Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochimica et Biophysica Acta 1747 (2005) 81–88

BIOCHIMICA ET BIOPHYSICA ACTA BBA

http://www.elsevier.com/locate/bba

Effect of genetic variation on the thermal stability of human serum albumin

Ulrich Kragh-Hansen[a,*], Shiori Saito[b], Koji Nishi[b], Makoto Anraku[b], Masaki Otagiri[b]

[a]*Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark*
[b]*Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan*

Received 30 April 2004; received in revised form 23 September 2004; accepted 27 September 2004
Available online 12 October 2004

Abstract

Reversible thermal denaturation of 33 genetic variants of human serum albumin (HSA) appeared to be a two-state process when studied by circular dichroism (CD). Fourteen single-residue variants have T_m values (midpoint of denaturation) higher than, and nine have T_m values lower than, their endogenous, wild-type counterpart. Nine single-residue variants have ΔH_v values (van't Hoff enthalpy) higher than, and 14 have ΔH_v values lower than, normal albumin. All types of combinations of positive and negative ΔT_m values and $\Delta(\Delta H_v)$ values were found. Good linear correlations between mutation-induced changes of α-helical content and $\Delta(\Delta H_v)$ values, but not ΔT_m values, were found especially for the variants mutated in domains I and III. The effect of altered chain length and glycosylation on T_m and ΔH_v was also studied. For all variants, no clear relationship was found between the changes in the thermodynamic parameters and the type of substitution, changes in protein charge or hydrophobicity. However, the protein changes taking place in domain I have a rather uniform effect (almost all of the nine variants have positive ΔT_m values and negative $\Delta(\Delta H_v)$ values, i.e., they denature more easily than normal albumin but they do so at a higher temperature). The present results can be of both protein chemical relevance and of clinical interest, because they could be useful when designing stable, recombinant HSAs for clinical applications.

Keywords: Human serum albumin; Genetic variant; Thermal stability; Midpoint of denaturation; van't Hoff enthalpy; α-Helical content

1. Introduction

Mutations of surface residues as well as of amino acids in the interior of proteins can effect their stability. Even single-residue substitutions can influence stability as has been observed for, e.g., intracellular fatty acid-binding proteins [1], lysozyme [2], apoflavodoxin [3], a thermophilic cold shock protein [4], crystallins [5] and staphylococcal nuclease [6]. The results referred to have all been obtained by using recombinant mutants. In the present work, we have studied the effect of genetic variation on the thermal stability of human serum albumin (HSA).

HSA is a single-chain protein synthesized in and secreted from liver cells. Normally, it is a simple protein, i.e., it lacks prosthetic groups and covalently bound carbohydrate and lipid. The protein has 585 amino acids and a molecular mass of 66.5 kDa [7]. According to X-ray crystallographic analyses of HSA and its recombinant version, the 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 [8,9]. HSA has 35 cysteine residues, and all of these but one, 34 Cys, are involved in the formation of stabilizing disulfide bonds.

Abbreviations: HSA, human serum albumin; Alb, albumin; proAlb, proalbumin; Alb A, normal (wild-type) albumin; CD, circular dichroism; T_m, midpoint of denaturation; ΔH_v, van't Hoff enthalpy

* Corresponding author. Tel.: +45 8942 2880; fax: +45 8613 1160.
E-mail address: ukh@biokemi.au.dk (U. Kragh-Hansen).

1570-9639/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2004.09.025

The temperature behaviour of albumin has been studied by several techniques but mostly by circular dichroism (CD) [10–14] and differential scanning calorimetry [12,15–21]. Among other things, the results showed increased thermal stability of the protein in the presence of aliphatic fatty acid anions of different chain length [13,15–17] or of *N*-acetyl-L-tryptophanate [13,17]. The effect of species differences has also been investigated [21]. Here, the effect of natural mutation of HSA has been examined. The work made use of 33 structurally different genetic variants which represent all kinds of known albumin isoforms, namely single-residue substitutions, proalbumin variants, chain termination mutants and glycosylated albumins. The thermal stability of these alloalbumins, as compared with that of wild-type albumin isolated from the same heterozygous carriers, was monitored by CD at 222 nm. Stability was quantitated in terms of midpoint of the denaturation curve (T_m) and van't Hoff enthalpy (ΔH_v). In addition, in the case of the 23 single-residue variants, the changes in T_m and ΔH_v were related to changes in α-helical content.

