–463.

Blood replacement is the basic therapeutic modality when a considerable amount of blood is lost because of trauma or major surgery. Despite the recent progress in transfusion medicine, enormous investments are still necessary to establish and sustain the systems from blood donation to transfusion. Donated blood inspections to avoid the side effects of homologous blood transfusion, such as transfusion associated infectious disease, alloimmunization, and graft *versus* host diseases are still essential.[1,2] To overcome these problems associated with transfusion, development of artificial blood substitutes is important. To this end, we have developed several types of artificial oxygen carriers and have evaluated the efficacy of these compounds in various animal models.[1] Among these compounds, hemoglobin vesicle (HbV), a form of liposome encapsulated hemoglobin, is rapidly approaching clinical trials. The cellular structure of HbV, similar to red blood cells, shields all of the physiologic effects of acellular Hb solutions.[3–5] We have studied the oxygen transporting capabilities of HbV, using several exchange transfusion and hemorrhagic shock models.[6–10] In these studies, we have shown that HbV effectively restores the systemic circulation in hemorrhagic shock.

It is known that gastrointestinal perfusion is compromised at a relatively early stage in hypovolemic shock to sustain the systemic circulation to other vital organs.[11] This, however, causes damage to the intestinal mucosa followed by systemic inflammatory response syndrome (SIRS) or sepsis, which is one of the leading causes of shock related morbidity and mortality.[12,13] In the present study, we examine the ability of HbV to sustain not only systemic but also intestinal perfusion to further evaluate the efficacy of HbV in hemorrhagic shock.

Materials and Methods

Animal Care

The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of Keio University, School of Medicine. It also complies with Guidelines for the Care and Use of Laboratory Animals of Keio University, School of Medicine. All rabbits and rats were housed in groups of two in standard cages and were provided with food and water in a temperature controlled room on a 12 hour dark/light cycle.

Preparation of Hemoglobin Vesicle Suspended in 5% Albumin

HbV suspension was prepared in a similar manner as previously reported in the literature.[14,15] In brief, a purified and concentrated human hemoglobin solution (40 g/dl) was obtained from outdated red blood cells.[16] Added to this purified hemoglobin solution were pyridoxal 5'-phosphate (18 mM, Merck Co., Frankfurter, Germany) as an allosteric effector and homocysteine (Aldrich Co., Milwaukee, WI) as a reductant of methemoglobin. The lipid bilayer of HbV was composed of Presome PPG-I (Nippon Fine Chem. Co., Osaka, Japan) containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, (DPPC), cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phos-

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