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Interaction of Hemoglobin Vesicles, a Cellular-Type Artificial Oxygen Carrier, with Human Plasma: Effects on Coagulation, Kallikrein-Kinin, and Complement Systems

Hideki Abe, Mitsuhiro Fujihara, Hiroshi Azuma, and Hisami Ikeda
Hokkaido Red Cross Blood Center, Sapporo, Japan

Kenji Ikebuchi
Department of Transfusion Medicine, Saitama Medical School Hospital, Saitama, Japan

Shinji Takeoka and Eishun Tsuchida
Department of Polymer Chemistry, Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, Japan

Hideyoshi Harashima
Laboratory for Molecular Design of Pharmaceutics and COE Program in the 21st Century, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

Abstract: Hemoglobin vesicles (HbVs), cellular-type artificial oxygen carriers containing human hemoglobin, were assessed for their biocompatibility by mixing with human plasma *in vitro*. Among three kinds of HbVs (PEG-DPEA-HbV, PEG-DPPG-HbV and DPPG-HbV), PEG-DPEA-HbV did not affect the extrinsic or intrinsic coagulation activities of the plasma, while PEG-DPPG-HbV and DPPG-HbV tended to shorten the intrinsic coagulation time. The kallikrein-kinin cascade of the plasma was slightly activated by PEG-DPPG-HbV

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Address correspondence to Hideki Abe, Ph.D., Hokkaido Red Cross Blood Center, Japanese Red Cross, Yamanote 2-2, Nishi-ku, Sapporo 063-0002, Japan. E-mail: abeh@hokkaido.bc.jrc.or.jp

and DPPG-HbV, but not by PEG-DPEA-HbV. The complement consumption of the plasma was observed by incubation with DPPG-HbV, but not with PEG-DPEA-HbV or PEG-DPPG-HbV. These results indicate that PEG-DPEA-HbV has a higher biocompatibility with human plasma.

Keywords: Oxygen carrier; Liposome; Hemoglobin vesicles; Complement; Kallikrein-Kinin

INTRODUCTION

Hemoglobin vesicles (HbVs) are human hemoglobin encapsulated into a lipid bilayer (*i.e.* liposome) with a polyethylene glycol (PEG) surface modification and have been developed as an artificial oxygen carrier. We have been evaluating the biocompatibility of the HbVs *in vitro* using human blood. So far, we have revealed that HbVs hardly activated platelets in terms of the aggregation response, p-selectine expression and the release of RANTES [1,2]. For neutrophils, HbVs have the least effect on the chemotactic activity, gelatinase B release, up-regulation of Mac-1 expression and superoxide production triggered by f-MLP [3]. On the basis of these experiments, HbVs appeared to have an acceptable biocompatibility to human blood.

In general, liposomes interact with various kinds of biological components *in vitro* and *in vivo*. The interaction depends on the liposome characteristics such as particle size, surface charge, lipid composition and surface modification. It is widely recognized that negatively charged liposomes activate complement in the rat, guinea pigs and humans [4–6], resulting in rapid removal from the blood circulation as opsonized liposomes by the reticuloendothelial system. A negatively charged surface triggers the intrinsic coagulation pathway and kallikrein-kinin cascade by activating coagulation factor XII (FXII) [7,8]. In addition, the cholesterol contents affects the complement activation [5,9], which is thought to be mediated by natural antibodies [10].

Incorporation of the PEG-conjugate lipid into liposomes (PEGylation) has been reported to be efficacious to avoid these biological responses [11–13]. Indeed, liposome-encapsulated hemoglobin (LEH), which was not PEGylated, has induced complement activation *via* both the classical and alternative pathways in human serum containing natural anti-phospholipid antibodies [14]. These phenomena become of great concern as a pseudoallergic reaction [15,16], which was already observed in a pig model [17]. Recently, however, it has been reported that not only LEH but also PEGylated-liposomes induce hypotension, flushing, respiratory distress, decrease of mean arterola pressure and chest pain [18,19].

In this study, we have used three modified HbVs and assessed their effects on plasma coagulation activity, the kallikrein-kinin cascade and the complement system using human blood. The results obtained here have reinforced the higher biocompatibility of HbVs.

MATERIALS AND METHODS

Liposomes

HbVs were prepared as previously described [20,21]. Briefly, hemoglobin solution prepared from outdated red blood cells for transfusion was heated under a CO gas atmosphere to inactivate possibly contaminated viruses and to remove the stroma and non-hemoglobin proteins [22]. After the removal of impurities by centrifugation and filtration, hemoglobin solution was encapsulated into liposomes by mixing with lipids. The liposomes were then extruded through membrane filters with a pore size of 0.22 μm. Three kinds of HbVs were prepared in this study and the lipid composition (mol%) was as follows: dipalmitoyl phosphatidylcholine (DPPC):cholesterol (CHOL):dipalmitoyl phosphatidylglycerol (DPPG):polyethylene glycol-conjugated distearoyl phosphatidylethanolamine (PEG5000-DSPE) = 5:5:1:0.033 (designated PEG-DPPG-HbVs); DPPC:CHOL:DPPG = 5:5:1 (DPPG-HbVs); DPPC:CHOL:dipalmitoyl-L-glutamate-*N*-succinic acid (DPEA):PEG5000-DSPE = 5:5:1:0.033 (PEG-DPEA-HbVs). All lipids were purchased from Nippon Fine Chemical Co. (Osaka, Japan) except PEG5000-DSPE, which was from NOF Co. (Tokyo, Japan). HbVs were suspended in saline and each of them contains 10 g of Hb/dL, 5.7 g lipids/dL and <0.1 endotoxin unit of lipopolysaccharide/mL. Empty liposome (Coatsome EL-A, 3.1 g lipids/dL) was purchased from NOF Co. and the lipid composition (mol%) was DPPC:CHOL:DPPG = 30:40:30. The particle size and surface charge as the zeta potential of HbVs suspended in saline were measured by Photal ELS-8000HO (OTSUKA Electronics, Tokyo, Japan).

Measurement of Coagulation Activity

Human plasma was prepared from voluntary donated whole blood at Japanese Red Cross blood centers and was stored at $-80°C$ until use. The prothrombin time (PT) and activated partial thromboplastin time (APTT) as an extrinsic and intrinsic coagulation system, respectively, were determined using a physical clotting assay on a coagulation analyzer

(KC10; Amelung, Lehbrinksweg, Germany) at several mixing ratios with plasma and liposomes. Reagents for these assays were purchased from Dade International Inc. (Miami, FL). PT and APTT were measured according to the manufacturer's instructions. Briefly, the mixtures at ratios of 20:80, 40:60 or 60:40 (v/v) of plasma:HbVs or saline were dispensed into a sample cup in duplicate. After the addition of reagents, time to coagulation was measured automatically.

