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[25]. HbV infusion into rats induced minor changes in aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase without deteriorative liver damage, a transient increase of cholesterol and phospholipids and reversible changes of amylase and lipase levels in the plasma [26]. Thus, HbV infusion transiently or reversibly affects biochemical parameters in the rat plasma.

In this study, we have investigated the effects of HbV on the hematological characteristics and complement titer using rats. In addition, the immune response after HbV infusion was also examined.

METHODS

HbV

HbV was prepared as previously described [27,28]. Briefly, hemoglobin solution prepared from out-dated red blood cells obtained from blood centers was heated under a CO gas atmosphere to inactivate any possibly contaminating viruses and to remove the stroma and non-hemoglobin proteins [29]. After the centrifugation and filtration, hemoglobin solution was mixed with lipids and then extruded through membrane filters with a pore size of 0.22 μm to make liposomes. The lipid composition (mol%) was as follows: dipalmitoyl phosphatidylcholine (DPPC):cholesterol (CHOL):dipalmitoyl-L-glutamate-*N*-succinic acid (DPEA):polyethylene glycol-conjugated distearoyl phosphatidylethanolamine (PEG5000-DSPE) = 5:5:1:0.033. The mean particle size was 250 nm. All lipids were purchased from Nippon Fine Chemical Co. (Osaka, Japan) except PEG5000-DSPE, which was from NOF Co. (Tokyo, Japan). HbV was suspended in saline and contained 10 g of hemoglobin/dl, 5.7 g lipids/dl and <0.1 endotoxin unit of lipopolysaccharide/ml. An empty vesicle (EV), which consisted of the same lipid composition as HbV without hemoglobin encapsulation, was also prepared.

Animals and HbV Infusion

WKAH male rats, 8–10 weeks old and weighing 220–300 g, were purchased from Japan SLC (Shizuoka, Japan). Under ether anesthesia, HbV or EV was intravenously infused into rats from the tail vein at top-load. As a control, saline was infused into the rats. The injection volume was 20% of the whole blood volume according to the estimation as follows that the whole blood volume is 56 ml per kg of body weight in rats. For the experiments investigating the anaphylactic reaction, Brown Norway male rats, 8 weeks old and weighing 200–220 g (Japan SLC),

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were used because of their highly sensitive behavior to allergic and anaphylactic reactions [30].

Hematological Analysis

Before and after the infusion of HbV, EV or saline, peripheral blood was collected from the tail vein into a plastic tube coated with EDTA. Numbers of red blood cells (RBC), white blood cells (WBC) and platelets (PLT) were measured using an automatic cell counter (AcT diff; Beckman Coulter, Miami, FL, USA). The leukocyte population was measured with a flow cytometer (EPICS XL; Beckman Coulter) based on forward-and side-scatter plot analysis. The lymphocyte subset was analyzed with the flow cytometer after staining with monoclonal antibodies; PE-labeled anti-CD4 (Beckman Coulter), FITC-labeled anti-CD8b (BD Biosciences, San Jose, CA, USA).

Complement Study

Before and after the infusion of HbV, EV or saline, peripheral blood was collected into the glass tube from the tail vein. Blood was clotted by standing for 1 h at room temperature and then for 1 h at 4°C. Serum was separated from the blood clot by centrifugation at 2,000 g for 20 min at 4°C, followed by additional centrifugation at 15,000 g for 45 min at 4°C to separate the serum from HbV or LE, and then stored at −80°C until assay.

In another complement experiment *in vivo*, HbV or saline was administered four times at two-day intervals from the first injection. Blood was collected before each injection and at subsequent time intervals. The serum was prepared as described above and stored at −80°C until assay.

In the *in vitro* study, rat serum was prepared from five non-treated rats. The serum was incubated with HbV, EV or saline at a ratio of 80:20 or 60:40 (v/v) at 37°C for 1 h. After centrifugation at 15,000 g for 45 min at 4°C, the supernatants were stored at −80°C until assay.

The complement titer was measured with a 50% hemolysis assay based on Mayer's method using a commercial kit (New One point CH50 (KW); Japan BCG Supply Co., Tokyo, Japan), which was approved to be applicable to the measurement of rat serum complement [31]. The unit of complement titer was expressed as CH50 (U/ml).

Examination of Anaphylactic Reaction

EV was used in this study to avoid interfering heterologous immune responses against human hemoglobin in HbV. Brown Norway rats were

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intravenously administered with EV (1–2 ml). The second infusion was carried out at 2 weeks, the third infusion was at 4 weeks, and the fourth infusion was at 8 weeks after the first infusion. As a positive control of the anaphylactic reaction model, Brown Norway rats were immunized subcutaneously with ovalbumin (OVA) in combination with the complete Freud's adjuvant. Two weeks after the immunization, OVA was intravenously infused from the tail vein. Clinical behavior, such as fluffiness, tear, blood drain speed and respiration, was observed during and after the infusion.

Statistical Analysis

Experimental differences from the controls (i.e. saline group) among the three groups were assessed with Non-repeated Measures ANOVA followed by the Dunnett test as a post-hoc test. The comparison of two groups was made with the Unpaired Student's t-test. A computer statistics package was utilized for statistical analysis (ystat2004, IGAKUTOSYO Press Co. Ltd., Tokyo, Japan). Values of $p < 0.05$ were considered significant.

RESULTS

Hematological Changes After HbV Infusion

Hematological markers were investigated at 6 h and 1, 3 and 7 days after the infusion of HbV, EV or saline. During the observation period, numbers of RBC, WBC and PLT varied in three groups. However, no significant differences were observed among the three groups at any time point (Fig. 1).

For the leukocyte population, the transient decrease of the lymphocyte ratio and the increase of the granulocyte ratio was significant in the HbV and EV groups at 6 h after the infusion. The 80.1% lymphocyte ratio in the control was decreased to 71.4% in EV and 56.7% in HbV. On the other hand, the 14.0% granulocyte ratio in the control was increased to 25.9% in EV and 40% in HbV. The degrees of the decrease and the increase were significantly greater in the HbV group than the EV group. The ratio of monocytes decreased 6 h after the infusion in three groups and significant differences were observed between the HbV and EV groups and the control group. The decrease of monocytes gradually returned to the initial level. The ratios varied from 2.7% to 10.4% (Fig. 2).