2. Materials and methods

2.1. Protein samples

The genetic variants of HSA and their normal (wild-type) counterparts (endogenous Alb A) were isolated from serum from heterozygous carriers by ion-exchange chromatography. After isolation, the albumins were checked by electrophoresis, and no denaturation or significant (no more than 5%) cross-contamination was detected. The proteins were put at our disposal 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. Tárnoky, University of Reading, Reading, UK; Dr. F.M. Salzano, Universidade de Federal do Rio Grande do Sul, Porto Alegre, Brazil; Dr. D. Donaldson, East Surrey Hospital, Redhill, UK and Dr. O. Sugita, Niigata University School of Medicine, Niigata, Japan. Before use, the albumins were delipidated by treatment with a hydroxyalkoxypropyl-dextran at pH 3.0 as previously described [22]. After defatting, the albumins were dialysed extensively against deionized water, lyophilized and stored at −20 °C until use. 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 (96–99% pure), assumed to be Alb A, was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and delipidated, dialysed and lyophilized in the same way as the other albumin samples.

2.2. Thermal denaturation measurements

The protein concentration was 10 μM, as determined by the method of Bradford [23], and the buffer was 67 mM sodium phosphate, pH 7.4. CD measurements were made with a Jasco J-720 spectropolarimeter (Tokyo, Japan), and CD melting curves were determined by monitoring the changes in the dichroic intensity at 222 nm as a function of temperature. The albumin solutions were heated by a Peltier effect in the CD-cuvette. The thermal denaturation was studied in the range 298–358 K using a heating rate of 1.0 K/min which was applied with a Jasco PTC-348 thermostat (Tokyo, Japan). Similar results (not shown) were obtained with a heating rate of 0.5 K/min. The calorimetric reversibility of the thermally induced transition was checked by reheating protein solutions in the calorimetric cell, flushed with nitrogen, after cooling from the first run. It was observed that heating to or above 358 K caused irreversible denaturation. This finding is in accordance with results of differential scanning calorimetry [20,21] and fluorescence spectroscopy [20].

The denaturation process was characterized by determining the midpoint of denaturation (melting temperature, T_m) and the van't Hoff enthalpy (ΔH_v). T_m is the temperature at which half of the protein is in a denatured state (D) and the other half is in the native state (N). ΔH_v was determined according to the following method, which is essentially the same as that of Budisa et al. [24]. At each temperature an equilibrium constant for the denaturation (K) was calculated from [D]/[N], where the squared brackets represent concentrations. Next, ln$K$ was plotted as a function of temperature (T) according to the van't Hoff equation:

$$\ln K = (-\Delta H_v/R) \times 1/T.$$

In this equation, R is the gas constant. Finally, ΔH_v was determined from the slope of the straight line obtained.

2.3. Far-UV CD spectra

The protein concentration was 1.5 μM, and the buffer was 67 mM sodium phosphate, pH 7.4, 25 °C. Far-UV intrinsic spectra were recorded from 200 to 250 nm using the Jasco J-720 spectropolarimeter. For calculation of the mean residue ellipticity, [θ], the molecular masses of normal albumin and of the albumins with single amino acid substitutions were assumed to be 66.5 kDa. The α-helical content of these proteins was estimated from the ellipticity values at 222 nm as described by Chen et al. [25].

3. Results and discussion

3.1. The genetic variants

The 33 alloalbumins used in this study have been named after the place from where the first detected carrier originates, and their molecular changes are summarized in Tables 1 and 2.

The majority of the albumin variants results from single-residue substitutions and almost all have net charges which

Table 1
Thermal denaturation of albumin variants with a single amino acid substitution[a]