Detection of Kallikrein Activation

The presence of kallikrein activation was evaluated as the degradation of high-molecular-weight kininogen (HMWK), which is substrate of kallikrein naturally existing in the plasma. HMWK was purchased from Enzyme Research Laboratories Inc. (South Bend, IN); anti-human HMWK light chain antiserum from Nordic Immunological Laboratories (Capistrano Beach, CA); plasma kallikrein from Sigma Chemical Co. (St Louis, MO). Plasma was incubated with HbVs or saline at ratios of 80:20, 60:40 or 40:60 (v/v) at 37°C for 24 h. After centrifugation at 15,000 g for 45 min at 4°C, supernatants were treated with 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol at 95°C for 10 min and then electrophoresed on a 5–20% gradient polyacrylamide gel containing 0.1% SDS. After proteins were transferred from the gel to nylon membrane, the membrane was treated primarily with anti-HMWK serum, and secondly with anti-goat IgG antibody labeled with horseradish peroxidase. HMWK was detected with a chemiluminescence detection system (ECL; Amersham, Buckinghamshire, UK). Single chain HMWK (S-HMWK) (33 μg) was digested with plasma kallikrein (6 mU) at 37°C for 2 h. Both digested and undigested S-HMWK were used as standards for Western blot analysis.

Measurement of Complement Titer

Human serum was prepared from whole blood of voluntary donors and stored at −80°C until use. Serum was incubated with HbVs, EL-A or saline at 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, supernatants were stored at −80°C until assay. The complement titer was measured using a 50% hemolysis assay based on Mayer's method with a commercial kit (New One point CH50 (KW); Japan BCG Supply Co., Tokyo, Japan). Therefore, the units of the complement titer were expressed as CH50.

Statistical Analysis

Data were analyzed with parametric Repeated Measures ANOVA following Dunnett post hock test. Statistical significance against saline in each group was established at $p < 0.05$.

RESULTS

Characteristics of Liposomes

The characteristics of HbVs and EL-A are shown in Table 1. The particle sizes of HbVs were 210–240 nm in diameter. The surface charge of PEG-DPPG-HbV and PEG-DPEA-HbV were –3.4 mV and –2.6 mV, respectively, in saline, and were considered to be neutral rather than negative. PEGylation of DPPG-HbV reduced the surface charge from –14.5 mV to –3.4 mV. EL-A, the negatively charged liposome used as a control, had a surface charge of –47.2 mV.

Coagulation Activity

Prolongation of both PT and APTT was observed as the increase of HbVs or the saline ratio to plasma (Table 2). Plasma dilution with HbVs or saline at 20% slightly prolonged or shortened the coagulation time in PT and APTT. Although a significant difference in PT was observed in three HbVs compared to saline at plasma ratios of 20% and 60%, the difference of the mean values was less than 1 second. No significant difference in APTT was observed in PEG-DPEA-HbV at any plasma ratio, but PEG-DPPG-HbV and DPPG-HbV significantly shortened the APTT. However, the shortened time was less than 2 seconds for the mean values.

Table 1. Characteristics of liposomes

Liposomes	Diameter (nm)	Zeta potential (mV)
DPPG-HbVs	239.7	−14.5
PEG-DPPG-HbVs	221.7	−3.4
PEG-DPEA-HbVs	209.9	−2.6
EL-A	162.0	−47.2

Table 2. Plasma coagulation activity as measured by PT and APTT

		Mixture ratio (additives:plasma)			
	Additives	0:100	20:80	40:60	60:40
PT (sec)	Saline	10.2 ± 0.17	10.6 ± 0.19	11.9 ± 0.20	15.1 ± 0.32
	DPPG-HbV	10.3 ± 0.26	10.8 ± 0.16*	11.9 ± 0.19	14.5 ± 0.27*
	PEG-DPPG-HbV	10.2 ± 0.17	10.8 ± 0.18*	11.8 ± 0.21	14.7 ± 0.25*
	PEG-DPEA-HbV	10.2 ± 0.20	10.8 ± 0.16*	11.8 ± 0.20	14.7 ± 0.25*
APTT (sec)	Saline	30.2 ± 0.63	29.3 ± 0.51	31.8 ± 0.58	40.8 ± 0.63
	DPPG-HbV	30.3 ± 0.33	28.1 ± 0.12*	31.3 ± 0.21	38.8 ± 0.37*
	PEG-DPPG-HbV	30.0 ± 0.38	27.7 ± 0.20*	30.4 ± 0.18*	38.4 ± 0.34*
	PEG-DPEA-HbV	30.0 ± 0.47	29.0 ± 0.44	31.2 ± 0.43	40.8 ± 0.63

Data are represented as the mean ± SEM using plasma from five individuals.
*Significantly different from saline ($p < 0.05$).

Kallikrein-Kinin Cascade

Degradation of HMWK was observed in the plasma incubated with DPPG-HbV and PEG-DPPG-HbV, but not with PEG-DPEA-HbV (Fig. 1). The decrease of intact HMWK and the increase of S-HMWK were evident at a ratio of 60% DPPG-HbV and PEG-DPPG-HbV.

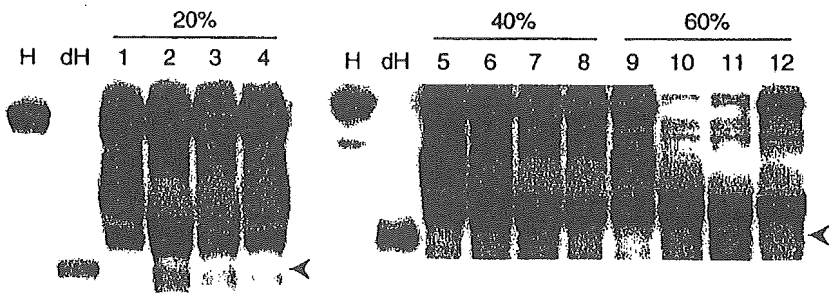

Figure 1. Activation of kallikrein-kinin cascade by HbVs. HbVs or saline were mixed with plasma as indicated ratio (v/v) at 37°C for 24 h. Appearance of digested S-HMWK was detected using western blot analysis as a result of kallikrein activation. Arrows indicate digested S-HMWK. A typical result of three independent assays is shown. H, S-HMWK; dH, digested S-HMWK; lanes 1, 5 and 9, saline; lanes 2, 6 and 10, DPPG-HbV; Lanes 3, 7 and 11, PEG-DPPG-HbV; lanes 4, 8 and 12, PEG-DPEA-HbV.

Complement Consumption

The residual complement titer in serum mixed with PEG-DPEA-HbV or saline was decreased in accordance with the dilution ratio of the additives (Fig. 2A). No difference in the complement titer was observed between