A subset of lymphocytes was analyzed by flow cytometry using monoclonal antibodies. Ratios of T cells and B cells were unchanged in all groups during the observation period (data not shown). As shown

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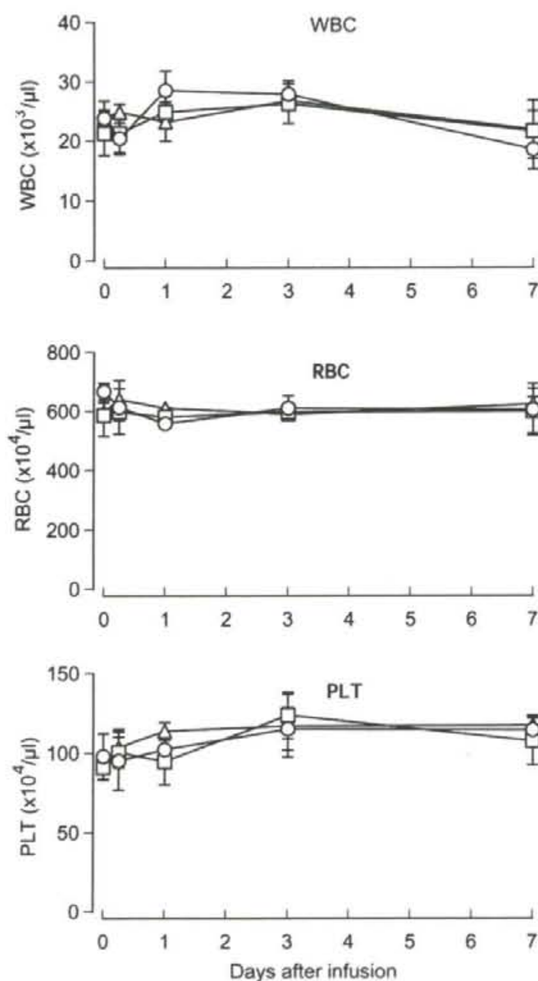

Figure 1. Effects of HbV infusion on peripheral blood cells. HbV, EV or saline was infused into rats at top-load from the tail vein. Blood was sampled and numbers of red cells, white cells and platelets were counted with an automatic cell counter. Triangles, HbV; squares, EV; circles, saline. N = 3–4, mean ± SD.

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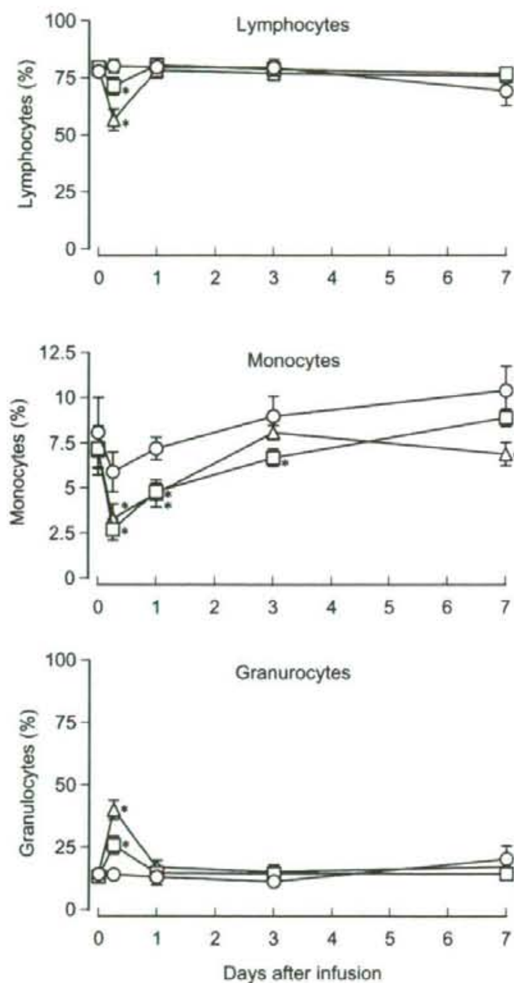

Figure 2. Effects of HbV infusion on the leukocyte population. HbV, EV or saline was infused into rats at top-load from the tail vein. Blood was sampled and ratios of lymphocytes, monocytes and granulocytes were analyzed with the flow cytometer. Triangles, HbV; squares, EV; circles, saline. N = 3–4, mean ± SD. $^{*}p < 0.05$.

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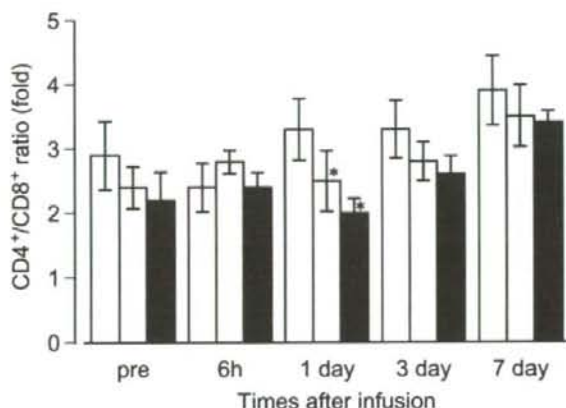

Figure 3. Effects of HbV infusion on the T cell subset. HbV, EV or saline was infused into rats at top-load from the tail vein. Blood was sampled and ratios of $CD4^+$ or $CD8^+$ T cells were analyzed as described in the Materials and Methods section. Black bars, HbV; gray bars, EV; white bars, saline. N = 3–4, mean ± SD. $^*p < 0.05$.

in Figure 3, ratios of $CD4^+/CD8^+$ T cells varied from 2.0 to 3.9 of the mean value in the series of experiments. However, statistical significance was not observed in the HbV and EV groups versus control group except on day 1. The mean values of the $CD4^+/CD8^+$ ratios in the HbV and EV groups were generally lower than those of the control group.

Effects of HbV on the Complement Titer

The complement titer in rat serum was assessed before and after the infusion of HbV, EV or saline. As shown in Figure 4, the complement titer dropped significantly three days after the infusion of HbV or EV, then afterwards it gradually returned to the levels before infusion and of the saline-infused group on day 14. No significant difference was observed between the HbV-infused group and the EV-infused group regarding the degree of decrease.

HbV, EV or saline was mixed with rat serum *in vitro* at a ratio of 20:80 or 40:60 and incubated for 1 h at 37°C. The complement titer decreased in accordance to the dilution of serum by mixing HbV, EV or saline (Figure 5). No significant difference was observed among three subjects at any mixing ratios, indicating that both HbV and EV did not consume complement.

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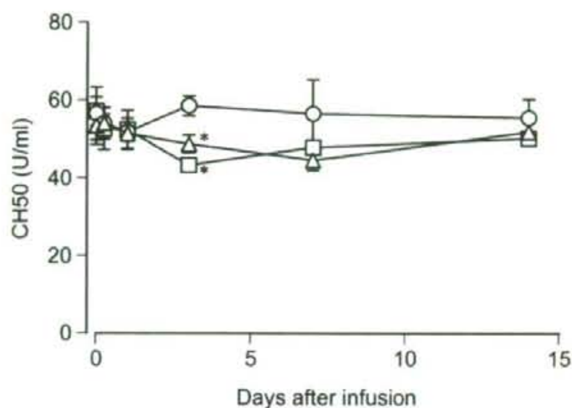

Figure 4. Changes of the complement titer in the rat serum after HbV infusion. HbV, EV or saline was infused into the rats at top-load from the tail vein. Blood was sampled, and sera were prepared as described in the Materials and Methods section. The complement titer was measured and indicated as CH50. Triangles, HbV; squares, EV; circles, saline. N = 3–4, mean $\pm$ SD. $^*p < 0.05$.