Variant (mutation[b])	Reference[c]	ΔT_m (K)[d]	$\Delta(\Delta H_v)$ (kJ/mol)[e]
Alb Blenheim (1 Asp→Val)	[26]	1.94	−132.61
Alb Malmö-95 (63 Asp→Asn)[f]	[27]	6.07	−163.15
Alb Vibo Valentia (82 Glu→Lys)	[28]	2.03	−35.48
Alb Tregasio (122 Val→Glu)	[29]	0.57	26.85
Alb Hawkes Bay (177 Cys→Phe)	[30]	−1.59	−17.66
Alb Tradate-2 (225 Lys→Glu)	[31]	−4.86	44.13
Alb Herborn (240 Lys→Glu)	[32]	−2.74	−71.99
Alb Niigata (269 Asp→Gly)	[33]	3.67	−70.90
Alb Caserta (276 Lys→Asn)	[31]	4.87	13.42
Alb Canterbury (313 Lys→Asn)	[34]	−7.16	6.84
Alb Brest (314 Asp→Val)	[35]	−0.38	24.09
Alb Roma (321 Glu→Lys)	[36]	1.42	28.98
Alb Sondrio (333 Glu→Lys)	[37]	−2.56	−21.89
Alb Trieste (359 Lys→Asn)	[38]	−6.56	−13.91
Alb Parklands (365 Asp→His)	[39]	0.89	58.06
Alb Milano Slow (375 Asp→His)	[38]	−0.09	−94.33
Alb Kashmir (501 Glu→Lys)	[40]	0.13	−1.52
Alb Ortonovo (505 Glu→Lys)	[41]	1.87	−83.36
Alb Maku (541 Lys→Glu)	[42]	6.12	−58.32
Alb Church Bay (560 Lys→Glu)	[43]	0.70	15.23
Alb Paris-2 (563 Asp→Asn)	[37]	4.17	−154.35
Alb Verona (570 Glu→Lys)	[44]	−6.53	83.92
Alb Milano Fast (573 Lys→Glu)	[45]	2.08	−41.45

[a] The table gives average values for two to three experiments, which coincided with each other within ±6%.
[b] The positions of Alb A are from 1 to 585.
[c] See the references for more information about, for example, isolation and sequencing.
[d] ΔT_m is T_m for the variant minus T_m for the corresponding Alb A.
[e] $\Delta(\Delta H_v)$ is ΔH_v for the variant minus ΔH_v for the corresponding Alb A.
[f] The unglycosylated form of the variant. The name of this variant, as well as of its glycosylated form (Table 2), was taken from Ref. [31].

differ from Alb A at physiological pH (Table 1): Alb Vibo Valentia, Roma, Sondrio, Kashmir, Ortonovo and Verona are all +2 variants (i.e., they have two positive charges more than Alb A); Alb Blenheim, the unglycosylated form of Malmö-95, Niigata, Brest, Parklands, Milano Slow as well as Paris-2 are +1 variants; Alb Tregasio, Caserta, Canterbury and Trieste are −1 variants; and, finally, Alb Tradate-2, Herborn, Maku, Church Bay and Milano Fast are −2 variants. Thus, all kinds of changes in net charge are represented in this work. The only variant with no change in net charge is Alb Hawkes Bay. This albumin is also special in the sense that it has one disulfide bond less than Alb A but one free sulfhydryl group more than Alb A. Most of the single amino acid substitutions are placed in domain II (residues 200–391), namely 11. Domains I (residues 1–199) and III (residues 392–585) are represented by five and seven examples, respectively.

Proalbumin (proAlb) is an albumin molecule to which the propeptide, Arg-Gly-Val-Phe-Arg-Arg-, is still bound at the N-terminus. Normally, this protein does not occur in detectable amounts in the circulation, because the propeptide is cleaved off by propeptidase within the liver cells. However, substitution of −2 Arg (as in proAlb Lille) or 1 Asp (as in proAlb Blenheim) inhibits the proteolytic cleavage of the propeptide but not the secretion of the protein, and proalbumin variants, in contrast to wild-type proalbumin, can be isolated from the serum. In the periphery, part of proAlb Blenheim (Table 2) becomes converted to Alb Blenheim (Table 1), i.e., the normal propeptide is hydrolysed [26].

Among the C-terminal variants most are truncated albumins (Table 2). Thus, Alb Catania is three amino acids shorter than Alb A, and the three last residues in the new C-terminal end are changed from Gln-Ala-Ala to Lys-Leu-Pro. Alb Venezia has been shortened by seven amino acids, and the new C-terminal end is changed from Gly-Lys-Lys-Leu-Val-Ala-Ala to Pro-Thr-Met-Arg-Ile-Arg-Glu. Alb Bazzano has been shortened by three amino acids, and 14 of the last 16 amino acids in the new C-terminal end have been substituted: from Cys-Phe-Ala-Glu-Glu-Gly-Lys-Lys-Leu-Val-Ala-Ala-Ser-Gln-Ala-Ala to Ala-Leu-Pro-Arg-Arg-Val-Lys-Asn-Leu-Leu-Leu-Gln-Val-Lys-Leu-Pro. Here the 567 Cys→Ala substitution has caused the loss of the C-terminal disulfide bridge. The most pronounced modification, however, is found in Alb Kénitra which is an extended, and not a truncated, variant [49]. The last 11 amino acids of Alb A have been changed from Leu-Val-Ala-Ala-Ser-Gln-Ala-Ala-Leu-Gly-Leu to Thr-Cys-Cys-Cys-Lys-Ser-Ser-Cys-Leu-Arg-Leu. The extension consists of Ile-Thr-Ser-His-Leu-Lys-Ala-Ser-Gln-Pro-Thr(596)-Met-Arg-Ile-Arg-Glu-Arg-Lys; in the circulation the two C-terminally placed Arg-Lys are cleaved off by basic carboxypeptidases. In Alb Kénitra