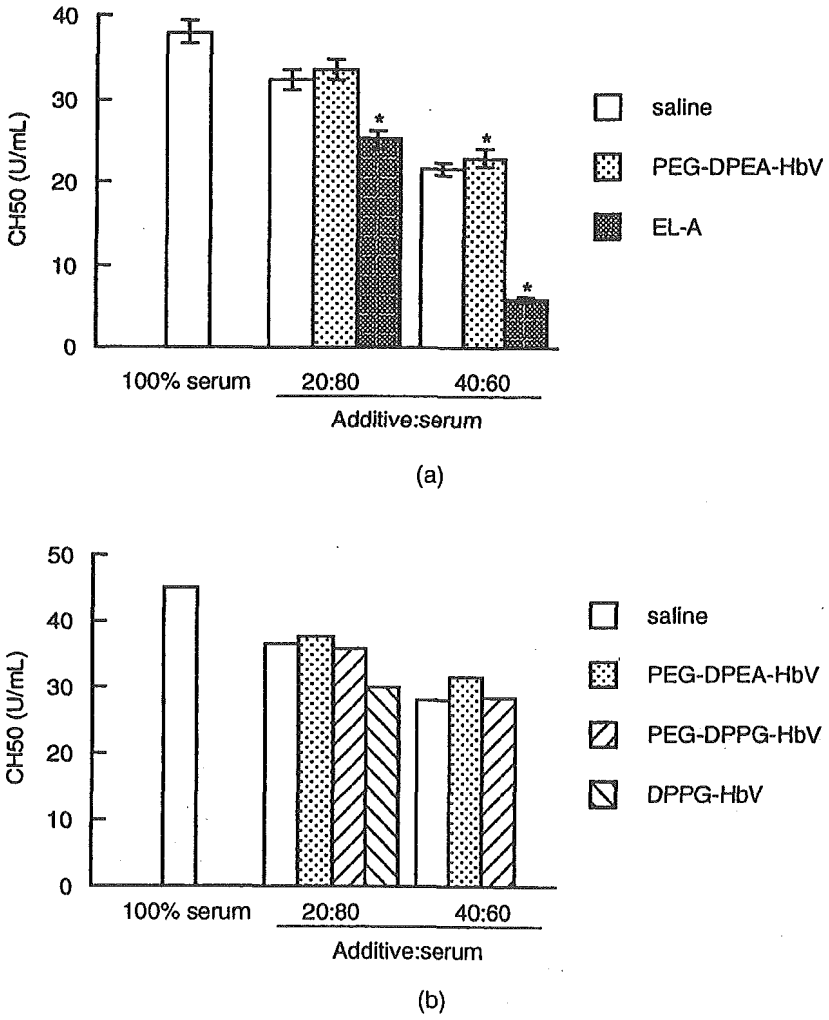

Figure 2. Consumption of complement by HbVs. HbVs, saline or EL-A were mixed with serum as indicated ratio (v/v) at 37°C for 24 h. A: Data are represented as the mean ± SEM using serum from five individuals. *Significantly different from saline ($p < 0.05$). B: Representative data are shown using one serum.

PEG-DPEA-HbV and saline, indicating that PEG-DPEA-HbV did not consume the complement component. The negatively charged liposome, EL-A, further reduced the complement titer, suggesting that the complement was activated by EL-A (Fig. 2A). No difference was observed between PEG-DPEA- and PEG-DPPG-HbV in the complement consumption (Fig. 2B). However, DPPG-HbV significantly consumed complement as no residual complement titer was detected with a mixing ratio of 40%.

DISCUSSION

We have evaluated the biocompatibility of HbVs using human plasma *in vitro*. We first investigated the effects of HbVs on the plasma coagulation activity. It is important for HbVs to have no effect on the coagulation activity of the plasma. As shown in Table 2, significant prolongations of both PT and APTT were observed as the increase of HbVs or saline ratio to plasma. However, even with the mixing ratio of HbVs or saline at 40%, PT and APTT were maintained in the normal range, 10–14 sec and 26–38 sec, respectively. Interestingly, the shortening of APTT was observed with the mixing ratio at 20% and its degree was significant for DPPG-HbV and PEG-DPPG-HbV compared to saline. The important observation was that only PEG-DPEA-HbV had no effect on APTT compared to saline at any plasma ratio. In addition, HMWK degradation was observed in the plasma incubated with PEG-DPPG-HbV and DPPG-HbV. Cleavage of HMWK reflects the activation of the kallikrein-kinin cascade and the release of bradykinin. HMWK cleavage by PEG-DPPG-HbV and DPPG-HbV may become of great concern because bradykinin is considered to be one of the causes of adverse hypotensive reactions in transfusion [23]. On the other hand, PEG-DPEA-HbV did not induce HMWK digestion. Both the intrinsic coagulation pathway and the kallikrein-kinin cascade are initiated by physical contact with FXII or FXII/HMWK with negatively charged surfaces, such as liposomes. Although PEG-DPPG-HbV has a negative charge (–3.4 mV) similar to PEG-DPEA-HbV (–2.6 mV), only PEG-DPPG-HbV affected the intrinsic coagulation system and kallikrein-kinin cascade. It is unclear as to the reason why PEG-DPPG-HbV shortened APPT and activated the kallikrein-kinin cascade. It should be noted that the lipid composition is different between PEG-DPPG-HbV and PEG-DPEA-HbV; PEG-DPPG-HbV and DPPG-HbV have a phosphate group and PEG-DPEA-HbV has a carbonyl group. It is unclear whether the difference in lipid composition affects the intrinsic coagulation system and kallikrein-kinin cascade.

Several kinds of adverse reactions have been reported in the administration of PEGylated liposomes, such as hypotension, flush, respiratory distress, decrease of mean arterola pressure and chest pain [18,19]. Similarly, the decrease of C3, B and C4 was observed in patients, suggesting complement activation by the liposome [24]. These reports suggest that PEGylation may be insufficient to avoid complement activation. In this study, however, PEGylation of DPPG-HbV dramatically decreased its reactivity to the complement (Fig. 2B). It is well known that negatively charged liposomes reduced the complement titer [4–6], and we successfully reproduced this phenomenon in this study (Fig. 2 A), indicating that our complement assay system adequately estimated the complement titer. Therefore, the discrepancy of the effect of PEGylation between our study and others was unclear and may depend on the differences regarding lipid composition, liposome size or surface charge.

In summary, PEG-DPEA-HbV has an excellent biocompatibility to human plasma regarding coagulation and kallikrein-kinin systems. Most importantly, the property of PEG-DPEA-HbV of its lacking the ability of complement activation is an essential advantage not only as an oxygen carrier, but also in the pharmaceutical field, such as in liposomal therapeutic drugs and diagnostic liposomes.

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Review

A Molecular Functional Study on the Interactions of Drugs with Plasma Proteins

Masaki OTAGIRI

Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan

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Summary: The binding of drugs to plasma proteins, such as albumin and α_1-acid glycoprotein (AGP) is a major determinant in the disposition of drugs. A topology analysis of drug binding sites on HSA and AGP was determined using various methods, including spectroscopy, QSAR, photoaffinity labeling and site directed mutagenesis. Recombinant albumin was found to be useful for rapidly identifying drug binding sites. The binding sites on AGP are not completely separated but are partially overlapped, and Trp, Tyr, Lys and His residues in the drug binding pockets play important roles in this process. Drug displacement is somewhat complex, due to the involvement of multiple effects. The reduced binding in uremic patients may be explained by a mechanism that involves a combination of direct displacement by free fatty acids as well as cascade effects of free fatty acids and unbound uremic toxins for significant inhibition in serum binding. Albumin-containing dialysate is useful for the extracorporeal removal of endogenous toxins and in the treatment of drug overdoses. Oxidized albumin is a useful biomarker for the quantitative and qualitative evaluation of oxidative stress. Interestingly, AGP undergoes a structural transition to a unique structure that differs from the native and denatured states, when it interacts with membranes.