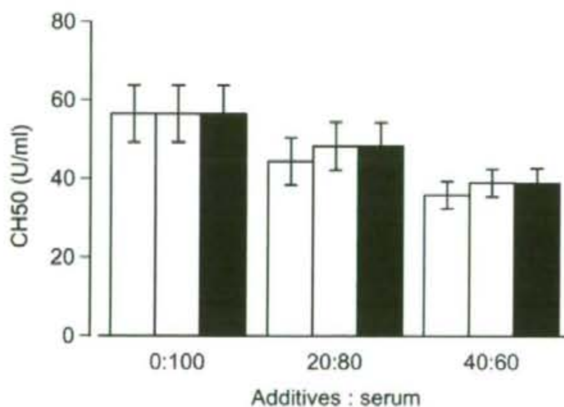

Figure 5. Effects of HbV on the rat complement *in vitro*. HbV, EV or saline was mixed with rat sera and incubated at 37°C for 1 hr. After centrifugation, the complement titer in the supernatant was measured. Black bars, HbV; gray bars, EV; white bars, saline. N = 5, mean$\pm$SD.

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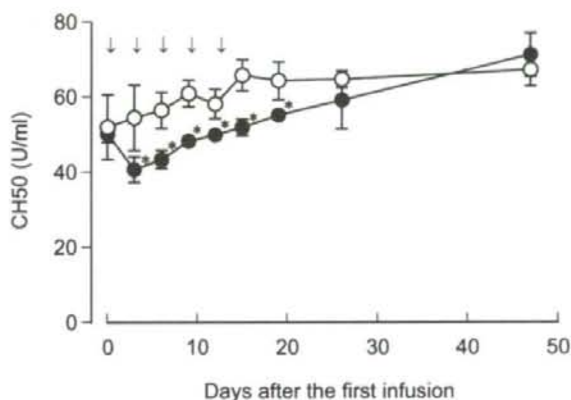

Figure 6. Effects of repeated infusion of HbV on the rat serum complement. HbV or saline was infused into rats at top-load from the tail vein at the time point as indicated by arrows. Blood sampling, sera preparation and complement measurement were carried out as described in the Materials and Methods section. Closed circles, HbV; open circles, saline. N = 3–5, mean ± SD. *$p < 0.05$.

HbV was sequentially infused five times with two-day intervals. The complement titer dropped on day 3 after the first injection (Figure 6). However, the additional infusion of HbV with 2-day intervals no longer further decreased the complement titer. Two weeks after the final injection, on day 26, no significant difference was observed in the complement titer between two groups.

Assessment of Anaphylactic Reaction Caused by EV

Because HbV contains human hemoglobin, which may act as an antigen in rats, EV was used to assess the potential to induce anaphylactic reaction caused by liposome components. No abnormal behavior, such as fluffiness and tear, was observed in the EV-infused rats and the respiration was kept normal. All animals were alive in the EV-infused group even after the final injection with symptomless (Table 1). OVA administration was performed to evaluate whether this rat model is appropriate to assess anaphylactic reaction. Rats were sensitized subcutaneously with OVA using complete Freud's adjuvant to obtain complete sensitization. Two weeks after the sensitization, OVA was intravenously infused from the tail vein. Immediately after the infusion, all rats were affected with respiratory distress and the blood drain speed dropped, resulting in death.

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Table 1. Lack of anaphylactic shock in rats infused with EV

Infusion	1st	2nd	3rd	4th
EV (n = 3)	Alive with symptomless	Alive with symptomless	Alive with symptomless	Alive with symptomless
Saline (n = 3)	Alive with symptomless	Alive with symptomless	Alive with symptomless	Alive with symptomless
OVA (n = 3)	Alive with symptomless	Dead within 3 min with respiratory distress		

DISCUSSION

In the previous study, Rabinovici et al. reported that LEH induced a hematological response in rats, such as an increase of WBC and a decrease of PLT within 5–25 min after the infusion, but these changes returned to basal levels 2 h after the infusion [32]. However, we did not observe the effects of HbV infusion in such a short period, and the numbers of RBC, WBC and PLT were unchanged in the three groups during the observation period of one week Figure 1. Although the numbers of WBC did not differ among the three groups, a significant change of the ratios of lymphocytes and granulocytes was observed at 6 h after the infusion (Figure 2). The degree of these changes was greater in the HbV group than in the EV group. Similarly, Rudolph et al. reported that the polymorphonuclear leukocyte counts were increased and lymphocyte counts were decreased 2 h after the LEH infusion, except that their change was correlated with the increase of WBC [33]. We also observed that the ratios of monocytes dropped 6 h after the infusion, even in the saline group, and gradually returned to the initial level. However, the reasons for these phenomena are unexplainable at present.

The ratio of $CD4^+$ T cells and $CD8^+$ T cells is an important indicator of the immune system in subjects. In immune compromised patients, such as AIDS, an inversion of the $CD4^+/CD8^+$ ratio is a typical event due to an increase in the absolute number of $CD8^+$ cells [34]. In this study, the $CD4^+/CD8^+$ ratio was not inverted and was constant in each group during the observation period (Figure 3). Although a significant difference between the HbV and EV groups and control group was observed on day 1, the implication of this significance for an immune response may be less important because the $CD4^+/CD8^+$ ratio was not inverted and lower in HbV and EV groups than in the control group, even at the pre-infusion time, due to unknown reasons.

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As mentioned in the previous study [19], the influence of liposomes on the complement is a considerable subject to assess the safety of liposomal therapeutics. The complement plays a role in subjects not only as a defense system, but also as causal agents for adverse reactions when it is activated in excess. The considerable characters of liposomes and other things are as follows: negatively charged surface [35–37], cholesterol contents [35,38], and the existence of natural anti-phospholipid antibodies in subjects [20]. Complement activation induces a pseudoallergic reaction in the pig model [21,22], and hypotension, flushing, respiratory distress, decrease of mean arterial pressure and chest pain in humans [39,40]. We previously reported that HbV does not activate the complement *in vitro* using human serum [19]. In this study, we examined the effect of HbV on the complement using rats *in vivo* and *in vitro*. The *in vitro* study showed that the complement titer in the HbV group was equivalent to that in the control group, indicating that HbV did not consume the complement of rat serum (Fig. 5). The *in vivo* study, however, showed that the complement titer dropped by 9% on day 3 and 14% on day 7 after the HbV infusion, and then returned to the initial level (Fig. 4). No allergic reaction was observed in these rats. In contrast, another study reported that complement consumption *in vivo* occurred immediately (i.e. within 10–120 min) in rats after the infusion of LEH, which has the ability to activate complement *in vitro* [41]. Therefore, the gradual drop of complement titer in rats in this study is unlikely to be due to the complement consumption, but some impairment in the metabolic or synthetic function of complement may be underlying the mechanisms of this phenomenon.