Table 2
Thermal denaturation of proalbumin variants, albumins modified at the C-terminal end and of glycosylated variants[a]

Variant (mutation[b])	Reference[c]	ΔT_m (K)[d]	$\Delta(\Delta H_v)$ (kJ/mol)[e]
proAlb Lille (−2 Arg→His)	[46]	4.64	−13.82
proAlb Blenheim (1 Asp→Val)	[26]	7.10	−118.49
Arg-Alb (Alb A having −1 Arg)	[47]	0.23	12.65
Alb Catania (580–582 substituted, 583–585 deleted)	[48]	0.13	−57.27
Alb Venezia (572–578 substituted, 579–585 deleted)	[48]	−5.74	99.43
Alb Bazzano (567–582 substituted, 583–585 deleted)	[31]	4.67	−8.54
Alb Kénitra (575–585 substituted, extended with 586–601)	[49]	−5.30	12.72
Alb Malmö-95 (63 Asp→Asn, glycosylated at 63 Asn)	[27]	4.06	−107.41
Alb Redhill (−1 Arg retained, 320 Ala→Thr, glycosylated at 318 Asn)	[50,51]	1.93	−9.52
Alb Casebrook (494 Asp→Asn, glycosylated at 494 Asn)	[52,53]	−1.11	54.36

[a] The table gives average values for two to three experiments, which coincided with each other within ±6%.
[b] The positions of proalbumin are from −6 to −1 (the juxtaposition to albumin itself), and those of Alb A are from 1 to 585.
[c] See the references for more information about, for example, isolation and sequencing.
[d] ΔT_m is T_m for the variant minus T_m for the corresponding Alb A.
[e] $\Delta(\Delta H_v)$ is ΔH_v for the variant minus ΔH_v for the corresponding Alb A.

the four additional cysteine residues form two new S–S bridges, and 596 Thr is partially *O*-glycosylated by a monosialylated oligosaccharide.

It is uncommon for an amino acid substitution to result in the formation of an oligosaccharide attachment sequence. However, that has happened to Alb Malmö-95 (partly), Redhill and Casebrook, which are glycosylated in domain I, II and III, respectively (Table 2). In all three cases, the glycan is a disialylated (mainly or totally) biantennary complex type oligosaccharide *N*-linked to an asparagine residue [51]. Alb Redhill is unique, because it is the only example so far of an albumin with two mutations. One is the 320 Ala→Thr, which leads to glycosylation of 318 Asn; the other is −2 Arg→Cys, which, in this case, results in abnormal hydrolysis of preproalbumin within the liver cells and to the formation of albumin still possessing an Arg at position −1 [50].

All the albumins were isolated from heterozygotes, who, except for two cases, had one proalbumin or albumin variant and Alb A in the circulation. In one exception, a member of a New Zealand family had both proAlb Kaikoura and Arg-Alb [47]. The explanation of this condition is that most of the modified preproalbumin (about 85%) is cleaved after the mutated residue, −2 Cys, giving rise to Arg-Alb (Table 2), with about 15% processed normally, i.e., hydrolysed before −6 Arg by signal peptidase, leading to the formation of proAlb Kaikoura. The liver excretes both alloalbumins, and both can be isolated from the serum. Unfortunately, the proalbumin variant was not available for this study. In the other example, a Swedish one, the person carried a variant (Alb Malmö-95) of which about 50% was glycosylated [27].

According to the literature cited [26–53], none of the mutations seem to affect the oligomeric state of albumin.