Key words: protein binding; albumin; α_1-acid glycoprotein; molecular aspect; interaction

Introduction

Many drugs bind reversibly to plasma proteins. Thus, drugs are transported in the circulation, either free, dissolved in the aqueous phase of the plasma, or bound in the form of complex with plasma proteins. Because of this, the binding of drugs to proteins is an important factor, which determines the pharmacokinetics and pharmacological effects of drugs. Hence, it is one of the most important areas of investigations in drug pharmacokinetics research. In fact, a large number of papers, including some reviews[1-3] have been published so far. While drug binding to plasma was first considered to represent a rather unspecific physicochemical phenomenon, similar to the adsorption of small molecules to charcoal or similar compounds, accumulated evidence indicates that drug binding to albumin and to α_1-acid glycoprotein (AGP) at low molar drug/protein ratios occurs at only a very few ligand binding sites of both proteins, respectively.[4,5] Therefore, a knowledge of the properties of these drug binding sites has become an important issue for understanding pharmacokinetically relevant binding phenomena such as displacement reactions between different drugs and the dramatically altered plasma protein binding of some drugs during several disease states as well.[6,7] Some general properties of important drug binding sites of human serum albumin (HSA) and AGP have been reviewed.[8,9] However, the detailed characteristics of the binding sites on HSA and AGP molecules are not yet clear.

Thus, the author initiated research on drug-plasma protein binding in order to investigate the factors that govern the binding specificity of HSA and AGP after his appointment as a professor of biopharmaceutics laboratory in 1983. Since then his research has been directed to biopharmaceutical issues, and has employed various biophysical and biochemical analytical methods such as spectrophotometry and protein engineering to investigate structural and functional aspects of HSA and AGP. He successfully used fluorescent probes to discover new ligand binding sites on major plasma proteins

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To whom correspondence should be adrressed: Masaki OTAGIRI, *Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. Tel. +81-96-371-4150, E-mail: otagirim@gpo.kumamoto-u.ac.jp*

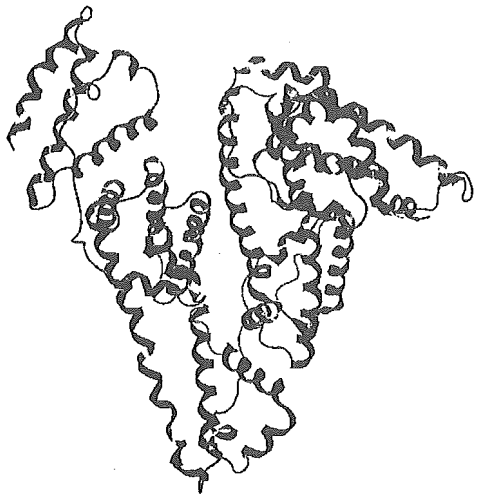

Fig. 1. X-ray structure of human serum albumin (HSA) (PDB ID: 1AO6)

such as HSA. Subsequently, a topology analysis of the drug binding site on HSA was carried out using various methods, including spectroscopy, QSAR, photoaffinity labeling, and site directed mutagenesis techniques. On the other hand, a characterization of the drug binding sites on AGP, which is known to interact with mainly basic drugs, has also been completed.

This article describes the some recent findings related to the mapping of drug binding sites on HSA, drug-plasma protein binding in certain disease states, the possible utility of HSA in clinical applications, and the drug-binding properties of AGP.

Drug Binding Sites of HSA

HSA contains 585 amino acids and has a molecular mass of 66,500 Da. According to an X-ray crystallographic analysis of HSA and its recombinant version (rHSA), the polypeptide chain forms a heart-shaped conformation with the approximate dimension of $80 \times 80 \times 80 \times 30$ Å and about 67% of it consists of α-helices, but no β-sheet is evident (**Fig. 1**). X-ray diffraction has also shown that the protein has three homologous domains (I-III), each of which is comprised of two subdomains (A and B).[10-12]

HSA interacts reversibly with a broad spectrum of therapeutic agents. Typically, drugs bind to one or a very few high-affinity sites with typical association constants in the range of 10^4–10^6 M^{-1}. The pioneering work of Sudlow *et al.*,[13] based on the displacement of fluorescent probes, revealed that most drugs bind with a high affinity to one of two sites, referred to as site I and site II.

Typical site I ligands appear to be dicarboxylic acids and/or bulky heterocyclic molecules with a negative charge localized in the middle of the molecule. Site I probes include warfarin, 5-dimethylaminonaphthalene-1-sulfonamide (DNSA), dansylamide, dansyl-L-glutamine, dansyl-L-asparagine, dansyl-L-lysine and *n*-butyl *p*-aminobenzoate. Site I appears to be capacious and flexible and to contain a large number of individual ligand-binding sites that sometimes are independent of each other but in other cases, mutually influence one another.[14] Ligands as large as bilirubin can be bound, and the independent binding of two different compounds to the site can occur. The fact that ligands with very different chemical structures bind to the region with high affinity indicates that the site is adaptable. Mutual interactions between bound ligands have been observed and this can be attributed to partially overlapping binding sites, or conformational changes in the albumin molecule (allosteric effect or anti-cooperativity). Single-residue mutations in this region of albumin have a significant effect on the conformational and thermal stability of the protein, much more than mutations in site II.[15] Site I is formed as a pocket in subdomain IIA and involves the lone tryptophan of the protein (Trp214).[16] The inside wall of the pocket is formed by hydrophobic side chains, whereas the entrance to the pocket is surrounded by positively charged residues. Both residues contribute positively (*e.g.*, Trp214 and Arg218) and residues that contribute negatively (*e.g.*, Lys199 and His242) to the binding have been identified in the high-affinity binding of ligands such as warfarin.

Ligands that bind to site II (also called the indole-benzodiazepine site) are often aromatic carboxylic acids with a negatively charged acidic group at one end of the molecule that is separate from a hydrophobic center. Dansylsarcosine, dansyl-L-proline, dansylglycine, and 7-alkylaminocoumarin-4-acetic acids all function as site II probes. Site II seems to be smaller, or more narrow, than site I, because no large ligands apparently bind to it. It also appears to be less flexible, because binding often is strongly affected by stereoselectivity. Furthermore, the substitution of ligands with a relatively small group strongly influences the binding. Site II is a pocket that is formed in subdomain IIIA principally the same way as site I. Among the individual amino acid residues of this subdomain, Arg410 and Tyr411 are usually assumed to be important.[17] On the basis of our mutagenesis data, we propose a new screening method, using mutant HSA, for the rapid identification of primary drug binding sites. The mutant used in such an approach must not influence the binding capability of HSA at binding sites other than the one in which the mutation has taken place. **Figure 2** outlines such a method using site II and the double-residue mutant R410 A/Y411A as an illustrative example.

Bhattacharya *et al.*[19] reported on the high-resolution crystal structures of rHSA with propofol or halothane. Propofol binds in subdomains IIIA (site II) and IIIB,

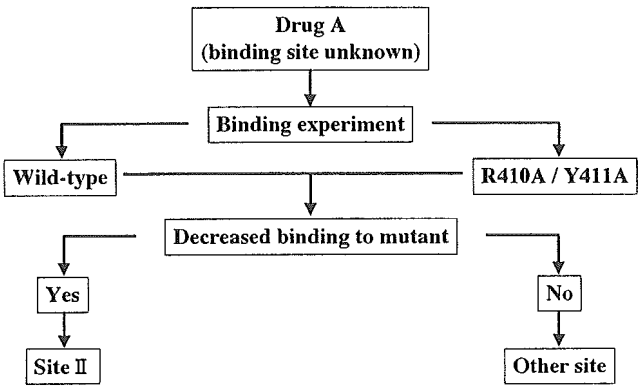

Fig. 2. New screening method for identifying the location of a primary drug binding site of HSA using site II as an illustrative example

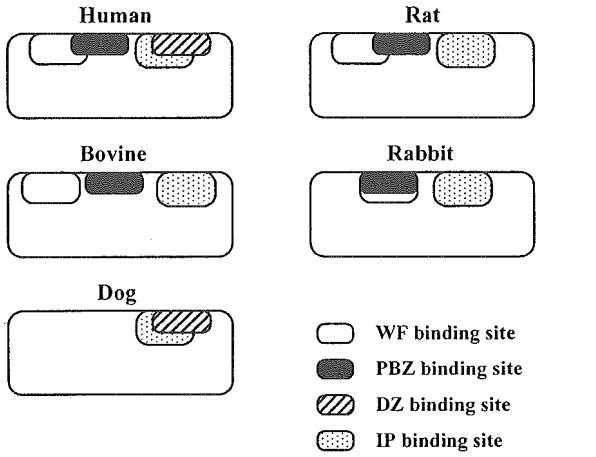

Fig. 3. Proposed model of drug binding sites on different albumins (from ref. 30)

with the former site having the highest affinity. It is probable that the latter site most also binds anions of long-chain fatty acids with a high affinity. In contrast, three primary sites were found for halothane, one in subdomain IIIA (site II) and two in a solvent-exposed trough at the outside interface between subdomains IIA and IIB. Eckenhoff *et al.*,[20] using solubilized HSA and rHSA mutants, observed that halothane, isoflurane, and 1-chloro-1,2,2-trifluorocyclobutane bind primarily in the interdomain cleft between sites IA and IIA, where Trp214, an important binding residue, is located. However, the binding of anesthetics to site II was also evident.

The cysteine residue at position 34 in subdomain IA is located in a crevice on the surface of the protein and does not participate in any disulfide bridges. Mercaptalbumin and probably also part of albumin forming mixed disulfides are able to bind nitric oxide and metal ions such as Ag, Hg, and Au as well as homocysteine and several drugs. Included among the latter are bucillamine derivatives, aurothiomalate, auranofin, D-penicillamine, captopril, ethacrynate, and cisplatin.[21-23] Covalent interaction between thiol-containing drugs and Cys34 of albumin usually also takes place *in vivo*, for example, *N*-acetyl-L-cysteine, D-penicillamine, captopril, *meso*-2, 3-dimercaptosuccinate and SA3786 (a bucillamine derivative).[24-26] Interestingly, the reactivity in patient sera is much higher than that found in albumin solutions and in sera from healthy volunteers.[27]

Although serum albumins are highly homologous, in terms of amino acid sequence (about 80% between human and other mammalian species), the drug binding properties differ considerably among the species. As a result, species differences with respect to drug binding to serum albumins have been examined extensively.[28,29] However, since these studies were not investigated systematically in terms of drug binding sites, the issue of whether or not these are specific drug binding sites on serum albumins is not clear, nor is the issue of the nature of such sites. For the purpose of obtaining additional information, we attempted to identify drug binding sites on different albumins using typical site I binding drugs on HSA, as well as site II of HSA. Human albumin site I appears to exist in rabbit and rat albumins, but not in the bovine and, especially not in dog albumin. However, bovine, rabbit and rat albumins lack binding sites for diazepam, and these are different from human albumin. Interestingly, the architecture of site II on dog albumin is quite similar to human albumin, although dog albumin lacks some of the important structure of site I (**Fig. 3**).[30]

The three recombinant domains (domain I, II, III) of HSA were recently expressed using *Pichia pastoris* and were purified in our laboratory. The ligand binding ability for these domains was then examined. Interestingly, all domains show a much lower ligand ability compared with rHSA, indicating the importance of interdomain interactions for maintaining the structural stability of ligand binding sites.[31]

Displacement and Drug-drug Interactions on HSA

In addition to competitive binding, drugs as well as other ligands can influence the simultaneous binding of another drug by electrostatic effects and/or binding-induced conformational changes in the protein.[32] Drug metabolites can also bind to albumin with a high affinity. The formation of acyl glucuronides is a major metabolic pathway for many compounds with a carboxylic acid function. This type of metabolite is a reactive electrophilic species and can therefore, in addition to participate in reversible binding, react covalently with HSA both *in vitro* and *in vivo*. Among the drugs that become glucuronidated and interact covalently with HSA are furosemide, salicylic acid, gemfibrozil, clofibric acid, fenofibric acid and nonsteroidal

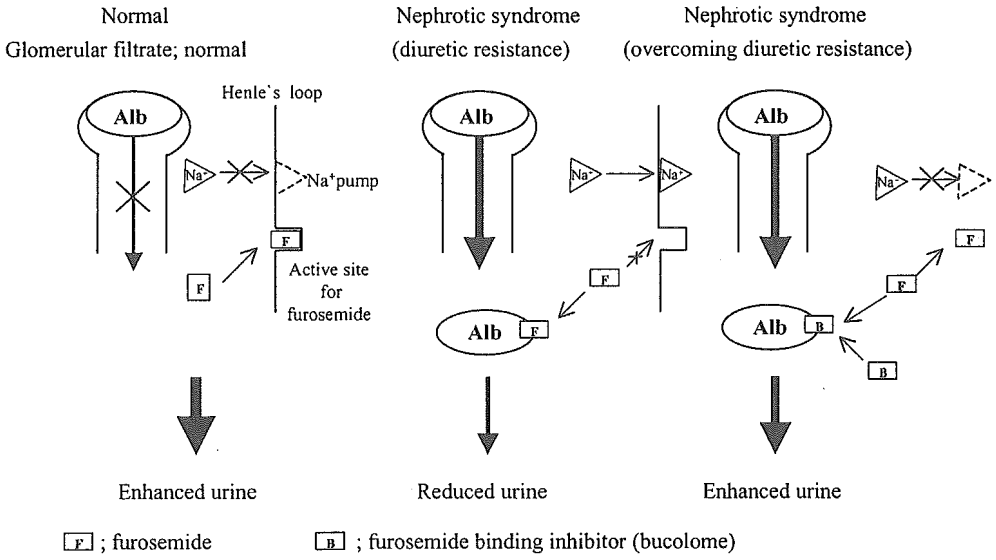

Fig. 4. A mechanism for overcoming the diuretic resistance to furosemide using drug displacement on albumin

antiinflammatory drugs like tolmetin, benoxaprofen, zomepirac, beclobric acid, ibuprofen, ibufenac, suprofen, ketoprofen and diflunisal.[33-37] Stereoselectivity was observed for, *e.g.*, carprofen, fenoprofen, and naproxen[33,38]; in all cases the *R*-forms exhibited the highest covalent binding. Photoactivated naproxen and frusemide, as well as photoactivated frusemide glucuronide, also bind covalently to HSA.[34]

Drug binding can be modulated by the simultaneous binding of endogenous compounds. Anions of long-chain fatty acids can bind at low ligand-to-protein molar ratios, most probably allosterically, and diminish the binding of *S*- and *R*-thiamylal to site II.[39] The effect on etodolac binding is dependent on the stereochemistry of the drug, because binding of the (*R*)-form is increased, whereas the binding of the (*S*)-form was decreased.[40] At higher relative concentrations, fatty acid anions often displace site II drugs by competition.

Alterations in the binding of a drug to HSA may lead to a change in the pharmacokinetic properties of the drugs. This risk is especially relevant if two or more drugs compete for the some high-affinity binding site. However, in certain cases, the increase in unbound drug concentrations is much less than would be expected, because a major portion of the displaced drug rebinds at another site (secondary site). For example, most of the carprofen and diclofenac that have been competitively displaced from site II (primary site) by ibuprofen becomes quantitatively rebound at site I (secondary site)[41,42]; and the effect is stereospecific.[41]

Drug-drug interactions at the protein-binding level are usually regarded as problematic secondary effects. However, such interactions can also be useful for therapeutic purposes. For example, an attenuated response to diuretics is frequently observed in patients with the nephrotic syndrome[43] and one of the possible contributors to diuretic resistance can be due to the binding of a drug to HSA in the urine.[44] This suggests that compounds that inhibit the binding of furosemide can normalize the natriuretic effects of drug in the renal tubules. For assess this, we examined the inhibitory effect of bucolome on the binding of furosemide to protein *in vitro* and *in vivo*, because bucolome has the following properties: 1) it is a potent inhibitor of the binding of furosemide to protein in the urine; 2) when administered in large doses, its plasma concentration reaches high levels; 3) it is excreted mainly via the urine; and 4) it should be highly safe and suitable for repeated administration. The results obtained clearly suggest that bucolome has a potent inhibitory effect on the protein binding of furosemide to protein in the urine and can partially restore the diuretic response of furosemide in patients with the nephrotic syndrome by increasing the free fraction of furosemide at the site of action (**Fig. 4**).[45] Drug displacement can also be useful in nonpathological conditions. Technetium-99m-labeled mercaptoacetylglycylglycylglycin binds strongly to albumin but it is displaced by coadministered bucolome leading to a higher unbound concentration, thereby making it more useful for renal scintigraphy.[46] The same study showed that 6-methoxy-2-naphthylacetate (a major metabolite of nabumetone), by principally the same mechanism, renders *N*-isopropyl-*p*-[^{123}I]iodoamphetamine more suitable for cerebral imaging.

Drug-albumin Binding in Disease States

The concentration or the properties of serum albumin are altered in a variety of disease states. Such changes

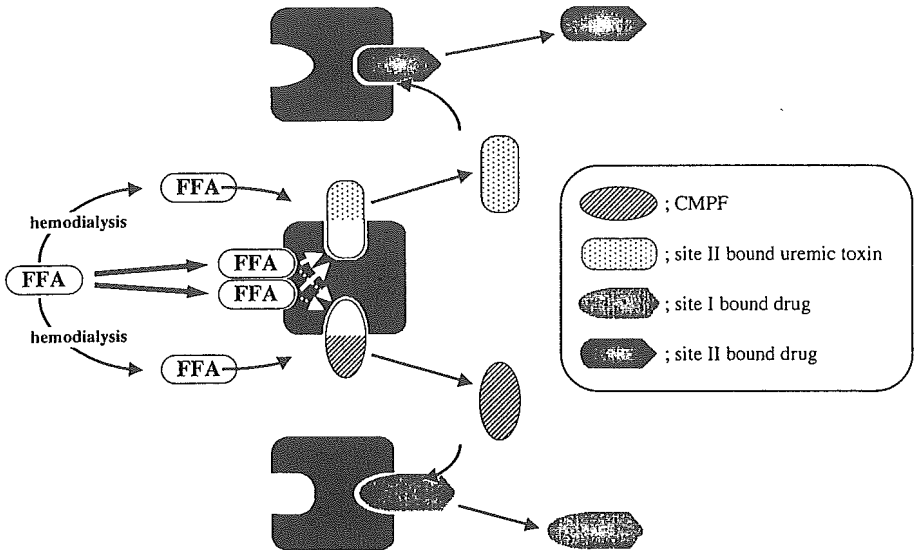

Fig. 5. Possible cascade displacement model in a fatty acid-uremic toxin-drug system (from ref. 51)
Heavy arrows: fatty acid binding to its high-affinity site; *thin arrows*: fatty acid binding to its low-affinity site; *white arrows*: allosteric effect of FFA at site II; *broken-line arrows*: allosteric effect of FFA on site I-bound drug.

decrease drug-albumin interactions and influence the fate of albumin-bound drugs in the body. It seems certain that the extent of drug-albumin interactions in patients with liver disease always decrease with serum albumin concentration.[6,47]

The binding of many drugs to albumin is decreased in patients with acute of chronic renal failure or nephrosis.[6,48] The increase in the free drug fraction in the serum of patients with renal disease is correlated with the degree of hypoalbuminemia, but this does not fully explained the issue. Other factors that may explain the decreased interaction of drugs with albumin in renal failure include conformational changes in the albumin molecule, thus decreasing its drug-binding capacity or the accumulation of endogenous substances such as free fatty acids or uremic toxins that compete with drugs for the binding site on albumin. To data, the albumin binding of uremic toxins such as indoxyl sulfate (IS), indole acetate (IA), hippuric acid (HA), and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) to albumin has been reported in human serum, and these compounds accumulate in uremic serum to levels of up to 202, 101, 883 and 370 mmol/L, respectively.[49] These compounds are largely responsible for the impaired binding of many drugs. To elucidate the mechanism of defective drug binding induced by uremic toxins, it is necessary to examine the relationship between drug and uremic toxin-binding sites. Uremic toxins with an indole ring and HA primarily bind to site II, which is located in subdomain IIA of the HSA structure, whereas the location of the CMPF-binding site is within specialized entities in subdomain IIA, corresponding to site I.[50] The reduced binding in uremic patients may be explained by the effect of a combination of direct and cascade effects of fatty acids and uremic toxins that result in a significant inhibition in drug binding in serum (**Fig. 5**).[51]

If albumin is regarded as an anion transport system then substances that interfere with binding may also be able to inhibit other anion transport processes. This hypothesis is supported by evidence that CMPF inhibits the uptake of *p*-aminohippuric acid in rat kidney slices. Very recently, we investigated the transporters responsible for the renal uptake of uremic toxins. Both OAT1 and OAT3 contribute almost equally to the uptake of IS. OAT3 mainly accounts for CMPF uptake by the kidney, while OAT1 mainly accounts for IA and HA uptake.[52-55]

It is known that the covalent modification of protein has a great influence on its conformational and functional properties. In fact, the decreased drug binding that is observed in diabetes, appears to depend on the extent of protein glycation and fatty acid concentration.[56] Interestingly, we recently found that advanced glycation end product albumins, produced by reacting albumin with glucose or reducing sugar are cleared from the kidney by scavenger receptor A-mediated endocytic uptake by mesangial cells.[57]

Extracorporeal Clearance using HSA

Albumin can be used to clear the body of certain endogenous toxins. Among these are bilirubin, bile acids, and uremic toxins. Toxins are usually removed from the patient by hemodialysis, but the treatment frequently has only a minor effect. Recently, this approach has been improved by the use of thinner

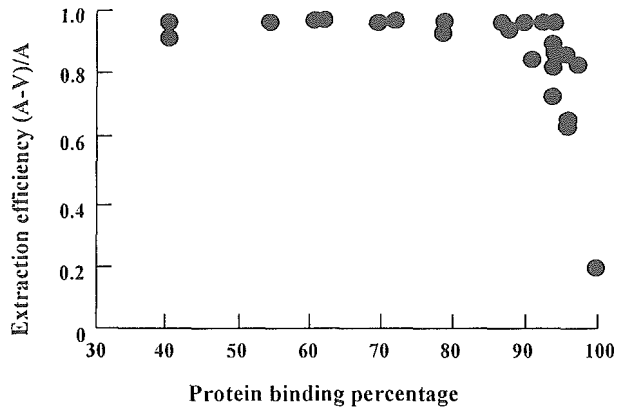

Fig. 6. Relation between extraction efficiency and the presence of protein bound (from ref. 64)

dialysis membranes with larger pore sizes and by adding albumin to the dialysate. In this method, albumin can be regenerated online by passing the dialysate through a charcoal column.[58] Another albumin-based extracorporeal detoxifier that has been developed recently is the molecular adsorbent recirculating system (MARS). MARS is a cell-free, liver assistance method for the selective removal of albumin-binding substances.[59] It also enables the removal of excess water and water-soluble substances *via* an inbuilt dialysis step. Technically, MARS is a modified and expanded dialysis system using an albumin-containing dialysate that is recirculated and regenerated online by dialysis against a bicarbonate-buffered solution, followed by passage through charcoal and anion-exchanger columns. This method of removing albumin-binding toxins and water-soluble substances was found to result in a decrease in hepatic encephalopathy, an increase in the mean arterial pressure, and in improvement in kidney and liver function.[60,61] Albumin dialysis in the form of a routine continuous veno-venous hemodiafiltration using a 44 g/l albumin-containing dialysate and a slow dialysate flow rate (1–2 l/h) has been shown to be effective in the treatment of fulminant Wilson disease, resulting in the removal of protein-binding toxins such as copper and bilirubin.[62] Endotoxins such as lipopolysaccharides and cytokines, and possibly other toxins as well, can be removed by passing blood through columns that contain immobilized albumin.

The above-mentioned methods, or modifications thereof, can also be used in the treatment of drug-overdoses. Charcoal hemoperfusion is a very effective extracorporeal treatment in cases of drug overdose.[63] It is generally accepted that charcoal hemoperfusion can not remove drugs that are largely bound to plasma proteins. We recently attempted to define a guideline based on the percentage of a drug that binds to protein for use in the treatment of drug-overdose cases. The relationship between the extraction efficiencies of a charcoal column and the plasma protein binding percentage of the drugs showed that drugs bound at levels of 90–95%, or less, could be effectively removed from the blood (**Fig. 6**).[64] However, the more tightly bound drugs were only partially removed. Our results show that, in addition to the volume of distribution, the degree of plasma protein binding can also be used as determinant for clearance by hemoperfusion, especially in the case of drugs with a binding percentage above 95%. The clinical decision as to whether to initiate hemoperfusion or not should not be made by taking only the volume of distribution of drug into consideration. The protein binding percentage is useful information in the choice of a suitable treatment for overdose patients.

Oxidized Albumin as a Biomarker in Uremic Patients

Recently, increasing attention has been paid to albumin as a biomarker of oxidative stress among biological substances because this molecule represents the major and predominant circulating antioxidant in plasma for continuous oxidative stress. The accessibility of plasma proteins for sampling, the relatively long plasma half-lives of many proteins, and the well-defined biochemical pathways that lead to protein oxidation can be used to detect more specific pathways of oxidative stress than plasma lipids. For example, oxidation of the amino acyl side chains of amino acids in proteins is an attractive biomarker of oxidative reactions because of the high specificity of the end products for specific oxidation pathways. In particular, detection of oxidized cysteine residues currently constitutes the most sensitive and specific means available for detecting end products of specific oxidative pathways.[65] Recent studies of some investigators have clearly demonstrated high levels of thiol group oxidation as well as carbonyl formation in plasma proteins from patients with uremia.[66] Since albumin is the most abundant protein in plasma, it could play a major role as an antioxidant in plasma at least with respect to thiol oxidation and carbonyl formation. Albumin is a mixture of mercaptalbumin (HMA; reduced form) and nonmercaptalbumin (HNA; oxidized form) i.e. a protein redox couple in serum.[67] HMA contains one highly reactive sulfhydryl group at position 34 (Cys34), while other serum proteins contain few or none.[10] HNA is composed of at least three types of molecules. The major HNA component is a mixed disulfide with cysteine or glutathione (HNA(Cys) or HNA(Glut)). The other is a more highly oxidized product than the mixed disulfide, such as sulfenic ($-SOH$), sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) states (HNA(Oxi)), the proportions of which are extremely small in extracellular fluids.[68] The high-performance liquid chromatographic (HPLC) analysis

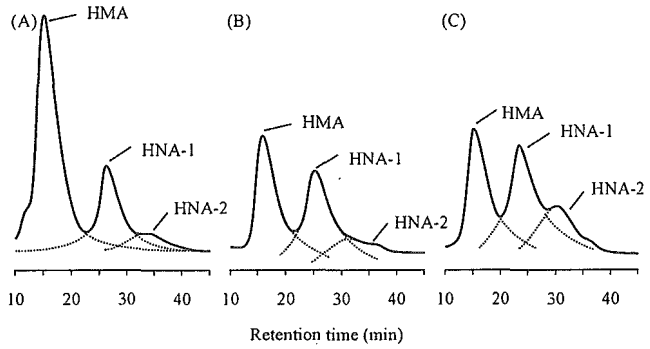

Fig. 7. HPLC profile of serum albumin from HD patients with or without IVIR (from ref. 70)
(A) A representative tracing of an HPLC profile of serum albumin from normal subjects.
(B) A representative tracing of an HPLC profile of serum albumin HD patients without IVIR.
(C) A representative tracing of an HPLC profile of serum albumin from patients treated with IVIR.

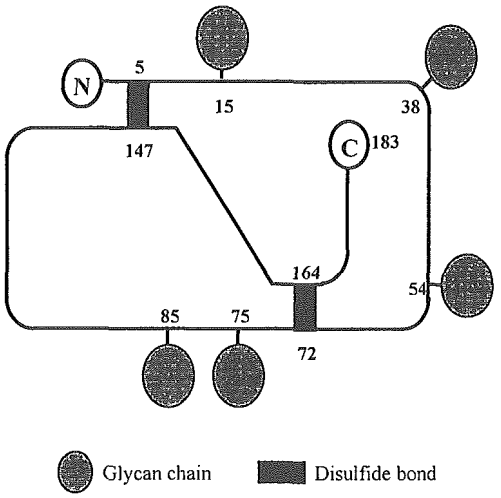

Fig. 8. Structure of human α_1-acid glycoprotein (AGP)

of albumin developed by Sogami *et al.* permits the clear separation of HSA into HMA and HNA,[67] and is used for the determination of the redox state for various pathophysiological conditions.[69,70] In this sense, one of the important physiological functions of serum albumin could be to participate in the maintenance of a constant redox potential in the extracellular milieu. We also recently demonstrated that the HPLC analysis of serum albumin represents a potentially useful method for the quantitative and qualitative evaluation of oxidative stress in chronic HD patients and the results strongly suggest the possibility that oxidative stress, generated by the *iv* administration of iron IVIR, enhances the oxidation of albumin in such patients (**Fig. 7**).[70]

In addition to oxidized cysteine residues, other important amino acyl groups subject to oxidation include tyrosine, amino groups (such as Lys), and alcohol groups. In particular, the oxidation of Tyr residues leads to the formation of 3-chlorotyrosine, 3-nitrotyrosine, or dityrosine.[65] Hemelliferb *et al.* recently demonstrated that plasma proteins from uremic patients contain elevated levels of 3-chlorotyrosine, but not 3-nitrotyrosine or dityrosine. Since this product (3-chlorotyrosine) is a specific product of the myeloperoxidase catalyzed reaction, it further suggests a specific and important role for phagocyte-initiated oxidative reactions as the cause of excess oxidative stress in uremia.[71] Witko-Sarsat *et al.* also demonstrated elevated levels of advanced oxidative protein products (AOPP) in plasma from uremic patients.[72] The contents of AOPP lead to the formation of dityrosine and account for almost all of the excess oxidation of albumin in uremic patients. Further, the contents of AOPP are good correlated with the levels of HNA. Collectively, these studies suggest that the oxidation of albumin in uremia mainly leads to the oxidation of cysteine and tyrosine residues and that these residues, when present on albumin, are useful biomarkers for the quantitative and qualitative evaluation of oxidative stress.

Characteristics of Drug-biding Site on AGP

AGP, a member of the lipocalin family, is a 40 kDa protein consisting of 183 amino acids with two disulfide bonds and five carbohydrate chains (**Fig. 8**).[73] Although the three-dimensional structure is still unknown, circular dichroism (CD) measurements and molecular modeling indicate that this protein, in aqueous solution, is mostly made up of β-sheets.[74,75] The interaction of many drugs with AGP have been reported, whereas the binding site of AGP has not been clarified.

AGP is one of the most glycosylated proteins, to extent of 40% of its total mass. The biological role of the oligosaccharide moiety was found to be exhibited in acute phase events,[76] whereas the moiety appears not to be involved in the binding properties of AGP. Friedman *et al.* found that sialic acids attached to oligosaccharides of AGP had no effect on its binding capacity and structural properties.[77] Other reports[73,78] that the removal of oligosaccharides from AGP has no effect on its structural properties also supports the theory that oligosaccharides of AGP are not associated with the binding properties of the protein. In addition, we established the AGP expression system in yeast, *Pichia pastoris*, for the first time.[79] The oligosaccharides of glycoprotein derived from yeast are the high mannose type. Recombinant AGP has almost the same binding capacity as human plasma derived AGP, but the type of oligosaccharide are different. These results strongly suggest that drug binding properties do not depend on the type and presence of oligosaccharide.

We previously classified the drug-binding sites on

AGP by displacement experiments using fluorescent probes.[80] Basic drugs not only efficiently displaced basic probes but acidic probes as well. On the other hand, acidic probes were displaced by acidic drugs such as phenylbutazone and sulfadimethoxine, which had no effect on most of the basic probes. This contradiction suggests that basic drugs do not completely share a binding site with acidic drugs. These displacement data suggest that the binding sites on AGP are not completely separated but are significantly overlapped and influence one another. In addition, we found that dicumarol-AGP and dicumarol-AGP-protriptyline form a 1:1 binary complex and a 1:1:1 ternary complex respectively, as evidenced by CD titration and equilibrium dialysis experiments.[81,82] Moreover, the binding experiments using dicumarol and chlorpromazine showed that the binding sites for basic and acidic drugs overlapped each other. Using chemically modified AGP, it was found that Trp, Tyr and Lys residues are involved in the dicumarol binding site, and that Trp and Lys residues are involved in chlorpromazine binding site.

7β-Hydroxystaurosporine (UCN-01) is a protein kinase inhibitor anticancer drug that is currently undergoing phase II clinical trials.[83,84] The low distribution volumes and systemic clearance of UCN-01 in human patients was found to be due to its extraordinarily high affinity binding to AGP.[85,86] We recently have reported on the binding properties of this drug to AGP.[87] The binding affinity of staurosporine, as well as UCN-02, 7α-hydroxystaurosporine, to AGP was lower than that of UCN-01 by 20- and 100-fold respectively. The percentage of UCN-01 that binds to AGP was low in an acidic pH region but increased with increasing pH, reaching a maximum at pH 7.4. The binding of UCN-01 to desialylated AGP was comparable to that of AGP. Chemical modification of the His, Lys, Trp, and Tyr residues caused a decrease in the percentage of bound UCN-01. Trp-modified AGP showed the largest decrease in binding. This result is consistent with the finding for the binding sites of dicumarol and chlorpromazine. Moreover, tryptophanyl fluorescence quenching results indicate that Trp residues play a prominent role in the binding of UCN-01 to AGP. These results indicate that a substituent at the C-7 position of UCN-01 appeared to influence the binding specificity of the drug, and Trp residues in AGP play a prominent role in the high affinity binding of UCN-01 to AGP. In addition, we photolabeled AGP with [^{3}H]UCN-01 without further chemical modification.[88] The photolabeling specificity of [^{3}H]UCN-01 was confirmed by findings that other AGP binding ligands inhibited the formation of covalent bonds between AGP and [^{3}H]UCN-01. The amino acid sequence of the photolabeled peptide was concluded to be SDVVYTDXK, corresponding to residues Ser153 to Lys161 of AGP. No PTH derivatives were detected at the 8th cycle, which corresponds to the 160th Trp residue (Trp160). This strongly suggests that Trp160 was photolabeled by [^{3}H]UCN-01. Three recombinant AGP mutants (W25A, W122A, and W160A) and wild-type recombinant AGP were photolabeled by [^{3}H]UCN-01. Only mutant W160A showed a marked decrease in the extent of photoincorporation. These results strongly suggest that Trp160 plays a prominent role in the high affinity binding of [^{3}H]UCN-01 to AGP. A docking model of UCN-01 and AGP around Trp160 provided further details of the binding site topology.

Moreover, we investigated the binding of flunitrazepam (FNZP) to AGP.[89] The photolabeling specificity of [^{3}H]FNZP was confirmed by findings that other AGP-binding ligands inhibit the formation of covalent bonds between [^{3}H]FNZP and AGP. A sequence analysis of the photolabeled peptide indicated a sequence corresponding to Tyr91-Arg105 of AGP. Kopecky *et al.* investigated the secondary and tertiary structures of AGP by infrared and Raman spectroscopies and homology modeling.[75] This model shows that the protein folds as a highly symmetrical all-β-sheet protein dominated by a single eight-stranded antiparallel β-sheet. A docking model of progesterone to the modeled AGP found progesterone-AGP interactions in the central hydrophobic pocket of the protein. This docking model and Raman difference spectroscopy suggest the involvement of Trp122 of AGP in progesterone binding. Halsall *et al.* reported that diethylpyrocarbonate (DEPC) specifically modified His97 and 100, as evidenced by high-performance liquid chromatography electrospray ionization-mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry.[90] Interestingly, the DEPC-modification to His97 was inhibited in the presence of warfarin. This indicates that His97 is involved in the binding site for acidic drugs. These inconsistent findings indicate that AGP contains a wide drug-binding region for basic, acidic and neutral drugs (**Fig. 9**). Thus, more attention should be paid to the characterization of drug binding sites on AGP.

Moreover, both dog and bovine AGP have a basic ligand binding site and a steroid hormone binding site, which significantly overlap and affect one another. However, dog and bovine AGPs do not contain an acidic ligand binding region.[91] The results of fluorescence experiments indicate that the hydrophobic nature of the ligand binding pockets on the human, dog and bovine AGP are similar, but that their microviscosities are markedly different.

AGP is a mixture of at least two genetic variants: the A variant and the F1 and/or S variant or variants, which are encoded by two different genes. Herve *et al.* reported on the fractionation of the genetic variants of