To further examine the decline of the complement titer by HbV, a repeated-infusion study was performed. Additional HbV was infused at two-day intervals because the decline of complement titer was remarkable on day 3 after the HbV infusion. Very interestingly, only the first infusion reduced the complement titer and, thereafter, the recovery of the complement titer occurred gradually despite additional HbV being infused (Fig. 6). Namely, the additional HbV infusion did not induce the further decline of the complement titer. These results also indicate that the transient drop of complement titer is not due to the complement consumption that resulted from the direct interaction between HbV and the complement components. As mentioned above, the gradual drop of the complement titer is probably due to yet unknown mechanisms other than consumption. However, such unknown mechanisms were not enhanced by the additional HbV infusion. Therefore, the precise mechanisms of the decrease of the complement titer induced by HbV infusion remain to be clarified with great interest.

To investigate whether HbV would induce anaphylactic or pseudoallergic reactions, rats were sensitized several times with EV at appropriate

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intervals. Because HbV would be administered intravenously in clinical settings, the first administration of EV was performed intravenously. On the other hand, OVA as a positive control was subcutaneously administered with the adjuvant to obtain a complete immunization. Although three administrations were carried out after the first EV infusion, no clinical modulation was observed while the OVA-sensitized rats resulted in death after the second administration. These results suggest that HbV may induce neither anaphylactic nor allergic reaction in subjects, possibly also including humans.

Finally, HbV induced a transient change in the leukocyte population, but not in the RBC, WBC or PLT counts. In addition, the $CD4^+/CD8^+$ ratio was maintained at a reasonable level without the inversion of the ratio. Although the complement titer transiently dropped on day 3 after the HbV infusion, the decline was not considered to be the complement consumption and the degree of the decline is unlikely to affect host defense. In addition, repeated HbV infusion also revealed that the influence of HbV on complement was transient and an additional four HbV infusions did not induce the further decline of the complement titer. Furthermore, no anaphylactic reaction was observed in multiple EV infusions. In conclusion, from a clinical point of view, HbV induced no serious side-effects regarding the subjects in this study, and is a promising material to be safely administrable as an artificial oxygen carrier.

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Oxidation of Arg-410 promotes the elimination of human serum albumin

Yasunori Iwao [a], Makoto Anraku [a], Keishi Yamasaki [a], Ulrich Kragh-Hansen [b], Keiichi Kawai [c], Toru Maruyama [a], Masaki Otagiri [a,*]

[a] *Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan*
[b] *Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark*
[c] *School of Health Sciences, Faculty of Medicine, Kanazawa University, Ishikawa 920-0942, Japan*

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Abstract

The effect of the oxidation of amino acid residues on albumin on its in vivo elimination was investigated using mutants and oxidized HSAs. The single-residue mutants (H146A, K199A, W214A, R218H, R410A, Y411A) and oxidized HSAs were produced by the recombinant DNA techniques and incubation with a metal ion-catalyzed oxidation (MCO) system for 12, 24, 48 or 72 h. Pharmacokinetics were evaluated in mice after labeling with ^{111}In. Structural and functional properties were examined by several spectroscopic techniques. Time-dependent increase in carbonyl group content resulted in increase in the liver clearance of oxidized HSAs. Slight decreases in α-helical content as the result of oxidation was induced by the increases in accessible hydrophobic areas and the net negative charge on the HSA molecule. No significant change in the pharmacokinetics and structural properties was observed for the W214A, R218H and Y411A mutants, but the properties for the H146A, K199A and R410A mutants were affected (extent of effect: R410A>K199A>H146A). The liver clearance of these proteins is closely correlated to hydrophobicity ($r=0.929$, $P<0.01$) and the net charge of the proteins ($r=0.930$, $P<0.01$). The rate of elimination of HSA is closely related to the hydrophobicity and net charge of the molecule. Further, the R410A mutants had a short half-life and structure similar to oxidized HSA after oxidation. Therefore, the modification of Arg-410 via oxidative stress may promote the elimination of HSA.
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Keywords: Human serum albumin; Oxidation; Arg-410; Elimination; Liver clearance

1. Introduction

In vivo studies with radioisotope-labeled human serum albumin (HSA) revealed the plasma half-life to be 19 days in humans [1]. However, it is well known that certain modifications of HSA shorten its half-life in circulating blood [2,3]. Such changes in the rate of elimination of HSA as well as of other serum proteins seems to depend on the individual elimination process, but the underlying mechanisms have not been clarified on a molecular basis.

Oxidative stress is increasingly thought to be a key element in some diseases [4–6]. Witko-Sarsat et al. reported the presence of elevated levels of oxidized protein products, termed advanced oxidation protein products (AOPP) such as oxidized albumin, in the plasma of hemodialysis (HD) patients [7]. It has been well documented that HSA is quite vulnerable to reactive oxygen species (ROS) [8]. In plasma, all amino acids in a protein are susceptible to oxidative modification by oxidants such as hydroxyl radicals and hypochlorous acid. Among them, amino acids, such as cysteine, histidine, lysine and arginine, are more vulnerable to oxidation than the others [9]. We recently found that the modification of these residues on HSA result in conformational changes in cases of uremia [10]. However, the issue of whether the modification of a specific amino acid residue affects the rate of elimination of HSA is not clear. Since changes in hydrophobicity and net charge on a protein surface affect the in vivo clearance of proteins [11,12], the consequences of HSA modification, such as oxidation and glycation, may increase its elimination rate. Sheffield et al. recently reported that the half-life of rabbit albumin was reduced to 2.87 days for the

Abbreviations: HSA, human serum albumin; rHSA, recombinant HSA; MCO_{12}-HSA, MCO_{24}-HSA, MCO_{48}-HSA and MCO_{72}-HSA, HSA oxidized by metal ion-catalysis for 12, 24, 48 and 72 h, respectively; bis-ANS, 1,1-bis-4-anilino-naphthalene-5,5-sulfonic acid

* Corresponding author. Fax: +81 96 362 7690.
E-mail address: otagirim@gpo.kumamoto-u.ac.jp (M. Otagiri).

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D494N variant [13]. This suggests that the modification of a specific amino acid residue of HSA could result in an increase in its rate of elimination.

In the present work, we examined the effect of oxidation on the plasma half-life and organ uptake of HSA in mice using mutants and oxidized HSAs. A metal ion-catalyzed oxidation (MCO) system was used for the oxidation, because ferrous ions in vivo are capable of generating radicals, which can oxidize proteins such as HSA [14]. On the other hand, in order to investigate the amino acids residues related to its elimination, we prepared six single-residue mutants of HSA, which are known to be elements of the three important binding sites, namely His-146 in subdomain IB, Lys-199, Trp-214 and Arg-218 in Site-I and Arg-410 and Tyr-411 in Site-II (rHSA) by site-directed mutagenesis. The structural and functional properties of the molecules were also examined by spectroscopic and electrophoretic analysis.

2. Materials and methods

2.1. Materials and animals

HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). Fluoresceinamine (isomer II) and the fluorescence probe 1,1-bis-4-anilino-naphthalene-5,5-sulfonic acid (bis-ANS) were obtained from Sigma (St. Louis, MO, USA). Potassium warfarin (Eisai Co., Tokyo, Japan) and ketoprofen (Sanwakagaku Co., Tokyo, Japan) were obtained as pure substances from the manufacturers. $^{111}InCl_3$ (74 MBq/mL in 0.02 N HCl) was a gift from Nihon Medi-Physics (Takarazuka, Japan). All other chemicals were also of the highest grade commercially available, and all solutions were prepared in deionized and distilled water.

Male ddY mice (24–26 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Synthesis of single-residue mutants

The recombinant DNA techniques used to produce wild-type rHSA and the single-residue mutants H146A, K199A, W214A, R218H, R410A and Y411A were essentially the same as described by Watanabe et al. [15]. The chimeric plasmid (pJDB-ADH-L10-HSA-A) having cDNA for the mature form of HSA along with an L10 leader sequence was a gift from Tonen Co. (Tokyo, Japan). The mutagenic primers used (underlined letters indicate mismatches) were 5′-GCCAGAAGA**GC**TCCTTACTTTTATGCCC-3′ for H146A, 5′-AAACAGAGACTC**GCC**TGTGCCAGTCTCC-3′ for K199A, 5′-GAGCTTTCAAAGCA**GCT**GCAGTAGCTCGCCTG-3′for W214A, 5′-GGG-CAGTAGCTC**AT**CTGAGCCAGAG-3′ for R218H, 5′-CTATTAGTT**TGC**-TACACCAAG-3′ for R410A, 5′-CTATTAGTTCGT**GC**CACCAAG-3′ for Y411A.

The L10-HSA coding region was amplified by PCR with a forward and a reverse primer carrying a 5′-terminal *Eco*RI site and cloned into the *Eco*RI-digested pKF19k vector (Takara Shuzo Co., Kyoto, Japan). Mutagenesis was performed with a site-directed mutagenesis kit (oligonucleotide-directed dual amber method), obtained from Takara Shuzo Co. The mutation was confirmed by DNA sequencing of the entire HSA coding region with the dideoxy chain termination method on a PerkinElmer ABI Prism 310 Genetic Analyzer. For constructing the HSA expression vector pHIL-D2-HSA, a L10-HSA coding region without or with the desired mutation site was incorporated into the methanol inducible pHIL-D2 vector (Invitrogen Co., San Diego, CA, U.S.A.). The resulting vector was introduced into the yeast species *P. pastoris* (strain GS115) for rHSA expression. The secreted rHSA was isolated from the growth medium by a combination of precipitation with 60% (w/v) $(NH_4)_2SO_4$ and purification on a Blue Sepharose CL-6B column (Amersham Pharmacia Co., Uppsala, Sweden). HSA, rHSA and the mutants were defatted using the charcoal procedure described by Chen [16], deionized, freeze-dried and then stored at −20 °C until used. Density analysis of protein bands stained with Coomassie Brilliant Blue showed the purity of the recombinant albumins to be in excess of 97%. The molecular mass of all the albumins was assumed to be 67 kDa.

2.3. Synthesis of oxidized HSAs

To prepare MCO-HSA, HSA (300 μM) was incubated in 67 mM sodium phosphate buffer (pH 7.4) at 37 °C in an oxygen-saturated solution containing sodium ascorbate (100 mM) and $FeCl_2$ (10 μM) [17]. Ascorbate was added to reduce the Fe^{3+} formed in the oxidation back to Fe^{2+}. Aliquots were withdrawn after different time intervals (12, 24, 48, 72 h), and the oxidative process was terminated by cooling and removing the oxidants by extensive dialysis against water. The MCO-HSAs and HSA were stored at −20°C until used.

2.4. Carbonyl content determination

Protein carbonyl content was quantified using the method of Climent et al. [18]. The groups were derivatized with fluoresceinamine and their modified levels were calculated from the absorbance of the complexes at 490 nm (Jasco Ubest-35 UV/VIS spectrophotometer).

2.5. In vivo experiments

All proteins were radiolabeled with ^{111}In using the bifunctional chelating regent DTPA anhydride according to the method of Hnatowich et al. [19], which has been described in a previous paper [20]. In previous works, we found no significant changes in pharmacokinetics properties among these albumins, when ^{111}In-labeled mouse, rat, bovine and human serum albumin was administered to mice (unpublished). Therefore, we chose the mouse as a reasonable model for the study of the pharmacokinetics of HSAs. Mice received tail vein injections of ^{111}In-labeled proteins in saline, at a dose of 1 mg/kg and were housed in metabolic cages to allow the collection of urine samples. Urine samples were collected throughout the 120 min of the experimental period. In the early period after injection, the efflux of ^{111}In radioactivity from organs is assumed to be negligible, because the degradation products of ^{111}In-labeled ligands using DTPA anhydride cannot easily pass through biological membranes [21]. This assumption was supported by the fact that no ^{111} In was detectable in the urine throughout the 120 min. At appropriate intervals after the injection, blood was collected from the vena cava under ether anesthesia and plasma was obtained by centrifugation. The liver, kidney, spleen, lung, heart and muscle were excised, rinsed with saline and weighed. The radioactivity of each sample was measured in a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo).

Pharmacokinetic analyses were performed as follows. The plasma ^{111}In radioactivity concentrations (C_p) were normalized with respect to the percentage of the dose per mL and analyzed using the nonlinear least-square program MULTI [22]. The two-compartment model was fitted according to the Akaike information criterion by Eq (1).

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \tag{1}$$

The half-lives of these HSAs were calculated by the β-phase. The tissue distribution patterns were evaluated using tissue uptake clearances (CL_{tissue}) according to the integration plot analysis. CL_{tissue} was calculated using Eq (2).

$$CL_{tissue} = \frac{AUC_{0-t}/C_t}{X_t/C_t}. \tag{2}$$

Where X_t is the tissue accumulation at time t, AUC $_{0-t}$ is the area under the plasma concentration time-curve from time 0 to t, and C_t is the plasma concentration at time t. CL_{tissue} was obtained from the slope of the plot of X_t/C_t versus AUC_{0-t}/C_t Mukai et al. [23] previously reported that ^{111}In is not suitable for evaluating the dynamic phase of a protein for which the in vivo half-life is long. Therefore, we estimated the plasma half-life and liver uptake clearance within a 120-min period.

2.6. Effective protein hydrophobicity

The effective hydrophobicity of all the albumins (1 μM), in 67 mM sodium phosphate buffer (pH 7.4), was estimated using the fluorescent characteristics of bis-ANS (10 μM) at 25 °C. The compound was excited at 394 nm, and fluorescence spectra were recorded on a Jasco FP-770 fluorescence spectrometer (Tokyo, Japan).

2.7. Changes in protein net charge

Changes in the net charge of albumin, as a result of oxidation or mutation, were determined by a modification of the capillary electrophoresis method described by Pande et al. [24]. One mL of a HSA sample (2 μM) was run in 100 mM borate buffer (pH 8.5 and 20 °C), and the migration time determined by using a CE990/990-10 type capillary electrophoresis from Jasco Co. (Tokyo, Japan) equipped with a 30-cm capillary having an aperture window of 100 × 200 μm.

2.8. Ligand binding experiments

Binding of warfarin (5 μM) and ketoprofen (5 μM) to HSA and oxidized HSAs (10 μM) in 67 mM sodium phosphate buffer (pH 7.4 and 25 °C) was studied by ultrafiltration. The unbound ligand fractions were separated using the Amicon MPS-1 micropartition system with YMT ultrafiltration membranes by centrifugation (2000×g, 40 min). The adsorption of warfarin or ketoprofen to the filtration membranes and apparatus was found to be negligible. The concentration of unbound ligand was determined by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump and a Hitachi F1000 variable fluorescence monitor or a Hitachi 655A variable wavelength UV monitor. LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as the stationary phase. The mobile phase consisted of 200 mM sodium acetate buffer (pH 4.5)/acetonitrile (40:60, v/v) for warfarin and of 200 mM sodium acetate buffer (pH 4.5)/acetonitrile (60:40, v/v) for ketoprofen. The flow rates in both cases were 1 mL/min. Warfarin was quantitated fluorometrically by using 300 nm and 400 nm for excitation and emission, respectively, and ketoprofen was detected at 257 nm by means of UV monitoring. The unbound fraction (%) was calculated as follows:

$$\text{Unbound fraction}(\%) = [\text{ligand concentration in filtered fraction} / \text{total ligand concentration(before ultrafiltration)}] \times 100.$$

2.9. Statistics

Statistical analyses were performed using the Student t-test. A probability value of $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Extent of carbonyl content on oxidized HSAs

The oxidation of a protein results in an increase in carbonyl content. This increase is due to the oxidation of Lys, Arg, or Pro residues. As seen in Fig. 1, the carbonyl content (nmol/mg protein) increased significantly with the time of incubation, compared with the level in a control sample of HSA. Data for the carbonyl content were as follows: MCO_{72}-HSA (5.72±0.23)> MCO_{48}-HSA (5.25±0.16)>MCO_{24}-HSA (4.92±0.32)> MCO_{12}-HSA (3.94±0.31)>HSA (2.36±0.24). These data suggest that the state of MCO-HSAs accurately reflects the oxidative state of serum proteins in uremia (3.12±1.11 nmol/mg protein, Fig. 1). Interestingly, the results of amino acid analysis showed that an exposure to MCO for 48 h incubation induced a significant change in content of arginine among the basic amino acids (Arg; MCO_{48}-HSA, 21.0±0.2, HSA, 23.9±0.4 ($P<0.05$), Lys; MCO_{48}-HSA, 57.3±0.5, HSA, 58.0±0.1, His; MCO_{48}-HSA, 14.4±0.4, HSA, 15.1±0.4, $n=4$).

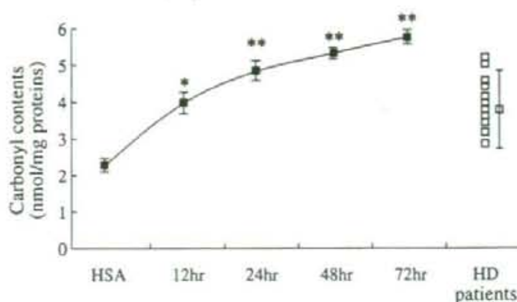

Fig. 1. Carbonyl content of MCO-HSAs as a function of incubation time and serum carbonyl content of HD patients ($N=20$, from Ref. [10]). The bars represent standard deviations ($n=4$). $^{*}P<0.05$ and $^{**}P<0.01$ as compared to HSA.

3.2. Pharmacokinetic properties of oxidized HSAs and mutated rHSAs

The time courses for radioactivity in mouse plasma after the intravenous administration of ^{111}In-labeled preparations of albumin are shown in Fig. 2. Table 1 shows the half-lives of these HSAs calculated by β-phase using the nonlinear least-square program MULTI (see Materials and methods). It can be seen from Fig. 2A that the more HSA is oxidized, the faster the radioactively labeled protein disappears from the circulation. Among the mutations (Fig. 2B), R410A had the most pronounced effect. Uptake by heart, lungs, spleen and kidneys were not significantly affected, either by the oxidation or mutations (data not shown). However, the rapid disappearance of oxidized HSA from the plasma is accompanied by a very pronounced increment in liver clearance. It is also apparent that the increment depends on the degree of oxidation. The mutations also showed an increased liver clearance. In particular, in the case of R410A, liver clearance was increased by more than 27-fold (Table 1).

3.3. Effect of oxidation and mutation on the net charge of albumin

According to the literature, the formation of MCO-HSA involves the modification of basic amino acid residues [14,25]. To test whether the oxidation procedure here affected basic amino acid residues, the net charge of the albumin preparations was investigated by determining their migration times in capillary electrophoresis. As seen in Fig. 3A, basic amino acid residues were clearly modified because an increase in migration time reflects an increase in the net negative charge of HSA. To identify specific amino acid residues that are oxidized in producing MCO-HSA, we modified some of the amino acids in HSA by site-directed mutagenesis. From Fig.

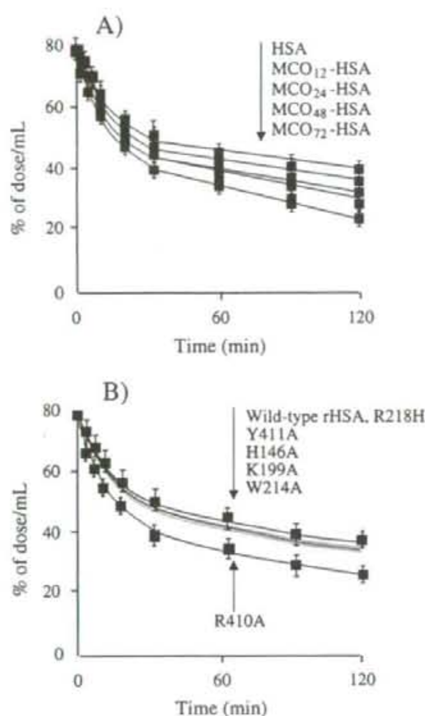

Fig. 2. Relative plasma concentrations of ^{111}In-labeled MCO-HSAs (A) and ^{111}In-labeled rHSA forms (B) after intravenous administration in mice. ^{111}In-albumin was injected as a bolus through the tail vein, and relative radioactivities are plotted against time after injection. The bars represent standard deviations (n=5–6).

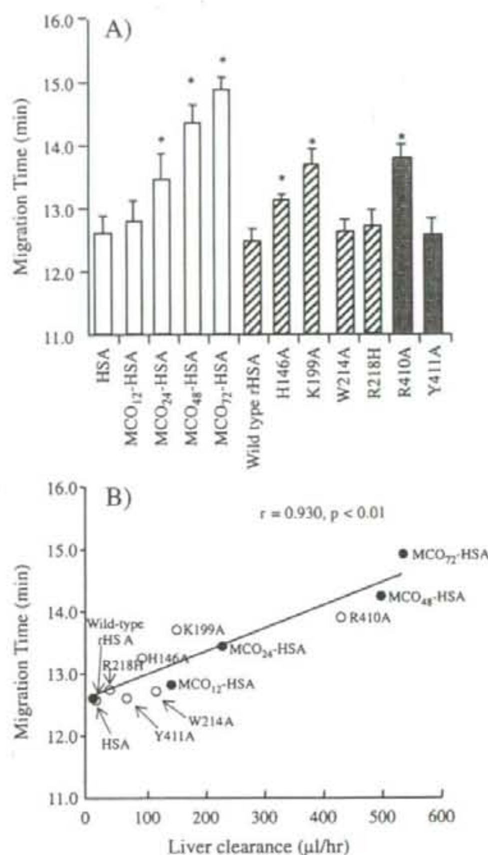

Fig. 3. Electrophoretograms of MCO-HSAs and single-residue mutants (A). The bars represent standard deviations (n=4). *P<0.05 as compared to HSA. Relationships between protein net charge and liver clearance (B).

3A, it can be seen, that the mutation of H146A and K199A situated in subdomain IB and the Site-I binding, domain led to an increased migration time (13.23±0.18 min and 13.83±0.26 min). In contrast, W214A and R218H had no significant effect on the migration of albumin. Of the mutations affecting site II binding, the mutation Y411A had no effect, whereas R410A (13.89±0.29 min) had a significant effect on migration. Furthermore, a change in the net charge on albumin is closely correlated with that of liver clearance (Fig. 3B; r=0.930, P<0.01). These results suggest that the modification of basic amino acid residue greatly affects the elimination rate of HSA.

Table 1
Half-life and liver clearances of oxidized and mutated rHSAs labeled with ^{111}In in mice

Protein	Half-life (min)	Liver clearance (μL/h)
HSA	273±6.25	13.37±2.57
MCO_{12}-HSA	211±3.31 **	136.0±3.72 **
MCO_{24}-HSA	187±8.05 **	222.1±2.02 **
MCO_{48}-HSA	170±8.34 **	498.3±8.02 **
MCO_{72}-HSA	150±5.09 **	534.7±4.13 **
Wild-type rHSA	272±9.85	15.73±5.57
H146A	238±6.22 *	98.31±5.75 *
K199A	221±5.78 **	144.4±6.01 **
W214A	206±5.26 **	126.7±6.51 **
R218H	274±9.29 *	36.92±8.56 *
R410A	162±9.26 **	426.2±8.12 **
Y411A	265±5.75 *	65.13±8.07 *

The data are average values of five or six experiments (± S.D.).
* P<0.05.
** P<0.01 as compared with HSA (oxidized HSAs) or with wild-type rHSA (mutated rHSAs).

3.4. Effect of oxidation and mutation on the hydrophobicity of albumin

The effects of oxidation and mutation on the exposure of hydrophobic areas were examined using the fluorescence probe bis-ANS. The spectra indicate that increased oxidation results in a parallel increase in accessible hydrophobic areas (Fig. 4A). In contrast, the mutations had a small effect, because only the mutations K199A and R410A mutants had an influence on bis-ANS fluorescence. We also found that the change in the hydrophobic areas on albumin is closely correlated with that of liver clearance (Fig. 4B; r=0.929, P<0.01). These results suggest that a change in hydrophobic areas greatly affects the elimination rate of HSA.

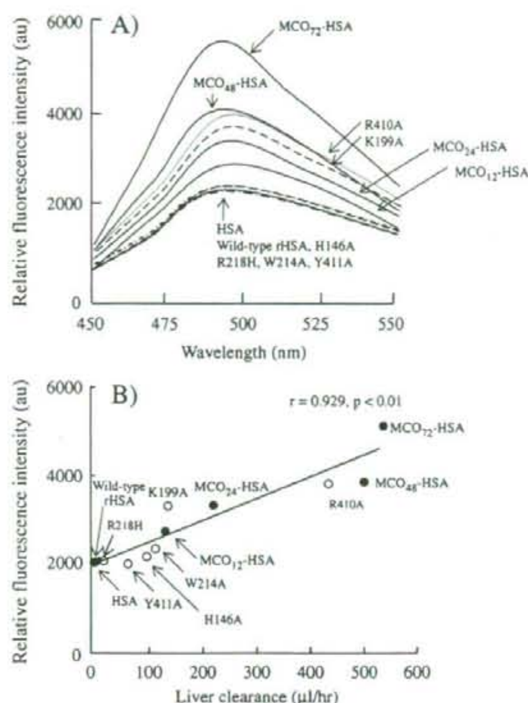

Fig. 4. Effect of oxidation and mutation on the fluorescence of albumin-bound bis-ANS (A). The spectra are averages of three determinations. Relationships between protein hydrophobicity and liver clearance (B).

3.5. Binding properties of oxidized HSAs

The unique ligand binding properties of albumin can, to a great extent, be explained by the presence of Site I and Site II located in subdomain IIA and IIIA, respectively [26]. The potential effect of oxidation on these sites was examined by using warfarin and ketoprofen as representative ligands. As seen from Table 2, high-affinity binding of warfarin, which takes place at Site I, was not significantly affected even after oxidation of HSA for 72 h. In contrast, high-affinity binding of the Site II-ligand ketoprofen to the MCO-HSAs was greatly diminished. The diminished binding was already observed for albumin, which had only been oxidized for 12 h, and the effect increased further with incubation time (Table 2).

4. Discussion

The long half-life of HSA ($t_{1/2}$ = 19 days) can be modified in disease states and/or age-related processes, most probably via the covalent modification of the protein. Our previous work showed that the oxidation of HSA in vitro results in a more rapid elimination of the protein from the circulation, a process which appears to be caused by the modification of basic amino acid residues such as lysine and arginine [3,27]. Furthermore, we recently found that the oxidation of HSA in vivo purified from HD patients was conformationally altered, and that its hydrophobic regions were more exposed and had a negative charge [10]. However, the issue of whether the modification of a specific amino acid residue is responsible for this is unclear. In the current studies, we investigated whether the oxidation of the amino acid residues on albumin affects its elimination in vivo via the use of mutants and oxidized HSAs.

A significant increase in the carbonyl content of MCO-HSAs was found with the time of incubation. Furthermore, the carbonyl content of MCO-HSAs was similar to those of serum proteins with uremia (Fig. 1). Thus, this result suggests that the state of MCO-HSAs in vitro reflects the uremic state of serum proteins in vivo. The rate of protein clearance from the blood was proportional to the time of oxidation (Fig. 2A). We also found an increased liver clearance of all of the oxidized albumins (Table 1). One reason for the shorter half-life and increased liver clearance of oxidized HSA could be due to conformational changes induced by changes in the net charge of the protein or its hydrophobicity. Another reason could be due to the modification of one or more essential amino acid residues. In this connection, it is noteworthy that Segal et al. [28] reported a correlation between protein hydrophobicity and the in vivo degradation rates of soluble rat liver proteins. In the present work, the oxidation also results in a slight decrease in α-helix content of the protein (MCO_{72}-HSA (58.6±2.9%)< MCO_{48}-HSA (60.3±2.35%)<MCO_{24}-HSA (62.1±3.21%)< MCO_{12}-HSA (65.3±2.15%)<HSA (68.3±1.18%). The minor conformational changes are accompanied by increases in the accessible hydrophobic areas of the protein (Fig. 4A). As evidenced by capillary electrophoretic measurements, oxidation also results in an increase in the negative net charge of HSA (Fig. 3A). Furthermore, taking the results for the oxidized albumins and the mutants into account, there is a good linear relationship between liver clearance and protein hydrophobicity (r=0.929, P<0.01, Fig. 4B) as well as between liver clearance and net charge of the protein molecules (r=0.930, P<0.01, Fig. 3B). Of the mutations that affect structural properties, only a mutation at K199A and R410A had a significant effect on the hydrophobicity and net charge of HSA. According to X-ray crystallographic analyses those residues are situated inside cavities or pockets in hydrophobic surroundings and are not in contact with the solvent [27,29]. Therefore, the effects of these mutations (Figs. 3A, 4A) on structural properties must be due to indirect effects such as changes in protein conformation and/or changes in electrostatic bonding patterns between residues.

Table 2
Binding of warfarin and ketoprofen to oxidized HSAs at pH 7.4 and 25 °C

Protein	Free fraction (%)	
	Warfarin	Ketoprofen
HSA	24.32±1.57	3.91±0.47
MCO_{12}-HSA	28.15±1.87	31.4±2.29 *
MCO_{24}-HSA	27.65±2.34	37.8±3.71 *
MCO_{48}-HSA	28.23±3.02	42.3±2.28 **
MCO_{72}-HSA	27.42±2.15	47.8±2.94 **

The data are average values of four experiments (± S.D.).
* P<0.01.
** P<0.001 as compared with HSA.

Thus, the increase in elimination rate may be due to not only to the modification of basic amino acid residues such as lysine and/or arginine but also to the modification of a specific site on HSA. The HSA molecule also contains a free cysteine residue and six methionine residues. We previously reported that these amino acid residues were oxidized in the early stage of the oxidation, but that this had no effect on the pharmacokinetics of HSA [3]. These results strongly support our view that the oxidation of lysine and/or arginine residues affected the pharmacokinetics of HSA, leading to rapid elimination.

In an attempt to determine which lysine and/or arginine residues were oxidized, we examined high-affinity binding to Site I and Site II on oxidized HSA. High-affinity binding at Site I was not altered significantly but Site II was significantly reduced, in the case of oxidized HSA (Table 2). Principally, the same observation was made when examining Site II binding for albumins in which Arg-410 and/or Tyr-411 [30] were mutated. It should be noted that the binding to R410A (Free fraction for ketoporofen binding; 35.85±4.03%, Ref. [30]) is similar to those for MCO_{12}-MCO_{48}-HSA (Table 2). Furthermore, amino acids analysis demonstrates that MCO results in the selective modification of Arg. At present, it is not possible to conclude for sure, whether the effect at Site II is caused by conformational changes at the site, a direct oxidative effect on Arg-410 or a combination of the two. In any event, the effect of mutating Arg-410 to alanine was dramatic. Ahmed et al. recently suggested that Arg-410 on HSA is modified by methylglyoxal in vivo [31]. Above findings suggest that Arg-410 on HSA may be a residue that is sensitive to physiological modification such as oxidation and glycation. Although this effect could be brought about by indirect means, such as changes in protein hydrophobicity and charge, it is tempting to suggest that such a pronounced effect is a specific one, implying that the integrity of Arg-410 is important for both the ligand binding properties and the plasma half-life of HSA.

Previous studies have shown that modified proteins, which contain oxidized albumin and advanced glycation end products (AGE) albumin, accumulate in liver extremely rapidly after intravenous administration, due to receptor-mediated endocytosis by nonparenchymal cells, particularly liver endothelial cells (LECs) [32,33]. Thus, we conclude that oxidized HSA prepared with the MCO system in this paper may also undergo scavenger receptor-mediated endocytosis by LECs. Several scavenger receptors that bind to modified proteins on LECs have been reported. These receptors include the following: SR-A, a scavenger receptor class A for oxidized LDL [34,35]; CD36 and SR-B1, scavenger receptor class B for AGE [36,37]; SREC, a scavenger receptor class F for modified LDL [38]; FEEL-1 and FEEL-2, endocytic receptors for AGE [39,40]; and gp18 and gp30, scavenger receptor for chemically modified of oxidized albumin [41,42]. On the other hand, Matsumoto et al. reported that SR-A is not involved in the endocytosis of AGE by LECs using SR-A knock-out mice [33]. Furthermore, Nakajou et al. reported that CD36 is not involved in the uptake of AGE by LECs using an anti-CD36 antibody [43]. Therefore, the molecular nature of the receptors involved in endocytic uptake of modified proteins is not well understood. In the future, the receptor responsible for the liver uptake of oxidized HSA needs to be identified.

In conclusion, the present study has clearly shown that the oxidative modification of a specific site on HSA may lead to conformational changes as well as the rate of elimination of HSA. In particular, the integrity of Arg-410 is important for the plasma half-life of HSA.

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