3.2. Thermal denaturation

The temperature behaviour of albumin is strongly dependent on the environmental conditions. For example, thermal denaturation has been reported to be dependent on protein concentration [10,16], the presence of ligands [13,17], pH [15,18], type of salt [19] and salt concentration in the medium [15,18]. In the present work, we have used our standard conditions with respect to medium and rate of heating; see Section 2.2. In this way we should be able to compare the results obtained in this work with those in which the effect of recombinant mutation [14] and species differences were examined [21]. Thus, the effect of temperature in the range 298–358 K (25–85 °C) on the reversible denaturation of the albumins was monitored by CD at 222 nm. In this temperature range, the thermal denaturation process can be regarded as a two-state transition, i.e., in these proteins, the probability of all the intermediate states between the native and denatured ones is very low, and the denaturation appears as a single cooperative system [21]. Fig. 1 shows examples of mutations having a moderate effect (Fig. 1A) and a small effect (Fig. 1B) on thermal stability. In all cases, the process was characterized by determining the midpoint of denaturation, T_m, and the van't Hoff enthalpy, ΔH_v, and the results are included in Tables 1 and 2. The average value of T_m for endogenous Alb A was 336.67 K. This value is similar to that determined for commercial HSA (337.02±0.21 K, n=4). The average value of ΔH_v for wild-type HSA was calculated as 262.55 kJ/mol, a value which is somewhat higher than that obtained for the commercial protein (237.49±0.18 kJ/mol, n=4). The differences between the average values determined for endogenous and commercial Alb A are most probably due to differences in isolation procedures.

3.2.1. Single-residue variants

From Table 1 it is seen that 20 of the 23 single-residue mutations resulted in ΔT_m values which are larger than two times the standard deviation determined for commercial Alb A (0.42 K). Thirteen of the ΔT_m values are positive, and seven are negative. Apparently, there is no simple relationship between the ΔT_m values and the domains in which the mutations are placed, or between the ΔT_m values and the

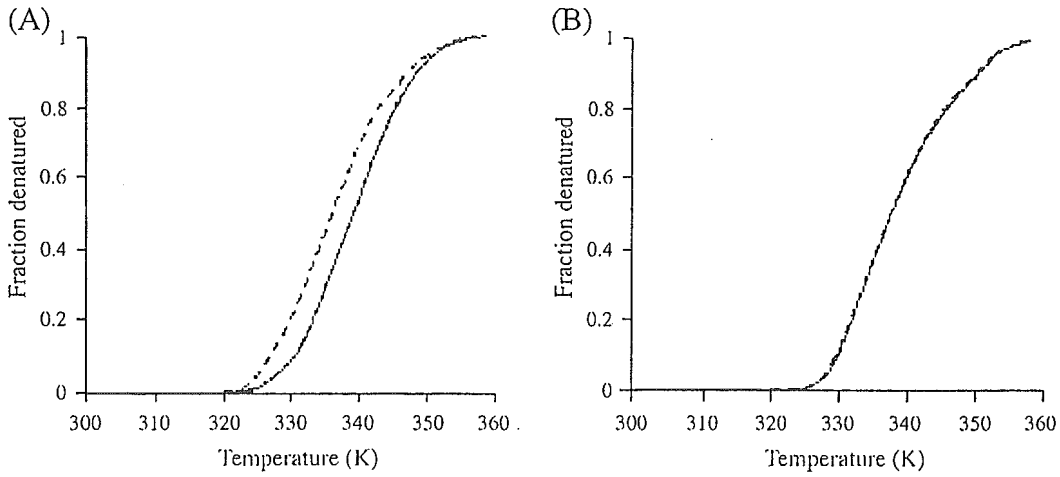

Fig. 1. Thermal denaturation of alloalbumins and their normal, endogenous counterparts. (A) Alb Herborn and (B) Alb Brest; the broken and full curves represent variant and corresponding Alb A, respectively. The curves are averages for three experiments.

change in protein charge (see Section 3.1). However, eight of the 20 mutations giving significant changes in ΔT_m are mutations resulting in more hydrophobic albumins. These include the albumins Blenheim, Malmö-95, Niigata, Caserta, Parklands and Paris-2, having positive changes in T_m, and albumins Canterbury and Trieste, which have very big negative ΔT_m values. For Alb Hawkes Bay, see below.

All of the mutations result in $\Delta(\Delta H_v)$ values larger than 0.36 kJ/mol (2×SD for commercial Alb A) (Table 1). Among these, 14 have ΔH_v values lower than their corresponding Alb A, i.e., the variants are easier to denature, more unstable, than Alb A. Nine of the 14 variants have positive ΔT_m values, i.e., the albumins denature more easily than Alb A but do so at a higher temperature. Although most of the positive $\Delta(\Delta H_v)$ values are placed in domain II, there seems to be no clear relationship between the $\Delta(\Delta H_v)$ values and the domains in which the mutations are placed. Finally, apparently there is no relationship between the $\Delta(\Delta H_v)$ values and the change in protein charge or between the $\Delta(\Delta H_v)$ values and protein hydrophobicity.

All the genetic variants included in this study were originally detected by electrophoresis performed under non-denaturing conditions. Among other things, this fact implies that the single-residue mutations are exposed to the solvent and result in modifications of albumins surface charges, a proposal which is supported by inspection of the crystal structure of HSA [8,29,31,35,38,43]. Alb Hawkes Bay is an exception, because the abnormal electrophoretic mobility of this alloalbumin was due to a gross conformational change caused by the mutation (177 Cys→Phe) [30]. The reason for the conformational changes is that this variant has lost the disulfide bond between 177 Cys and 168 Cys. The new SH-group of 168 Cys seems to bind to the nearby 124 Cys leaving 169 Cys unbound [30]; i.e., the variant has two free cysteine residues, namely the usual 34 Cys and 169 Cys. Surprisingly, the molecular rearrangements do not effect the thermal denaturation much (Table 1).

By using recombinant mutants, Watanabe et al. [14] studied the effect of single-residue mutations placed in subdomain IIA (Sudlow's binding site I) and IIIA (site II) on the thermal stability of HSA. These authors observed that mutations in subdomain IIA have a larger impact on the stability than those placed in subdomain IIIA, which only had marginal effects on the thermodynamic parameters. The mutation with the biggest effect was 214 Trp→Ala, which resulted in a ΔT_m value of −3.0 K and a $\Delta(\Delta H_v)$ value of −178.7 kJ/mol. The first value is within the range of the results found in this study, whereas the numeric value of the latter is higher than the numeric values of those found here (Table 1).

3.3. Relation between thermal stability and α-helical content

The molecular changes of the albumin variants had in themselves an impact on the CD spectrum. Fig. 2A shows that the mutation of Alb Herborn has a fairly large effect, whereas that of Alb Brest (Fig. 2B) has a small effect on the far-UV CD spectrum of albumin. Comparison of these observations with the results illustrated in Fig. 1 suggests that there exists a correlation between the mutation-induced effects on α-helical content and thermal stability of albumin. Therefore, a more detailed study was performed involving all the single-residue variants. Alloalbumins with more extensive molecular changes were not included in these experiments, because the effects on α-helical content were assumed to be more complex. Fig. 3 relates changes in α-helical content (abscissas) and $\Delta(\Delta H_v)$ (ordinates), and it is seen that there exists a good linear correlation between these parameters. Apparently, the correlation is best for mutations in domain III. However, if the results for Alb Hawkes Bay are ignored, an excellent correlation (r=0.998) is obtained for the domain I variants. The results of Fig. 3A also support the information in the literature [30], that the 177 Cys→Phe

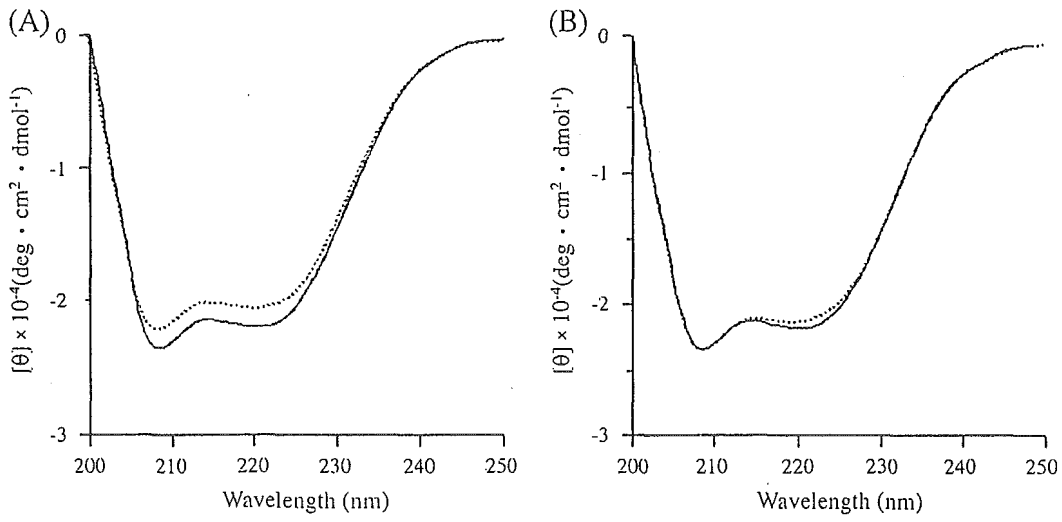

Fig. 2. Far-UV CD spectra of alloalbumins and their normal, endogenous counterparts. (A) Alb Herborn and (B) Alb Brest; the dotted and full curves represent variant and corresponding Alb A, respectively. The curves are averages for three experiments.

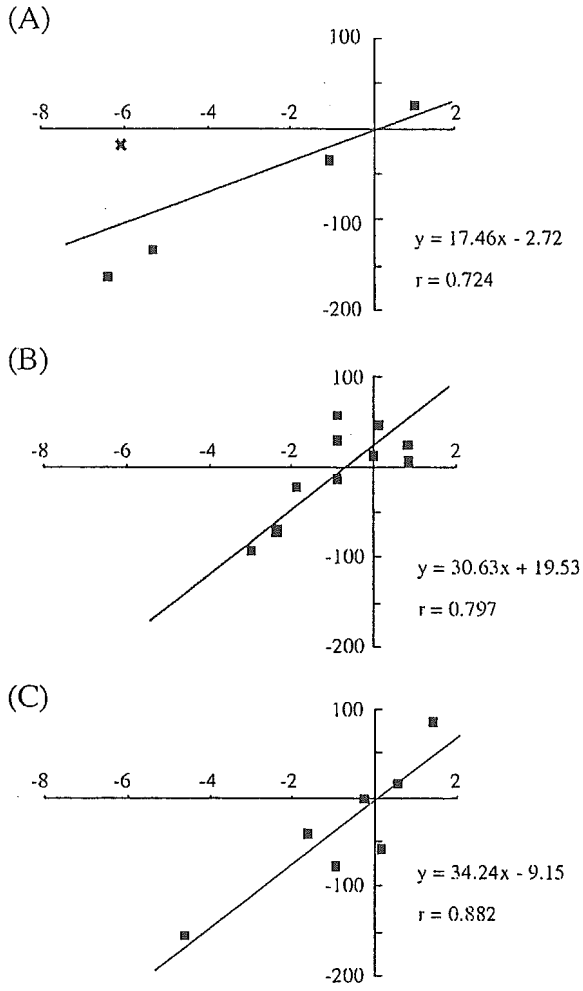

Fig. 3. Abscissas: Changes in percentages of the α-helical content of HSA caused by single amino acid substitutions. Ordinates: Corresponding $\Delta(\Delta H_v)$ given as kJ/mol, taken from Table 1. Results are shown for domain I (A), domain II (B) and domain III (C). In all three cases, the relation between changes in α-helical content and $\Delta(\Delta H_v)$ has been characterized by regression lines, and the expressions of these are given in the panels. The × in (A) represents the single variant in which a cysteine residue has been mutated (177 Cys→Phe). Without this protein, the domain I variants can be described by the following regression line: $y=25.00x-3.16$, $r=0.998$. The figure shows average values for two to three experiments, which coincided with each other within ±2%.

mutation results in a large conformational change, because the α-helical content of Alb Hawkes Bay is diminished by ca. 6%.

The changes in α-helical content were also related to ΔT_m (results not shown). However, this relationship was poor: the regression coefficients for the lines were only 0.242 (domain I), 0.319 (domain II) and 0.548 (domain III).

3.4. Effect of other types of mutation on thermal stability

3.4.1. Effect of altered chain lengths

The thermal stability of albumin is not effected much by an extra arginine residue at the N-terminus (Table 2). By contrast, the presence of a propeptide has a pronounced influence, and proAlb Blenheim is among the alloalbumins mostly effected. For both proalbumins, T_m is increased, and ΔH_v is decreased (Table 2).

A relatively small molecular change of the C-terminus (Alb Catania) has no effect on T_m (Table 2). By contrast, a relatively large decrease in ΔH_v was found. Alb Bazzano has lost its C-terminal disulfide bridge, namely the one between 558 Cys and 567 Cys. Surprisingly, this molecular change, as well as the relative large number of amino acid substitutions (see Section 3.1), has a pronounced increasing effect on T_m (ΔT_m=4.67 K), whereas ΔH_v is decreased somewhat. Alb Kénitra has only one free SH-group (34 Cys) but two new disulfide bridges, both of which are placed in the modified C-terminal end (see Section 3.1). Apparently, the two additional disulfide bonds have only a minor stabilizing effect because T_m is decreased by −5.30 K and ΔH_v is only slightly increased. However, the results are most probably a combined effect of the unusually many changes, which have taken place in this variant [49]. Finally, Alb Venezia has the mostly modified parameters, because T_m is much decreased, and the ΔH_v value is very high (Table 2).

3.4.2. Effect of N-glycosylation

All three variants have disialylated oligosaccharides linked to an asparagine residue (Table 2). Alb Malmö-95 has its glycan in domain I, and the molecular change causes a big increment of T_m and a large decrease of ΔH_v. Alb Redhill is so far the only example of an albumin with two mutations but they have only a relatively small impact on the proteins thermal stability. Finally, attachment of an oligosaccharide to domain III (Alb Casebrook) results in a more stable protein.

4. Concluding remarks

Apparently, there is no clear relation between changes in thermal stability of HSA and the type of substitution, change in protein charge or hydrophobicity. These observations probably reflect the fact that the effects on stability are strongly dependent on the molecular environment of the individual mutations. However, the protein changes taking place in domain I have a fairly uniform effect, because eight of the nine variants have positive ΔT_m values and seven of them have negative $\Delta(\Delta H_v)$ values (Tables 1 and 2); i.e., almost all of the alloalbumins modified in domain I denature more easily than normal albumin but they do so at a higher temperature. Furthermore, four of the five mutations resulting in very big changes in ΔH_v [$\Delta(\Delta H_v)<-100$ kJ/mol] are placed in domain I. This finding suggests that domain I is the most thermally unstable domain.

A good correlation was found between changes in α-helical content and $\Delta(\Delta H_v)$. Ignoring the variant with a modified disulfide bond pattern, the correlations were best for the peripheral domains. For all three domains, the trend is that a diminished α-helical content results in a negative

value of $\Delta(\Delta H_v)$. However, examples of increased α-helical content were also found. These increments were generally associated with positive $\Delta(\Delta H_v)$ values, i.e., more stable albumins.

Kosa et al. [21] have determined the T_m values for five species of serum albumin, namely human, bovine, dog, rabbit and rat. Of these, HSA was the most stable, and rat serum albumin was the least stable, and T_m for the former minus T_m for the latter was 2.0 K. Even though the sequence homologies between the five species of mammalian albumins are greater than 70% [21], many differences exist between them with respect to amino acid sequence. Therefore, it is surprising that half of the genetic variants with single amino acid substitutions have ΔT_m values larger than 2.0 K or lower than −2.0 K (Table 1).

Mutations resulting in change of size or shape of nonpolar residues in the hydrophobic core almost always modify thermal and chemical stability as well as structural properties of a protein [54,55]. However, the presence of cavities in the protein can to some extent neutralize the effect of single site mutations [55]. On the other hand, it has also been argued that filling of cavities leads to better packing and thereby helps stabilize the native state of the protein [56]. In general, it is expected that replacements on the surface of a protein have little or moderate effect on protein stability unless either the original or the introduced residue has specific roles. However, mutations of residues on the protein surface can also affect the proteins stability by more unspecific means. For example, the effect can be brought about by the alteration of charge–charge interactions [57], the packing and thereby the stability of the protein can be modified by changing the number of van der Waals interactions throughout the protein [56]. Thus, the observed changes in albumin stability could in some cases be due to structural alterations localized to the surface of the protein, whereas in other examples interior parts of albumin could be affected as well.

The present results can be of both protein chemical relevance and of clinical interest. HSA is a very widely used therapeutic agent. However, a major problem at present in the usage of the protein for clinical applications is viral contamination by, for example, human immunodeficiency virus, or vira causing herpes or hepatitis. Therefore, solutions of HSA are pasteurized by heating at 60 °C for 10–11 h before use [7]. Such a prolonged treatment can cause both reversible and irreversible denaturation of the protein. In the near future, the supply of recombinant HSA with high stability as well as inexpensive cost will be awaited. The results obtained here will be basically useful for designing these recombinant albumins.

Acknowledgements

We are grateful to the persons mentioned in Materials and methods for the generous gifts of genetic variants of human serum albumin and proalbumin. This work was supported in part by Aarhus University Research Foundation and by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (14370759).

References

[1] A.W. Zimmerman, M. Rademacher, H. Rüterjans, C. Lücke, J.H. Veerkamp, