stabilizing disulfide bonds. In the circulation, normally about half of the Cys-34 residues are freely accessible, i.e., not oxidized or involved in ligand binding, and they represent the largest fraction of free thiols in blood.

HSA is the most abundant protein in blood plasma and serves, among other things, as a transport and depot protein for numerous endogenous and exogenous compounds [10]. We studied the effects of the strongly bound ligands oleate (OA), bilirubin (BR) and Cu^{2+} and the weakly bound ligands L-tryptophan, progesterone, ascorbate, Zn^{2+} and Fe^{2+} on S-nitrosylation of HSA by S-nitrosoglutathione (GS-NO), 1-hydroxy-2-oxo-3-(N-3-methyl-aminopropyl)-3-methyl-3′-triazene (NOC-7) and stimulated RAW264.7 cells.

Materials and methods

Materials. Non-defatted HSA (96% pure) was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan), and it was defatted by treatment with charcoal as described by Chen [11]. Sephadex G-25 (φ1.6 × 2.5 cm), Blue Sepharose CL-6B (φ2.5 × 20 cm), and RESOURCE PHE columns (φ0.64 × 3 cm) were from Amersham Pharmacia Biotech (Tokyo, Japan). Enzymes for DNA assays were from Takara (Kyoto, Japan). The Pichia Expression kit was from Invitrogen (Carlsbad, CA). L-Tryptophan, ascorbic acid, $FeCl_2$, $(CH_3COO)_2Zn$, $CuSO_4{\cdot}5H_2O$, BR, progesterone, OA, 1,4-dithiothreitol (DTT), and glutathione (GSH) were purchased from Sigma–Aldrich (St. Louis, MO). Sulfanilamide, naphthylethylenediamine–hydrochloride, $HgCl_2$ and $NaNO_2$ were obtained from Nakalai Tesque (Kyoto, Japan). GS-NO, NOC-7, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), diethylenetriaminepentaacetic acid (DTPA), and ethylenediaminetetraacetic acid (EDTA) were obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of the best grades commercially available, and all solutions were made in deionized and distilled water.

Synthesis and purification of recombinant HSA. Wild-type HSA and the H3A mutant were synthesized, using *P. pastoris* GS115 his4, and purified as previously described [9]. The mutagenic primers (sense and antisense) for making the mutant were:

5′-GCTCATCCGATGGCCACAAGAGTGAGG-3′, and 3′-CCTCA CTCTTGTGGCCATCGGATGAGC-5′.

The albumins were deionized and defatted via charcoal treatment, freeze-dried, and then stored at −20 °C until used. According to density analysis of Coomassie Brilliant Blue-stained protein bands on 12.5% SDS–PAGE, the purity of the protein samples were more than 97%.

Preparation of ligand-HSA solutions. First, HSA was treated with DTT as follows. HSA (300 μM) was incubated with DTT (molar ratio, protein:DTT = 1:10) for 5 min at 37 °C. After that, DTT was quickly removed by Sephadex G-25 gel filtration using 10 mM phosphate-buffered saline (pH 7.4) (PBS; Ca^{2+}, Mg^{2+} free). Stock solutions of 20 mM OA and 20 mM progesterone were made in methanol-H_2O (1:1, v/v) and ethanol-H_2O (1:1, v/v), respectively, whereas 20 mM BR was made in 0.1 N NaOH and protected against light. Later, these stock-solutions were diluted with PBS. Other ligands were directly dissolved in PBS. In all cases, the resulting solutions were mixed with PBS containing HSA. The ligand–protein solutions, except for those having $CuSO_4$, were incubated for 30 min at 37 °C in the dark. Freshly prepared $CuSO_4$-HSA solutions were also incubated for 30 min in the dark but at 4 °C, because the SH-group of HSA easily undergoes oxidation in the presence of Cu^{2+}. To remove free ligands, mixtures were applied to a Sephadex G-25 column, quickly eluted with PBS and concentrated by ultrafiltration. The protein content of all protein preparations used in this study was determined by the Bradford assay.

Accessibility of Cys-34. In reduced HSA this was estimated with Ellman's reagent, DTNB. Briefly, the accessibility was evaluated as A_5/A_{60}, where A_5 and A_{60} is the sample absorbance at 405 nm after 5 min and 60 min (maximal absorbancy), respectively, of incubation with DTNB [12].

S-Nitrosylation of HSA in cell-free reaction systems. SNO-HSA was prepared with protection against light and according to previous reports [13,14]. HSA (100 μM) with and without ligand was incubated with GS-NO or NOC-7 as NO donor (molar ratio, protein:NO donor = 1:5) in PBS for 10 min at 37 °C. To remove NO donors, S-nitrosylated products were applied to a Sephadex G-25 column, eluted with PBS containing 0.5 mM DTPA, and concentrated by ultrafiltration. These samples were stored at −80 °C until analyzed.

Determination of S-nitrosylation efficiency. The amounts of the S-nitroso moiety of SNO-HSA were quantified by HPLC coupled with a flow-reactor system, as previously reported [13,15]. The HPLC column was a gel filtration column for S-nitrosylated proteins (φ8 × 300 mm), Diol-120, YMC, Kyoto, Japan. Briefly, the eluate from the HPLC column was mixed with a $HgCl_2$ solution to decompose S-nitrosylated compounds to yield NO_2^- (via NO^+). The NO_2^- generated was then detected after reaction with Griess reagent in the flow-reactor system.

SNO-HSA production by cells in culture. RAW264.7 cells were cultured in 24-well plates (16-mm diameter; Falcon, Lincoln Park, NJ) with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and nonessential amino acids (Life Technologies, Inc.). Cells at saturation density (1×10^6 cells/well) were stimulated with interferon-γ (Genzyme, Cambridge, MA) at 100 U/ml and lipopolysaccharide (*Escherichia coli* 026B; Difco) at 10 μg/ml for 12 h at 37 °C in a CO_2 incubator (5% CO_2, 95% air (v/v)). The culture medium was removed, and the cells were washed three times with PBS (pH 7.4). Cells were further incubated in the CO_2 incubator at 37 °C with 200 μl of PBS containing 0.5 mM L-arginine and 100 μM HSA alone or with bound ligand. After incubation for 10 min, the reaction medium was mixed with 1/10 volume of 5 mM DTPA dissolved in PBS (pH 7.4), followed by centrifugation at 10,000*g* for 10 min at 4 °C. The resultant supernatants were stored at −80 °C until applied to the HPLC-flow reactor system.

Statistical analysis. The statistical significance of collected data was evaluated using the ANOVA analysis followed by Newman–Keuls method for more than 2 means. Differences between groups were evaluated by the Student's *t* test. $P < 0.05$ was regarded as statistically significant.

Results and discussion

S-Nitrosylation of mercaptalbumin with bound ligands

HSA purified from serum has bound endogenous ligands, in particular fatty acids, and perhaps also exogenous ligands. Any effect of these ligands on the S-nitrosylation of HSA was examined by incubating non-defatted and charcoal-treated albumin with GS-NO. The S-nitroso moiety of the former preparation was 0.41 ± 0.02 ($n = 4$), whereas that of the latter was only 0.19 ± 0.01 ($P < 0.01$). Thus, the presence of ligands greatly enhanced the efficiency of S-nitrosylation. In order to identify ligands of importance for S-nitrosylation, individual ligands were added to HSA, which had been delipidated by charcoal and dialyzed extensively against deionized water. In these experiments, two kinds of S-nitrosylating agents were used, namely GS-NO which S-transnitrosylates via NO^+, and NOC-7 which S-nitrosylates mainly via NO and N_2O_3. The results obtained with equimolar amounts of protein and ligand are given in Figs. 1A and 2A. It can be seen that OA and BR enhances the efficiency of GS-NO, but not that of NOC-7, whereas Cu^{2+} increases the S-nitrosylation by NOC-7 but not that caused by GS-NO. In contrast, no significant effect was observed when adding L-tryptophan,

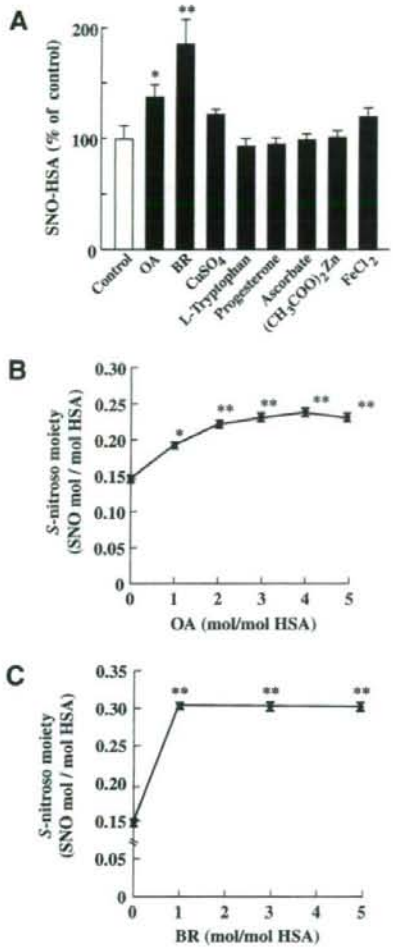

Fig. 1. Effect of ligand binding on S-nitrosylation of HSA by GS-NO. (A) 100 μM DTT-treated HSA was incubated with 100 μM of different ligands. (B) DTT-treated HSA was incubated with different molar ratios of OA. (C) DTT-treated HSA was incubated with different molar ratios of BR. In all cases, the GS-NO concentration was 500 μM. Data are expressed as means ± SEM ($n = 4$–6). $^{*}P < 0.05$, $^{**}P < 0.01$, as compared with control.

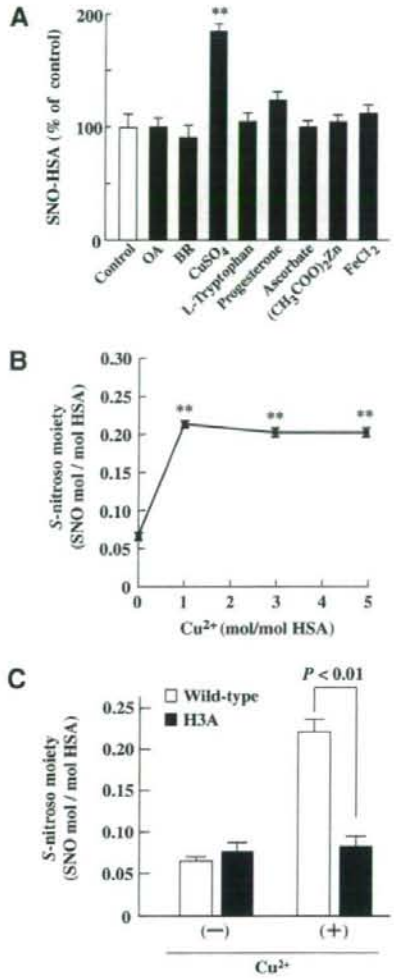

Fig. 2. Effect of ligand binding on S-nitrosylation of HSA by NOC-7. (A) Hundred micromolars of DTT-treated HSA was incubated with 100 μM of different ligands. (B) DTT-treated HSA was incubated with different molar ratios of Cu^{2+}. (C) Wild-type HSA and the H3A mutant, without or with Cu^{2+}, were S-nitrosylated by NOC-7. In all cases, the NOC-7 concentration was 500 μM. Data are expressed as means ± SEM ($n = 4$-6). $^{**}P < 0.01$, as compared with control.

progesterone, ascorbate, $(CH_3COO)_2Zn$ or $FeCl_2$. In the following, we have studied in more detail the positive effects of OA, BR and Cu^{2+}, which bind to different high-affinity sites of HSA [10,16] (Fig. 3).

Fig. 1B shows an increasing effect of OA on S-nitrosylation of HSA by GS-NO. The increment is dose-dependent until a OA:HSA molar ratio of 3; increasing the molar ratio further to 4 or 5 did not result in additional S-nitrosylation. Because OA does not bind to Cys-34 (Fig. 3), the effect observed could be due to binding-induced conformational changes of HSA making Cys-34 more accessible to GS-NO [12,17]. Actually, the data given in Table 1 propose such a mechanism, because OA binding results in an almost linear increment in binding of the test-compound DTNB to Cys-34.

The effect of BR binding on S-nitrosylation by GS-NO was also studied at different molar ratios of ligand to protein (Fig. 1C). Without BR the amount of S-nitroso moieties was 0.15 ± 0.02 ($n = 3$), and with BR it was approximately 0.30 ($P < 0.01$). The latter value was obtained, whether the BR:HSA molar ratio was 1, 3 or 5. Thus, only high-affinity BR binding increases S-nitrosylation. Because this kind of binding takes place to another region of HSA than that housing Cys-34 (Fig. 3) the improving effect must be the result of conformational

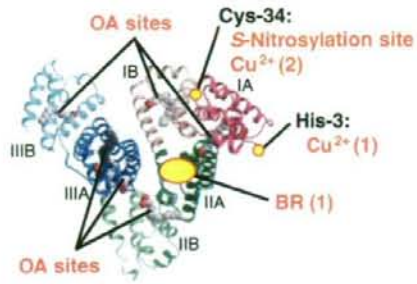

Fig. 3. Crystal structure of HSA showing locations of OA binding sites, high-affinity binding sites for BR (BR(1)) and Cu^{2+} (Cu^{2+}(1)) and Cys-34 which also is the site for secondary Cu^{2+} binding (Cu^{2+}(2)). The subdivision of HSA into domains (I-III) and subdomains (A and B) is indicated. The structure was simulated on the basis of X-ray crystallographic data for HSA-OA (PDB ID code 1gni) and modified with the use of Rasmol (downloaded from http://www.openrasmol.org).

Table 1
Effect of binding on the accessibility of Cys-34

Ligand/HSA	0	1	3	5
OA	0.17 ± 0.01	$0.22 \pm 0.02^{*}$	$0.67 \pm 0.03^{**}$	$0.84 \pm 0.04^{**}$
BR	0.17 ± 0.02	N.D.	N.D.	N.D.
Cu^{2+}	0.17 ± 0.02	0.18 ± 0.03	0.20 ± 0.04	0.19 ± 0.04

The accessibility was evaluated as A5/A60, where A5 and A60 is the sample absorbance at 405 nm after 5 min and 60 min (maximal absorbancy), respectively, of incubation with DTNB [12]. Results are given as means ± SEM ($n = 5$). N.D., not determined. $^{*}P < 0.05$, $^{**}P < 0.01$, as compared with Ligand/HSA = 0.

changes in the protein related to accommodation of the large BR molecule. That such conformational changes take place has previously been detected by techniques such as fluorescence spectroscopy [10]. By contrast to GS-NO, high-affinity binding of BR does not influence S-nitrosylation by NOC-7 (Fig. 2A). For testing whether this lack of effect could be caused by an interaction between NO and HSA-bound BR, we performed spectrophotometric experiments. These experiments showed that exposure of HSA-BR to NOC-7, but not to GS-NO, results in a fast decrease of the absorbancy at 470 nm (representing λ_{max} for HSA-BR) and a concomitant and pronounced increase at 650 nm (representing λ_{max} for HSA-biliverdin) (data not shown). Therefore, the following reaction seems to have taken place: (^{34}Cys-SH)-HSA-BR + ·NO → (^{34}Cys-SH)-HSA-BV + NO_2^-. Thus, the lack of effect of BR is due to a conversion to biliverdin (BV), and neither that ligand nor the NO_2^- formed can improve S-nitrosylation.

In contrast to the S-nitrosylating effect of GS-NO, the effect of NOC-7 was significantly increased by the presence of Cu^{2+} (Fig. 2A). The increasing effect was the same, whether the molar ratio of Cu^{2+} to protein was 1:1, 3:1 or 5:1 (Fig. 2B). Cu^{2+} binds with a very high affinity to a specific site in the N-terminal region of HSA, and His-3 is an essential element of that site [10]. In order to test whether high-affinity binding of Cu^{2+}, which takes place at a distance from Cys-34 (Fig. 3), is responsible for the improving effect of NOC-7, or whether the effect is caused by other means, e.g. secondary binding, we mutated His-3 for an alanine. The results of Fig. 2C show that the positive effect of Cu^{2+} disappears when mutating His-3. This finding strongly proposes high-affinity binding as the reason for the improving effect of Cu^{2+} on the S-nitrosylation by NOC-7. The positive effect of high-affinity Cu^{2+} binding is most probably caused by conformational changes induced in the HSA molecule, which render the SH-group of Cys-34 more reactive. Such a mechanism also seems to be supported by the results of Zhang and Wilcox [18]. These authors, using isothermal titration calorimetry and different spectroscopic techniques, found evidence for an interaction between the first Cu^{2+} binding site and Cys-34 in bovine serum albumin. However, these conformational changes are different from those caused by OA, because in contrast to OA binding of Cu^{2+} does not affect the accessibility of Cys-34 (Table 1). In contrast to the present findings Stubauer et al. [19] found no effect of high-affinity bound Cu^{2+} on RS-NO formation. RS-NO formation was only initiated, when that binding site was saturated, and the authors proposed S-nitrosylation of Cys-34 when also Cu^{2+} binds with a low affinity to the same residue. However, they used bovine serum albumin and NO gas in their studies.

S-Nitrosylation of mercaptalbumin-ligand complexes by NOC-7 and RAW264.7 cells

For studying S-nitrosylation of HSA in a biological system, we investigated the process caused by the murine macrophage cell line RAW264.7 (Fig. 4). The cell line had been activated by interferon-γ and lipopolysaccharide for expressing the inducible NO synthase. Binding of OA or BR does not affect S-nitrosylation of HSA by the cells. By contrast, Cu^{2+} binding facilitates S-nitrosylation (Fig. 4B). Fig. 4C shows that binding of Cu^{2+}, but not binding of OA or BR, decreases significantly the production of NO_2^-. These results propose that the formation of SNO-HSA by the cell line takes place via NO. This proposal was supported by findings showing that the effects of GS-NO in a similar experiment were different from those of NOC-7 and the RAW cells (data not shown).

Concluding remarks

Normally, the molar ratio of endogenous fatty acids to HSA is about 1.5 or lower [10], and those of BR and Cu^{2+} are below unity. Strenuous exercise or other adrenergic stimulation can rise the molar ratio for fatty acids to about 4 [10]. The molar ratios of all three ligands can be elevated in pathological conditions, e.g., metabolic syndrome, Type II (non-insulin-dependent) diabetes (fatty acids), increased catabolism of hemoglobin or hepatic

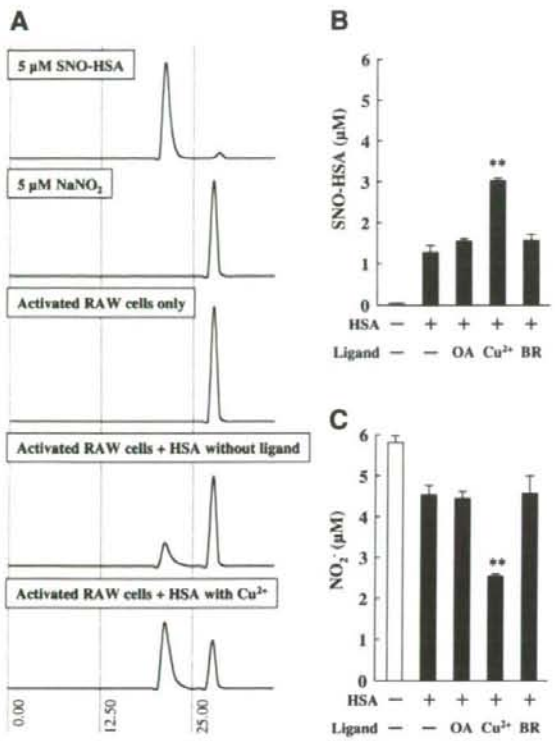

Fig. 4. Effect of high-affinity binding of OA, Cu^{2+} and BR on S-nitrosylation of HSA by activated RAW264.7 cells. (A) Elution profiles for the standard solutions (5 μM SNO-HSA and 5 μM $NaNO_2$) and for different cell media made by the HPLC-flow reactor system. The retention time for SNO-HSA and NO_2^- is about 21 and 27 min, respectively. (B) Production of SNO-HSA from HSA with and without bound ligand. (C) Production of NO_2^- in the presence of HSA with and without bound ligand. Data are expressed as means ± SEM ($n = 4$–6). $^{**}P < 0.01$, as compared with HSA without bound ligand.

disorders (BR) and Wilson's disease (Cu^{2+}). Thus, several physiological, clinical and pathological situations can affect the concentration of the quantitatively important SNO-HSA via modified high-affinity binding of OA, BR or Cu^{2+}.

In addition to HSA, other proteins possessing a free cysteine residue can be targets for S-nitrosylation, e.g. α_1-protease inhibitor [20]. Many of these proteins also bind ligand(s) the amounts of which can change in different biological or pathological conditions. Therefore, the present observations made with HSA could be general effects also applying to other proteins binding ligands and NO.

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Changes of net charge and α-helical content affect the pharmacokinetic properties of human serum albumin

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Abstract

The pharmacokinetics of 17 genetic variants of human serum albumin with single-residue mutations and their corresponding normal albumin were studied in mice. In all cases, the plasma half-life was affected, but only variants with +2 changes in charge prolonged it, whereas changes in hydrophobicity decreased it. Good positive and negative correlations were found between changes in α-helical content taking place in domains I+III and domain II, respectively, and changes in half-lives. No correlation was found to type of mutation or to changes in heat stability as represented by ΔH_v. Liver and kidney uptake clearances were also modified: α-helical changes of domains I+III showed good negative correlations to both types of clearances, whereas changes in domain II only had a good positive correlation to kidney uptake clearance. No correlation between the other molecular changes and organ uptakes was observed. The relatively few correlations between changes in molecular characteristics and the organ uptakes of the variants are most probably due to different handling by plasma enzyme(s) and the various types of cell endocytosis. Of the latter, most lead to destruction of albumin, but at least one results in recycling of the protein. The present information should be useful when designing recombinant, therapeutical albumins or albumin products with a modified plasma half-life.

Keywords: Human serum albumin; Genetic variant; Pharmacokinetics; Half-life; Hepatic uptake; Renal disposition

1. Introduction

Human serum albumin (HSA) is produced in the parenchymal cells of the liver and after removal of its pre–pro sequences, it is secreted into the circulation, where it is the most abundant plasma protein. It is an important circulating carrier of endogenous and exogenous ligands in the blood, and contributes to the maintenance of osmotic pressure, plasma pH and to the Donnan-effect in the capillaries [1,2]. The protein is formed by a single polypeptide chain of 585 amino acids and has a molecular mass of approximately 67,000 [2]. According to X-ray crystallographic analyses of HSA and of its recombinant version, albumin has about 67% α-helix but no β-sheet. The analyses also showed that the polypeptide chain forms a heart-shaped protein with three homologous domains (I–III), each comprised of two subdomains (A and B) with distinct helical folding patterns that are connected by flexible loops [3,4]. HSA has 35 cysteine residues, and all of these but one, Cys 34, are involved in the formation of stabilizing disulfide bonds.

Clinically, HSA is used to treat severe hypoalbuminemia or traumatic shock [2], and the usual dosages of HSA are in excess of 10 g/dose. To date, albumin has been produced by fractionation of whole blood. However, there is the potential risk of HSA contamination with blood-derived pathogens. In addition, human plasma is in limited supply in countries like Japan. Therefore, the development of an alternative method of industrial production of HSA is desired as this would greatly assist in the general movement of, for example, Japan toward self-sufficiency in blood and blood products. Because of these

Abbreviations: HSA, human serum albumin; Alb, albumin; Alb A, normal (wild-type) albumin; ΔH_v, van't Hoff enthalpy

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problems, recombinant HSA, that is highly expressed by *Pichia pastoris*, most probably will be commercially available in the near future. Protein engineering will also enable the creation of recombinant HSAs with modified properties such as extended half-life in the circulation. A modified half-life will also be beneficial, when HSA is used as a fusion protein to extend the half-life of small proteins like soluble CD4 and hirudin [5,6]. Despite these possibilities, not much has been done to clarify the effects of mutations on the pharmacokinetics of HSA.

Previous studies with chemically modified albumin have demonstrated that changes in hydrophobicity and net charge on the protein surface affect its in vivo clearance [7,8]. In a previous study in which we produced six recombinant HSAs, our findings suggested that even single-residue mutations of surface or more internally placed amino acids can affect both the structural properties and the in vivo elimination of HSA [9]. Even though these studies indicated that the elimination of HSA is dependent on its structural properties more detailed studies of the relationships between structural and pharmacokinetic properties are needed to design useful recombinant HSAs with modified half-lives in the blood.

In our search for such candidates, we paid our attention to HSA genetic variants. Until now, more than 60 inherited variants of HSA have been identified and structurally characterized [10]. Usually, these genetic variants are expressed in heterozygous form and without any known association to disease [2]. Therefore, unlike lethal mutations, such as may occur for hemoglobin and coagulation factors, studying the pharmacokinetic properties of HSA variants is a good way of gaining information which can be used when designing recombinant HSAs, because we can consider the effects of molecular variation without worrying about complications such as antigenic effects.

Previously, we have shown that inherited single-residue substitutions affected the structure (α-helical content) and thermal stability (van't Hoff enthalpy (ΔH_v)) of HSA [11]. In addition, good linear correlations between mutation-induced changes of α-helical content and ΔH_v were found. In the present study, we examined the pharmacokinetics of 17 structurally different genetic variants with single-residue mutations isolated from heterozygote carriers, namely Alb Malmö-95 (D63N), Alb Tregasio (V122E), Alb Hawkes Bay (C177F), Alb Herborn (K240E), Alb Niigata (D269G), Alb Caserta (K276N), Alb Canterbury (K313N), Alb Brest (D314V), Alb Roma (E321K), Alb Sondrio (E333K), Alb Parklands (D365H), Alb Milano Slow (D375H), Alb Kashmir (E501K), Alb Maku (K541E), Alb Church Bay (K560E), Alb Verona (E570K) and Alb Milano Fast (K573E). We labeled the variants and their corresponding endogenous normal albumin with ^{111}In, and examined the pharmacokinetic properties of these albumins in mice. Then, we determined the effects of the natural mutations on plasma half-life and organ uptakes. Finally, we related these results to the previously reported changes in α-helical content and ΔH_v.

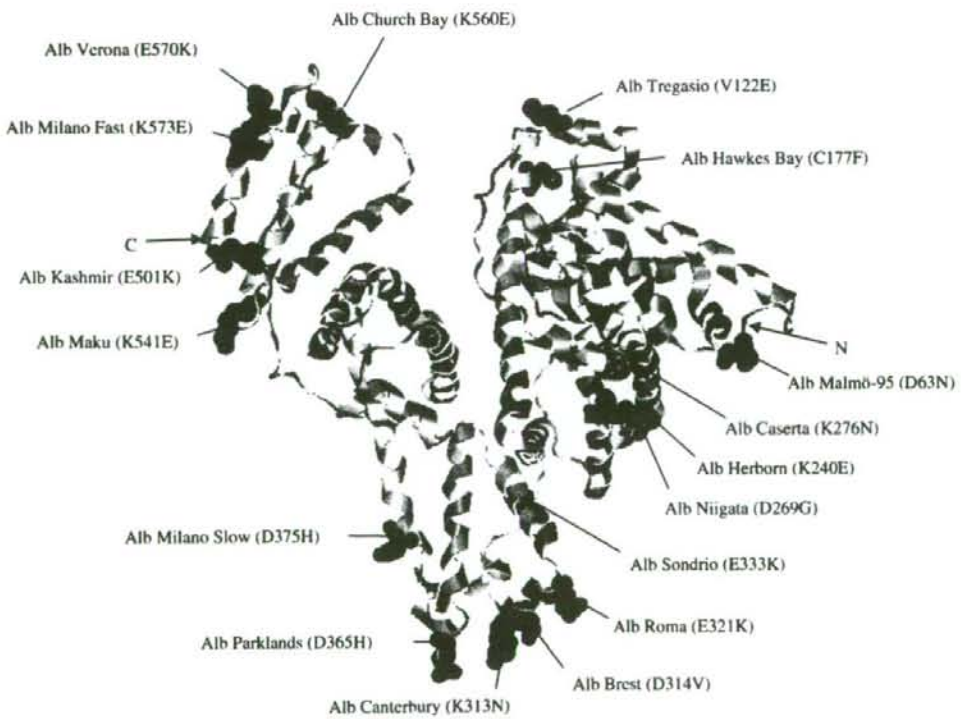

Fig. 1. The crystal structure of HSA showing the locations of the single-residue mutations of the 17 genetic variants used in this study. N and C stand for the N-terminal and the C-terminal ends, respectively.

2. Materials and methods

2.1. Protein samples

The 17 HSA genetic variants used in this study have been named after the place from where the first detected carrier originates. The variants have single-residue substitutions, and the location of the mutations is shown in Fig. 1. All variants, except one, have net charges which differ from that of normal (wild-type) HSA (endogenous Alb A) at physiological pH: Alb Roma (E321K) [12], Sondrio (E333K) [13], Kashmir (E501K) [14] and Verona (E570K) [15] are all +2 variants (i.e., they have two positive charges more than Alb A); Alb Malmö-95 (D63N) [16], Niigata (D269G) [17], Brest (D314V) [18], Parklands (D365H) [19] as well as Milano Slow (D375H) [20] are +1 variants; Alb Tregasio (V122E) [21], Caserta (K276N) [22] and Canterbury (K313N) [23] are −1 variants; and, finally, Alb Herborn (K240E) [24], Maku (K541E) [25], Church Bay (K560E) [26] and Milano Fast (K573E) [27] are −2 variants. The only variant with no change in net charge is Alb Hawkes Bay (C177F) [28]. Most of the substitutions are placed in domain II (residues 200–391), namely 9. Domains I (residues 1–199) and III (residues 392–585) are represented by three and five examples, respectively.

After isolation from heterozygous sera by ion-exchange chromatography, the individual albumins were checked for homogeneity by native electrophoresis, and no denaturation or significant (no more than 5%) cross-contamination between variant and Alb A was detected. The proteins were donated to us by Drs. M. Galliano and L. Minchiotti, University of Pavia, Pavia, Italy; Dr. S.O. Brennan, Canterbury Health Laboratories, Christchurch, New Zealand; Dr. A.L. Tarnoky, University of Reading, Reading, UK; and Dr. O. Sugita, Niigata University School of Medicine, Niigata, Japan. Before use, the albumins were delipidated by treatment with hydroxyalkoxypropyldextran at pH 3.0, as described elsewhere [29]. After defatting, the albumins were dialysed extensively against deionized water, lyophilized and stored at −20 °C until used. Thus, the albumins from a donor have been exposed to exactly the same conditions from the time the blood samples were taken until the present experiments were performed.

Fraction V HSA (96% pure), assumed to be Alb A, was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and defatted using the charcoal procedure described by Chen [30], deionized, freeze-dried and then stored at −20 °C until used.

2.2. Chemicals and animals

$^{111}InCl_3$ (74 Mbq/mL in 0.02 N HCl) was donated by Nihon Medi-Physics (Takarazuka, Japan). All chemicals were of the highest grade commercially available, and all solutions were prepared using deionized, distilled water.

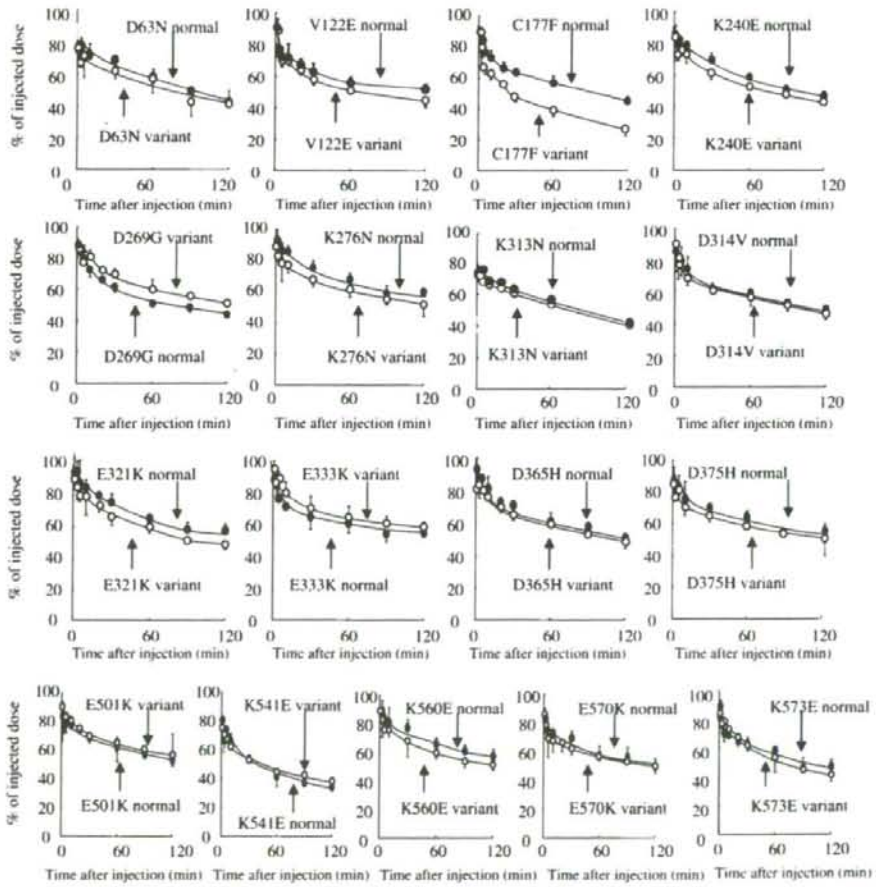

Fig. 2. Relative plasma amounts of ^{111}In-labeled HSA variants and their corresponding Alb A after intravenous administration in mice. ^{111}In-albumin was injected as a bolus dose into the tail vein. Relative amounts are plotted against time after injection. Each point represents an average value obtained for 3–6 mice (±S.D.). The open and closed circles represent variant and normal albumin, respectively.

Male ddY mice (26–32 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan), and 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.

2.3. In vivo experiments

All proteins were radiolabeled with ^{111}In using the bifunctional chelating reagent DTPA anhydride according to the method of Hnatowich et al. [31], which has been described in detail in a previous paper [32]. In previous works, we found no significant differences in pharmacokinetic properties among these albumins, when ^{111}In-labeled mouse, rat, bovine or human serum albumin was administered to mice (unpublished), suggesting that immunogenic behavior does not occur in mice. Therefore, we chose the mouse as a reasonable model for the study of the pharmacokinetics of the HSAs. Mice received tail vein injections of ^{111}In-labelled proteins in saline, at a dose of 0.1 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 proteins using DTPA anhydride cannot easily pass through biological membranes [33]. This assumption was supported by the fact that no ^{111}In was detectable in the urine after 120 min. At 1, 3, 5, 10, 30, 60, 90 or 120 min after injection, blood was collected from the vena cava under ether anesthesia and plasma was obtained by centrifugation. After blood collection, the animals were sacrificed, organs were excised, rinsed with saline and weighed. The radioactivity of each blood and tissue 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 injected dose and analyzed using the nonlinear least-square program MULTI [34]. The two-compartment model was fitted according to the Akaike information criterion by Eq. (1).

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

The half-lives of the HSAs were determined as β-phase elimination within a 120-min period. The tissue distribution patterns were evaluated using tissue uptake clearances (CL_{uptake}) according to the integration plot analysis. CL_{uptake} was calculated using Eq. (2).

$$CL_{uptake} = \frac{X_t/C_t}{AUC_{0-t}/C_t} \quad (2)$$

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_{uptake} was obtained from the slope of the plot of X_t/C_t versus AUC_{0-t}/C_t. We estimated the organ uptake clearances within a 30-min period.

Table 1
Half-lives and organ uptake clearances of ^{111}In-labeled variants and corresponding Alb A in mice

Variant name (mutation)		Half-life[a] (min)	Clearance (μL/h)[a]	
			Liver	Kidney
Alb Malmö-95 (63 Asp→Asn)	Variant	259.4±7.26	53.14±12.09*	65.09±18.83*
	Alb A	264.2±6.14	79.72±11.74	111.64±18.18
Alb Tregasio (122 Val→Glu)	Variant	250.3±6.65**	73.22±18.70*	71.42±12.74
	Alb A	270.4±1.23	129.13±11.87	104.87±24.94
Alb Hawkes Bay (177 Cys→Phe)	Variant	176.3±8.68**	494.40±38.82**	374.08±43.19**
	Alb A	250.8±5.58	23.99±2.68	69.11±11.67
Alb Herborn (240 Lys→Glu)	Variant	265.8±6.97	132.45±22.19**	137.86±25.61
	Alb A	269.3±4.51	41.10±5.37	110.69±12.94
Alb Niigata (269 Asp→Gly)	Variant	269.7±1.68	14.12±2.39	83.69±19.97
	Alb A	275.2±4.68	18.00±3.27	97.37±23.26
Alb Caserta (276 Lys→Asn)	Variant	254.8±4.89*	45.86±6.29**	203.48±35.37**
	Alb A	269.3±7.64	21.77±5.03	81.00±13.51
Alb Canterbury (313 Lys→Asn)	Variant	221.6±4.65	119.37±12.12	331.98±60.07**
	Alb A	231.6±4.64	128.80±29.97	64.36±20.01
Alb Brest (314 Asp→Val)	Variant	260.8±7.64*	35.48±11.53	102.87±13.27
	Alb A	276.4±5.46	25.31±6.10	99.75±16.32
Alb Roma (321 Glu→Lys)	Variant	269.4±4.51	56.18±5.89**	117.48±11.06*
	Alb A	266.1±3.65	13.34±2.91	83.49±14.23
Alb Sondrio (333 Glu→Lys)	Variant	251.1±4.98	44.72±10.58	172.70±40.37
	Alb A	247.6±7.89	28.92±8.71	169.51±16.82
Alb Parklands (365 Asp→His)	Variant	267.3±6.51	30.99±4.11**	119.35±14.93*
	Alb A	273.1±4.49	63.58±6.54	75.63±20.15
Alb Milano Slow (375 Asp→His)	Variant	268.7±7.61	43.88±10.33*	47.34±9.81
	Alb A	272.9±1.43	17.57±8.33	59.65±22.73
Alb Kashmir (501 Glu→Lys)	Variant	273.2±4.62	44.21±6.34*	76.48±16.91*
	Alb A	270.6±5.48	64.36±9.46	138.53±18.94
Alb Maku (541 Lys→Glu)	Variant	245.9±7.32	144.86±15.01**	285.50±44.74**
	Alb A	251.3±6.51	276.01±47.95	90.25±20.42
Alb Church Bay (560 Lys→Glu)	Variant	270.3±3.56*	13.37±4.39	77.06±16.01*
	Alb A	276.3±1.03	17.61±8.08	136.67±30.62
Alb Verona (570 Glu→Lys)	Variant	274.1±3.98*	99.12±18.83	128.12±20.68
	Alb A	262.4±4.14	63.85±11.72	121.06±16.44
Alb Milano Fast (573 Lys→Glu)	Variant	263.9±2.46	119.41±18.90	218.05±33.41**
	Alb A	263.5±6.45	85.88±11.35	89.64±10.02

[a] The data are average values of 3–6 experiments (±S.D.). *$P<0.05$, **$P<0.01$ as compared with endogenous Alb A.

2.4. Analysis of experimental data

The effects of the single-residue mutations were evaluated by using the following relationship:

$$\text{Percent change} = \frac{(\text{Result for variant}) - (\text{Result for Alb A})}{(\text{Result for Alb A})} \times 100\% \quad (3)$$

In Eq. (3), the result can be a value determined for plasma half-life, organ uptake clearance, α-helical content or for ΔH_v.

2.5. Statistical analysis

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

3. Results

3.1. Pharmacokinetic properties of HSA variants

Fig. 2 shows the time courses for radioactivity in mouse plasma after intravenous administration of ^{111}In-labeled preparations of the variants and their corresponding Alb A. For all 17 variants, the mutation affected the elimination of HSA. In particular, Alb Hawkes Bay (C177F) showed rapid elimination as compared with Alb A. However, in this example the variant has undergone a major conformational change, see below, because it has lost a disulfide bond [28]. Table 1 gives the plasma half-lives, calculated by β-phase using the nonlinear least-square program MULTI and Eq. (1), and liver and kidney uptake clearances, determined by an integration plot analysis (Eq. (2)). As a control we have compared the pharmacokinetic results obtained for endogenous Alb A (Table 1) with those obtained with commercial HSA (not illustrated), because both types of preparations are assumed to represent the normal protein. The average half-life for Alb A is 264.2 min. This value is comparable to that determined for commercial HSA, i.e., 268.2±7.2 min ($n=6$). The liver and kidney uptake clearances for Alb A are on an average 64.64 μL/h and 100.2 μL/h, respectively, whereas those for commercial HSA are 54.31± 8.23 μL/h and 83.12±5.43 μL/h, respectively. The slightly lower values found for commercial HSA are most probably due to differences in isolation and/or defatting procedures.

Table 2 shows the effects of the mutations on plasma half-lives and organ uptake clearances as determined by Eq. (3). As seen, 12 of the mutations diminished plasma half-life, i.e., negative percent changes, whereas one mutation had almost no effect (Alb Milano Fast). By contrast, the remaining variants, all of which are +2 variants, had a prolonged half-life. These findings are in accordance with those of Iwao et al. [9], who, by using oxidized and recombinant mutants, observed that increasing the net negative charge of HSA increased its elimination from the circulation. The results of Table 2 also propose that a change in hydrophobicity diminish plasma half-life. Thus, Alb Malmö-95, Niigata, Caserta, Canterbury, Brest, Parklands and Milano Slow all are more hydrophobic than Alb A, and all of them have negative values for the effect of mutation. Principally the same was found to be the case for Alb Tregasio which is less hydrophobic than its normal protein.

All genetic variants also have a modified liver and kidney uptake clearance (Table 2). About half of the mutants have diminished liver uptake clearance, and about half of them have a reduced kidney uptake. In general, the increases in organ uptake clearance are more pronounced than the decreases. Thus, all percent changes of organ uptake clearances of 100% or more are associated with increases in uptake. For liver uptake it is Alb Hawkes Bay (1961%), Roma (321%), Herborn (222%), Milano Slow (150%) and Caserta (111%), and for kidney uptake it is Alb Hawkes Bay (441%), Canterbury (416%), Maku (216%), Caserta (151%) and Milano Fast (143%). However, no clear correlation could be found between changes of protein net charge and hydrophobicity, respectively, and liver and kidney uptake clearance, respectively.

3.2. Relationships between structure, stability and pharmacokinetic properties of HSA variants

Previously, we have studied the effects of the single-residue substitutions on the α-helical content and thermal stability of

Table 2
Percent changes of half-life and organ uptake clearances of HSA variants in mice

Variant name (mutation)	Half-life (%)	Liver clearance (%)	Kidney clearance (%)	α-helical content[a] (%)	ΔH_v[a] (%)
Alb Malmö-95 (63 Asp→Asn)	−1.82	−33.34	−41.69	−9.68	−46.71
Alb Tregasio (122 Val→Glu)	−7.43	−43.30	−31.89	2.02	9.76
Alb Hawkes Bay (177 Cys→Phe)	−29.70	1960.58	441.31	−10.00	−8.53
Alb Herborn (240 Lys→Glu)	−1.30	222.26	24.54	−4.12	−21.59
Alb Niigata (269 Asp→Gly)	−2.00	−21.56	−14.05	−3.92	−18.83
Alb Caserta (276 Lys→Asn)	−5.38	110.68	151.21	0.68	5.74
Alb Canterbury (313 Lys→Asn)	−4.32	−7.32	415.82	1.47	3.28
Alb Brest (314 Asp→Val)	−5.64	40.17	3.13	1.57	10.53
Alb Roma (321 Glu→Lys)	1.24	321.26	40.70	−1.77	11.31
Alb Sondrio (333 Glu→Lys)	1.41	54.66	1.88	−1.57	−12.14
Alb Parklands (365 Asp→His)	−2.12	−51.26	57.81	−3.13	29.19
Alb Milano Slow (375 Asp→His)	−1.54	149.77	−20.63	−5.30	−32.23
Alb Kashmir (501 Glu→Lys)	0.96	−31.30	−44.79	0.37	−0.60
Alb Maku (541 Lys→Glu)	−2.15	−47.52	216.36	0.40	−20.76
Alb Church Bay (560 Lys→Glu)	−2.17	−24.12	−43.62	1.18	5.28
Alb Verona (570 Glu→Lys)	4.46	55.24	5.83	2.55	36.24
Alb Milano Fast (573 Lys→Glu)	0.17	39.03	143.24	−2.95	−16.64

[a] The values for α-helical content and ΔH_v are taken from Kragh-Hansen et al. [11].

albumin [11], and the results of that study are included in Table 2. The α-helical contents were estimated from far-UV intrinsic circular dichroism spectra by using the ellipticity values at 222 nm. The effect of the mutations on the reversible thermal denaturation (25–85 °C) was also monitored by circular dichroism at 222 nm. These additional data allow for a more detailed comparison between molecular characteristics and pharmacokinetic properties. Fig. 3 gives the correlations between mutation-induced changes in α-helical content and pharmacokinetic parameters. For the mutations in domain I and III, i.e., positions 63–177 and 501–573, respectively (Fig. 1), the same tendencies between the data were observed; therefore they have been gathered in Fig. 3A–C. For these mutations, an increase in α-helical content implies increased plasma half-lives, but decreased liver and kidney uptake clearances. Surprisingly, mutations in domain II, i.e., positions 240–375 (Fig. 1), have the opposite effects. In these cases, an increase in α-helical content results in decreased plasma half-lives (Fig. 3D) but in an increment of kidney uptake clearances (Fig. 3F). By contrast, there is no correlation between changes in α-helical content and liver uptake clearances (Fig. 3E).

The effect of the mutations on albumin stability was examined in terms of heat stability, which was quantified by determining van't Hoff enthalpies (ΔH_v-values) (Table 2). Fig. 4 shows that no correlation was found between the percent changes in ΔH_v and any of the pharmacokinetic parameters. Principally the same observations were made when pooling all the results for domains I–III; in that case the P-values were in the range 0.79–0.97 (not shown).

4. Discussion

From a clinical point of view it would be beneficial, if protein engineering could result in the production of HSA with a prolonged half-life in the circulation. In addition, because of its half-life of 19 days in humans, its ease of synthesis and its known structure albumin is an attractive candidate for use in recombinant fusion proteins. These proteins combine a therapeutic protein (that would otherwise be rapidly cleared) and a plasma protein with a slow clearance in a single polypeptide chain. Furthermore, HSA has been proposed as a carrier in drug delivery systems. Also in the two latter types of examples it

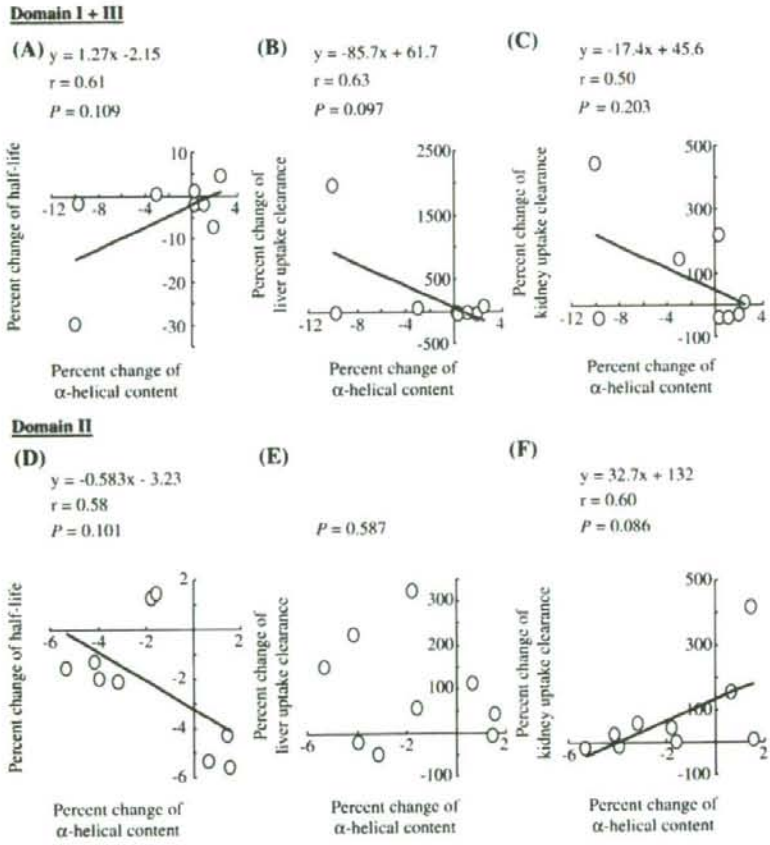

Fig. 3. Relationships between percent change of mutation-induced alterations of α-helical content and percent changes of half-life (A and D), liver uptake clearance (B and E) and kidney uptake clearance (C and F).

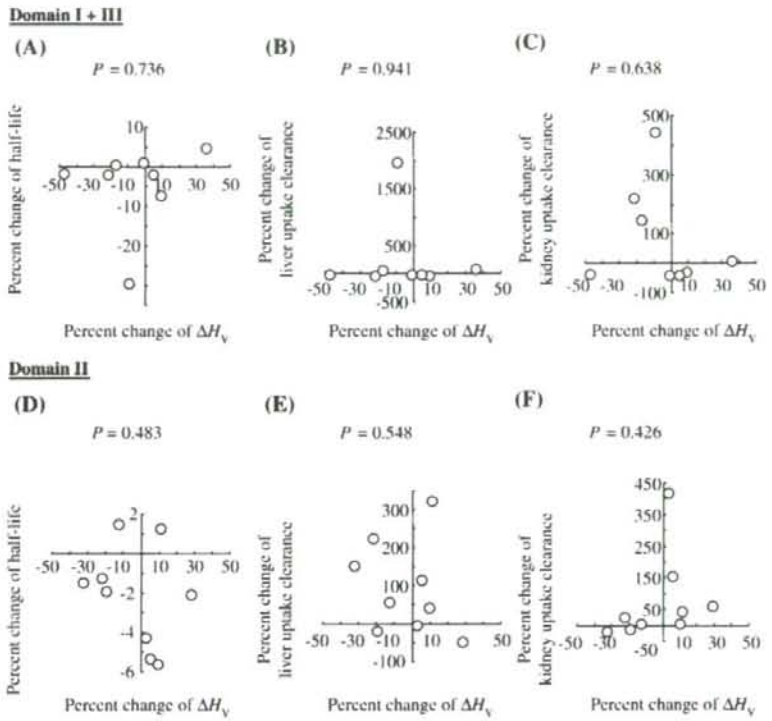

Fig. 4. Relationships between percent change of mutation-induced alterations of ΔH_v and percent changes of half-life (A and D), liver uptake clearance (B and E) and kidney uptake clearance (C and F).

would be advantageous to be able to modify the plasma half-life of the protein product. Therefore, in the present study, we investigated the half-life and organ uptakes of a series of genetic HSA variants. Since the isoforms have known single-residue mutations, we tried to identify molecular characteristics responsible for any modified pharmacokinetics.

The present variants have been detected by electrophoresis performed under native conditions. Therefore, their changes in charge are located on the surface of the protein, where they are exposed to the solvent. The only exception is Alb Hawkes Bay which rather is detected due to major conformational changes caused by the loss of the disulfide bond between Cys 168 and Cys 177 [28]. Thus, the amino acid substitutions affect the hydrophilic/hydrophobic characteristics of accessible regions of the protein. It should be mentioned that we did not quantify these effects experimentally. Instead, we used the knowledge of the amino acid substitutions, and when doing that we found that pronounced decreases in net charge (i.e., +2 variants) resulted in increased plasma half-lives. By contrast, increases in hydrophobicity implied shorter half-lives (Tables 1 and 2). These observations are in full accordance with those of Iwao et al. [9], who studied the effects of oxidation and recombinant mutations. Furthermore, Sheffield et al. [35], who used rabbit serum albumin, found that the recombinant mutant D494N, but not the charge-neutral mutant V14T, had a shorter half-life than the wild-type protein.

In addition to change in net charge and surface hydrophobicity we considered whether the modified plasma half-lives could be correlated to other molecular parameters. Therefore, in Fig. 3A and D the half-lives have been related to changes in α-helical content, which in this case were caused by single-residue substitutions; the results showed a good correlation between the two parameters ($P=0.1$). However, surprisingly, the effect depended on the location of the molecular change, because changes in α-helical content taking place in domains I and III showed a positive correlation to changes in half-life, whereas changes situated in domain II had the opposite effect. It can also be seen that most changes in α-helical content resulted in faster elimination from the circulation. The changes in half-life were also compared to mutation-induced changes in heat stability; quantified by using ΔH_v. However, in this case there was no correlation between the two parameters (Fig. 4A and D).

Iwao et al. [9] found that the diminished half-lives of their HSA preparations were accompanied by increased liver uptake clearances. Nakajou et al. [36] reported that a recombinant mutant of HSA in which the lysine residues in positions 199, 439 and 525 all had been substituted for alanine disappeared faster than the wild-type protein from the circulation. The authors suggested a faster than normal loss of the mutant to extravascular spaces. However, the triple-residue mutant also exhibited increased liver and kidney clearances. For being able to explain, at least in part, the modified plasma half-lives of the present

genetic variants we also investigated their liver and kidney uptake clearances (Table 1). No clear correlation could be found between organ uptakes and changes in protein charge or hydrophobicity, respectively. With respect to the effect of changes in α-helical content the domains again behaved differently. For changes in domains I and III good correlations were found ($P=0.1–0.2$) between increases in percent change of α-helical content and decreases in the change of liver (Fig. 3B) and kidney (Fig. 3C) uptakes. For domain II changes, a good correlation ($P=0.1$) was observed for kidney uptake (Fig. 3F), whereas no correlation was registered between the molecular alterations and changes in liver uptake clearance (Fig. 3E). As with the plasma half-lives, no correlations were found between changes in organ uptakes and changes in ΔH_v (Fig. 4B, C, E and F).

Uptake of HSA by liver and kidneys is mainly due to the presence of cell membrane receptors which recognize the protein and then internalize it by endocytosis. Hepatocytes and the nonparenchymal cells of the liver are involved in galactosyl receptor-mediated and mannose receptor-mediated endocytosis, respectively. Due to the specificities of these receptors, it is not likely that the effects of the present single-residue mutations on liver uptake clearance can be explained by one of these mechanisms. The liver also possesses receptors for rapid uptake of oxidized albumin and albumin with advanced glycation end products. Whether small molecular changes such as single-residue mutations can initiate endocytosis by scavenger receptors such as gp18 or gp30 is at present only speculative. On the other hand, liver uptake by adsorptive endocytosis could be influenced by the amino acid substitutions, because this type of uptake is dependent on the net charge of the protein.

Normally, glomerular filtration of HSA in the kidneys is followed by its return into the venous circulation without degradation (the albumin retrieval pathway). However, a small fraction is degraded in proximal tubular cells most probably after uptake by the endocytic receptors megalin and cubulin. Whether genetic modification of HSA results in increased glomerular filtration and increased uptake by this receptor-complex remains to be clarified. It should be noted that no radioactivity was detected in the urine during the present experimental time. An alternative explanation for the increased uptake of the albumin isoforms by the kidney could be uptake by tubular receptors for advanced glycation end products (RAGE-receptors). As with the scavenger receptors of the liver, it is not known whether single-residue mutations of HSA can initiate uptake by these receptors.

Fujino et al. [37] have found that oxidized bovine serum albumin, in contrast to the native protein, can be cleaved by oxidized protein hydrolase. Because this endopeptidase is found in the blood, it could partly hydrolyse some of the genetic variants in the mouse circulation and thereby render them more exposed to organ uptake. The enzyme selectively recognizes hydrophobic regions in its substrate. Therefore, this mechanism can be especially relevant for the genetic variants with increased hydrophobicity, which all have shorter plasma half-lives (Tables 1 and 2).

More recently, another type of endocytosis of HSA has been identified in virtually all nucleated cells which results in reuse of the protein [38]. After pinocytosis, albumin binds intracellularly and in a pH-dependent manner to the receptor FcRn. Thereby the protein is diverted from the lysosomal degradation pathway and exocytosed back to the circulation in an intact form extending its plasma half-life. Chaudhury et al. [39] have proposed that the intracellular binding of HSA to FcRn is caused by interaction(s) between histidine residue(s) in the receptor and histidine residues in domain III of albumin. By contrast, Andersen et al. [40] suggested that FcRn interacts with negatively charged and surface exposed residues on domain III of HSA. Thus, especially genetic variants with domain III substitutions could have modified plasma half-lives due to a modified HSA-FcRn recycling process.

In conclusion, the plasma half-life of HSA can be modified by single-residue mutations on its surface. No clear relation exists with respect to type of mutation, but +2 variants increase the half-life, whereas increased hydrophobicity decreases it. Changes in the proteins α-helical content have a positive effect on the half-life, if they take place in domain I or III, else they have a negative effect (domain II). By contrast, no correlation was found between the half-lives and changes in ΔH_v representing thermal stability. All mutations modified liver and kidney uptake clearances, and good correlations were found when relating liver (partly) and kidney uptakes to changes in α-helical content. No correlations were found to type of mutation, changes in charge or hydrophobicity or to changes in ΔH_v. Organ uptakes are brought about by different types of endocytosis with different characteristics. Some of these lead to protein destruction in the lysosomes, whereas interaction with FcRn results in recycling of HSA. The relatively few correlations between molecular albumin parameters and organ uptakes could be due to different effects of the mutations on the various forms of endocytosis. However, substitutions of domain III of HSA could modify binding to FcRn and thereby alter its recycling. Although several details with respect to organ uptakes still have to be shed light on, the present information should be useful when designing recombinant HSA mutants with a modified plasma half-life.

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Original Article

Effect of Olmesartan on Oxidative Stress in Hemodialysis Patients

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The effect of olmesartan, an inverse angiotensin II type 1 receptor blocker (ARB), on oxidative stress in hemodialysis (HD) patients is not fully understood, and has not been widely investigated *in vitro* or *in vivo*. We determined the amount of oxidized albumin and albumin hydroperoxides formed during incubation in the absence and presence of olmesartan by high-performance liquid chromatography (HPLC) and by a ferrous oxidation xylenol assay in an *in vitro* study. Six hypertensive HD patients were treated with 40 mg of olmesartan once daily, and blood pressure monitoring (BPM) was performed after 0, 4, and 8 weeks of treatment. The ratio of oxidized to unoxidized albumin was also determined. The oxidized albumin ratios and levels of albumin hydroperoxides were significantly decreased in a concentration-dependent manner in the presence of olmesartan, compared with the absence of olmesartan ($p<0.05$) in *in vitro* studies. In HD patients, olmesartan also significantly reduced systolic and diastolic blood pressure after 4 weeks, with a further significant decrease after 8 weeks. The ratio of oxidized to unoxidized albumin was markedly decreased after 4 weeks and these lower levels were maintained at 8 weeks. Olmesartan effectively lowered the extent of oxidation of albumin in both *in vitro* and *in vivo* studies, and this effect might confer benefits beyond a reduction in blood pressure. (*Hypertens Res* 2007; 30: 395–402)

***Key Words*: olmesartan, blood pressure, oxidative stress, hemodialysis, albumin**

Introduction

The renin-angiotensin-aldosterone system (RAAS) plays an important role in regulating blood pressure (BP). Angiotensin II type 1 (AT1) receptor blockers (ARBs) inhibit the RAAS and have been shown to be effective for treating hypertension (*1*, *2*). Independent of their ability to lower BP, these compounds have also been reported to reduce the progression of nephropathy in patients with diabetes mellitus (DM) and chronic kidney disease (CKD) (*3–5*). Although much of the renal protective effects of ARBs might be due to the lowering of BP, some protection may be due to their effects in reducing oxidative stress. In support of this idea, the blocking of AT1 receptors in hypertensive patients has been shown to reduce oxidative stress, inflammation, and endothelial dysfunction (*6*).

The mechanisms associated with hypertension in hemodialysis (HD) patients are complex, but the RAAS is generally thought to be an important contributor. Angiotensin II, *via* AT1 receptor, stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and enhances the production of reactive oxygen species (*7*), which in turn contributes to endothelial dysfunction and vascular inflammation (*8*, *9*). Thus, the combination of hypertension and oxidative stress induced by stimulation of the RAAS results in the accelerated progression of atherosclerosis in HD patients (*10*).

Olmesartan is an orally active nonpeptide ARB that lowers

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BP when administered daily. Due to its long duration of action, BP control is maintained throughout 24 h. The antihypertensive efficacy and excellent tolerability of olmesartan have been demonstrated in short-term and long-term controlled trials (*11–14*). Miyata *et al.* recently reported that olmesartan, unlike a calcium channel blocker (CCB), inhibited the formation of advanced glycation end-products (AGE) in an *in vitro* study. Olmesartan is a biphenyl tetrazole derivative with a common core structure, 5-(4′-methylbiphenyl-2-yl)-1H-tetrazol, and this core structure is probably responsible for the inhibitory effect on oxidative stress (*15*). Thus, this effect suggests that olmesartan exhibits antioxidant activity in addition to reducing BP. However, the antioxidant effects of olmesartan have not been extensively studied either *in vitro* or *in vivo*.

The aim of this study was to examine the possible antioxidant and free radical-scavenging properties of olmesartan in *in vitro* studies. We also evaluated the effect of once daily administration of 40 mg of olmesartan on BP and on oxidized serum albumin, a marker of protein oxidation, in HD patients (*16–18*).

Methods

Patients

The study protocol was approved by the Institutional Review Board of Kumamoto University. Patients who met each of the following criteria were included in the study: 1) predialysis BP > 140/90 mmHg for 6 consecutive dialysis sessions; 2) no prior treatment with RAAS inhibitors; 3) stable weight for at least 3 months before enrollment; 4) weight gain of less than 5% between dialysis sessions. Written informed consent was obtained from each of 6 stable HD patients (4 men, 2 women) aged 37–80 (mean, 55.5±6.4) years with a duration of dialysis under 1 year. The cause of end-stage renal disease was glomerulonephritis in all cases. At enrollment, all patients were on regular bicarbonate HD for 4–5 h 3 times weekly using high-flux polysulfone hollow-fiber dialyzers. They were not treated with antioxidants such as vitamin E and C or with intravenous iron supplements during the 3 months before inclusion in the study.

Study Design

The study consisted of a 4-week placebo baseline period followed by an 8-week, open-label active treatment period during which the patients received olmesartan once daily, in the morning, at a dose of 40 mg. In patients already on CCB therapy, olmesartan was added to the previous drug. After 0, 4, and 8 weeks of olmesartan therapy, blood samples were obtained from each patient before the first HD session of the week for measurement of the ratio of oxidized to unoxidized albumin. In addition, blood pressure monitoring (BPM) was performed for each patient after 0, 4, and 8 weeks. To avoid any bias with respect to body fluid condition and dialysis efficiency, BPM was performed on the first non-dialysis day after the first HD session of the week. The drugs, dialysis conditions and dry weight of each patient were not changed during either the 4-week placebo period or 8-week treatment period.

Materials and Reagents

Human serum albumin (HSA; Cohn fraction V, fat free) and catalase (EC 1.11.1.6; 65,000 U/mg) were supplied by Boehringer-Mannheim (Mannheim, Germany). Xylenol orange (*o*-cresosulfonaphthalein-3,3-bis-[sodium methyliminodiacetate]) was from Sigma (St. Louis, USA). Olmesartan was obtained from Sankyo Pharmaceutical (Tokyo, Japan).

Chromatography of Serum Albumin

High-performance liquid chromatography (HPLC) was used to analyze serum albumin as described previously (*16*). Samples obtained from the *in vitro* study and from each patient were immediately frozen and stored at −80°C until used for analysis. Then 5 μL aliquots of serum were analyzed on a Shodex Asahipak ES-502N column (Showa Denko Co., Ltd., Tokyo, Japan). From the HPLC profile, the content of each albumin fraction (human mercaptalbumin, f[HMA]; human nonmercaptalbumin-1, f[HNA-1]; human nonmercaptalbumin-2, f[HNA-2]) was estimated as the area of the HNA fraction divided by the HMA fraction of the serum albumin peak (*17*).

Antioxidant Activity of Olmesartan *In Vitro*

Incubation Assay

Fresh heparinized plasma samples were obtained from uremic patients, with informed consent, before the dialysis session. To achieve adequate volumes of plasma and to obtain a stable baseline in experiments in which multiple results were generated, *in vitro* experiments were performed with plasma pooled from several donors ($n=3$ to $n=5$). Pooled plasma (900 μL) was incubated with olmesartan in the presence of air at 37°C. The drug was dissolved in ethanol to obtain a stock solution of 200 μmol/L and further diluted to the required concentrations. One milliliter samples of the plasma were incubated in the presence of air at 37°C for 7 days in the presence of the tested compounds (final concentrations: 2.0 and 20 μmol/L). At the end of the incubation, the ratio of oxidized to control albumin was measured, as described before.

Protein Hydroperoxides

Protein hydroperoxides were generated by irradiating 20 μmol/L HSA solutions with ^{60}Co γ-rays at a dose rate of 36 Gy/min. Protein hydroperoxides were measured by the perchloric acid–xylenol orange assay (*19*). The radiation-generated H_2O_2 was removed by treatment with catalase (154 U/mL). After the addition of the assay reagents and standing at

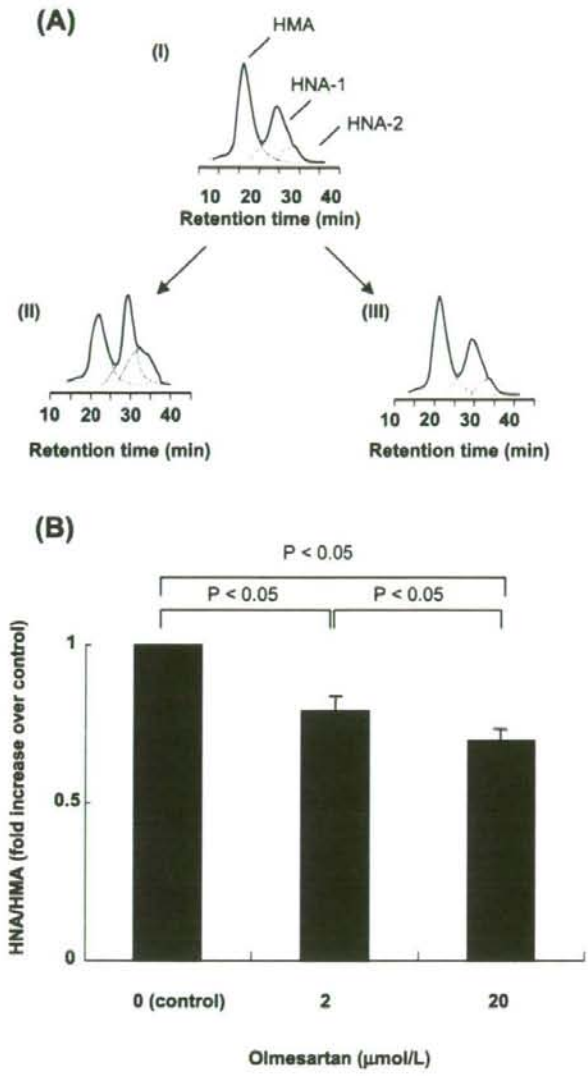

Fig. 1. *A: HPLC profile of in vitro–oxidized serum albumin. HPLC profile of albumin from a uremic subject under control conditions before incubation (I). In II and III, plasma was incubated without or with 20 μmol/L of olmesartan for 7 days. HMA, mercaptalbumin (reduced form); HNA-1, nonmercaptalbumin (disulfide form); HNA-2, nonmercaptalbumin (oxidized form). B: Effect of olmesartan on the HPLC profiles of serum albumin. The calculated ratio of oxidized to reduced albumin ([HNA-1+HNA-2]/[HMA]). Values are expressed as the fold-increase over the control (without olmesartan) (mean ± SEM).*

room temperature for 30 min, absorbances were measured at 560 nm and converted to concentrations using the molar absorption coefficient of 3.70×10^4 mol/L/cm (*17*).

Antioxidant Activity of Olmesartan *In Vivo*

Individual Plasma Carbonyl Contents Measurement

The oxidation of individual plasma proteins was measured by Western blot analysis as described by Shacter *et al.* (*20*). Plasma was diluted to 2 mg/mL of total protein with phosphate-buffered saline (PBS) and derivatized with anti-2,4-

dinitrophenylhydrazine (DNP) using an OxyBlot Kit (Serologicals Corporation, Norcross, USA). Samples were diluted to 1 mg/mL of total protein by the addition of an equal volume of nonreducing sample buffer, and 15 μL samples were electrophoresed on duplicate SDS-PAGE gels. Following electrotransfer to a PVDF membrane, one blot was stained for DNP using the OxyBlot Kit reagents. The second blot was stained with Coomassie brilliant blue G for proteins. The bands were visualized with chemiluminescent chemicals and captured on film at 10 min. Each Western blot included samples from both HD patients and healthy controls. These data were recorded as DNP area/protein area, and are reported as densitometry units. The means for each subject group are calculated from each blot.

Statistics

Statistical significance was evaluated by the 2-tailed paired Student's *t*-test for comparison between 2 mean values and by ANOVA followed by the Newman-Keuls test for comparison among >2 mean values. For all analyses, $p<0.05$ was regarded as being statistically significant. The results are reported as the mean±SEM.

Results

Inhibition of Oxidized Albumin Formation by Olmesartan

We determined the HPLC profile of serum albumin with or without olmesartan before and after the *in vitro* incubation. The HPLC profile of plasma for mercaptalbumin (HMA) and nonmercaptalbumin-1 and -2 (HNA-1 and HNA-2) in uremic patients before incubation is shown in Fig. 1A (I). One week after the incubation, in the absence of olmesartan, HMA was reduced and both the HNA-1 and HNA-2 fractions were further increased (Fig. 1A (II)). In the presence of olmesartan, the oxidation of albumin was decreased (Fig. 1A (III)). The ratio of the HNA fraction (HNA-1 and HNA-2) to the HMA fraction was calculated and the results are summarized in Fig. 1B. Treatment with olmesartan caused a significant decrease (21.7±4.1%) in the HNA/HMA ratio at 2 μmol/L of olmesartan ($p<0.05$ *vs.* control), with a further reduction (31.2±3.7%) at 20 μmol/L of olmesartan. These results demonstrate that olmesartan inhibits the oxidation of serum albumin in a concentration-dependent manner.

Inhibition of Albumin Hydroperoxides Formation by Olmesartan

The ability of olmesartan to inhibit the generation of protein hydroperoxides by hydroxyl radicals was measured by irradiating HSA with or without olmesartan by a γ-source, followed by an assay for hydroperoxides. The results showed that olmesartan lowered the amount of HSA hydroperoxides generated and that the effect was concentration- and time-dependent (Fig. 2). These results are consistent with albumin oxidation being inhibited by olmesartan, as shown in Fig. 1.

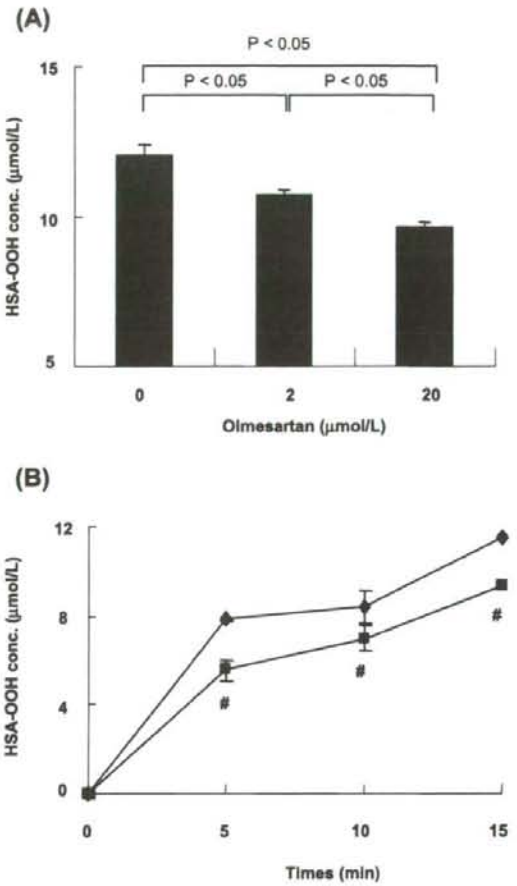

Fig. 2. *Effect of the presence of olmesartan on HSA-OOH formation after γ-irradiation. Concentration (A)- and time (B)- dependent formation of HSA-OOH with olmesartan (20 μmol/L) (■) and with olmesartan (2 μmol/L) (◆). The concentration of HSA was 20 μmol/L and the cobalt-60 radiation dose rate was 36 Gy/min. Hydroperoxide concentrations were measured using the perchloric acid–xylenol orange assay method. Each bar represents the mean±SEM from triplicate samples. HSA-OOH, human serum albumin hydroperoxide. #$p<0.05$ vs. 2 μmol/L olmesartan.*

Carbonylation of Plasma Protein from HD Patients with or without Olmesartan

We also investigated the antioxidant effects of olmesartan *in vivo*. As shown in Fig. 3, oxidized proteins were derivatized

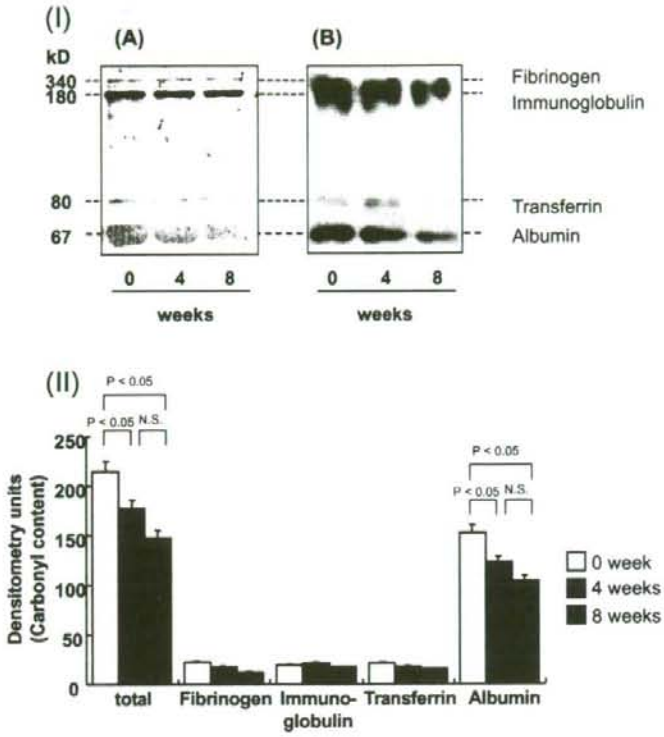

Fig. 3. *Carbonyl content of major plasma proteins from HD patients treated with olmesartan at different times. (I) Plasma samples from HD patients with or without olmesartan were derivatized with DNP after 4 and 8 weeks of treatment and subjected to duplicate SDS-PAGE gels. Following electrotransfer, one blot was stained with Coomassie brilliant blue G for protein (A) and the second blot was stained for DNP using OxiBlot kit reagents (B). (II) Carbonyl formation of major plasma proteins (albumin, transferrin, immunoglobulin, and fibrinogen) was determined as the densitometry ratio of the DNP area and the protein area, and is reported in densitometry units. Values are expressed as the mean±SEM; n=6 patients per group.*

with DNP, separated by SDS-gel electrophoresis, and screened with antibodies against dinitrophenyl groups. HSA was the only major plasma protein that was significantly oxidized in HD patients without olmesartan and, in the group treated with the drug, the oxidation of HSA was decreased. There was no significant difference in the carbonyl contents of the other plasma proteins (transferrin, immunoglobulin, and fibrinogen). These findings show that the decrease in plasma protein carbonyl contents in HD patients was largely due to a decrease in the level of oxidized HSA. Therefore, it would be expected that characterization of the oxidation status of serum albumin might provide useful information regarding the redox state of the human body, prompting us to examine the effect of olmesartan on the oxidation of albumin.

Oxidation of HSA from HD Patients with or without Olmesartan

The ratio of each HSA fraction to total HSA (f[HMA], f[HNA-1], and f[HNA-2]) was calculated and the results are summarized in Fig. 4. Treatment with olmesartan caused a significant decrease (20.5±3.3%) in the HNA/HMA ratio at 4 weeks ($p<0.05$ *vs.* ratio at 0 weeks), and this effect was maintained up to 8 weeks.

BPM Profile from HD Patients with or without Olmesartan

We investigated the mean BPM profiles (systolic blood pressure [SBP] and diastolic blood pressure [DBP]) after 0, 4, and 8 weeks of treatment with olmesartan. Olmesartan therapy significantly reduced the SBP and DBP at 8 weeks *vs.* the baseline ($p<0.05$) (data not shown). These findings strongly

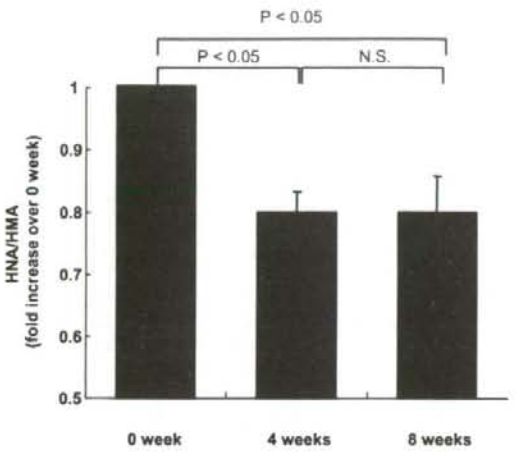

Fig. 4. *Effect of olmesartan on HPLC profiles of serum albumin in vivo. Aliquots (5 μL) of serum were obtained at 0, 4, and 8 weeks after the start of olmesartan therapy and subjected to HPLC using a Shodex Asahipak ES-502N column. The ratio of oxidized albumin to reduced albumin was then calculated ([HNA-1+HNA-2]/[HMA]). Values are expressed as the fold-increase over the control (0 week) (mean±SEM).*

suggest that olmesartan has a significant long-acting BP-lowering effect in HD patients at a daily dose of 40 mg and that ≥8 weeks are required to reach the maximum antihypertensive effect. Olmesartan levels were not significantly different during the experimental periods, suggesting that olmesartan did not accumulate in HD patients when administered at a dose of 40 mg daily for 8 weeks (data not shown). These results indicate that, although the maximum antihypertensive effect was reached at 8 weeks, treatment with olmesartan caused a significant decrease (20.5±3.3%) in oxidative stress at 4 weeks. Thus, this effect might result in benefits by the clinical use of olmesartan.

Discussion

Oxidative stress has long been incriminated in the development of dialysis complications, such as β_2-microglobulin amyloid arthropathy and the acceleration of atherosclerosis (*21*). Until recently, direct evidence for *in vivo* oxidative stress in HD patients was almost entirely limited to the measurement of lipid peroxidation by-products such as malondialdehyde and other thiobarbituric acid–reactive substances (*22*). Despite the observation that proteins are highly susceptible to oxidative stress, there have been few reports of the production of oxidatively modified proteins in HD procedures. Measurement of markers of protein oxidation such as advanced oxidation protein products and carbonyl content have recently been performed to assess oxidative stress under pathological conditions (*23–26*). In 2001, Himmelfarb *et al.* (*27*) reported that the oxidation of albumin accounts for almost all of the excess plasma protein oxidation in uremic patients as demonstrated by SDS-PAGE and an immunoassay using a DNP antibody. In this study, we showed that the decrease in plasma protein carbonyl content in HD patients was largely due to a decrease in the level of oxidized albumin, and that olmesartan substantially decreased the plasma protein carbonyl content by oxidizing albumin (Fig. 3). Given the fact that, in extracellular fluids, serum albumin plays a major antioxidant role (*28, 29*), we expected that characterization of the oxidation status of serum albumin might provide useful information regarding the redox state of the human body, prompting us to examine the effect of olmesartan on the oxidation of albumin *in vivo*. Previously we reported that purified albumin from HD patients triggered oxidative bursts in neutrophils, and thus appeared to act as a true inflammatory mediator (*30*). Furthermore, the binding of ligands to albumins was found to decrease by oxidative modification of albumin (*25, 30*). Therefore, management of the oxidation status of serum albumin is an important issue in cases of CKD and medicine therapy.

Serum albumin can be separated into HMA and HNA by HPLC (*31*) and is used to determine the redox state under various pathophysiological conditions (*32–35*). We also recently demonstrated by HPLC that serum albumin shows high levels of oxidation in HD patients compared with age- and gender-matched healthy subjects, and that HPLC analysis of serum albumin can be useful for the quantitative and qualitative evaluation of oxidative stress in HD patients (*16*). However, until the present study, HPLC analysis of serum albumin had not been used to determine whether the antioxidant activity of olmesartan is expressed in *in vivo* and *in vitro* systems.

In a previous study, Miyata *et al.* (*15*) also had suggested that the antagonist olmesartan inhibited the formation of two AGE, pentosidine and carboxymethyllysine, during incubation with uremic plasma or bovine serum albumin. This effect is unlike that of the calcium channel blocker nifedipine. These results suggest that olmesartan has antioxidant activity in HD patients. However, they applied a relatively higher concentration of olmesartan (mmol/L order) to demonstrate the antioxidant effects. Therefore, the anti-oxidant effects of olmesartan at a clinical concentration (around 2 μmol/L) remain to be determined. In the present study, using a highly sensitive HPLC method, a clinical concentration of olmesartan (0–20 μmol/L) was found to attenuate the oxidized albumin ratio *in vitro* based on the redox states of Cys-34 of albumin (Fig. 1) (*36, 37*). We also examined the possibility that olmesartan can inhibit such damage by preventing the formation of protein peroxides or by decreasing any peroxide groups that are generated by the radicals. The tests showed that irradiated olmesartan did not form stable peroxides (data not shown). However, when HSA (20 μmol/L) was exposed to radiation-generated hydroxyl radicals in the presence of 2.0 or 20 μmol/L of olmesartan, the HSA damage was inhibited, as evidenced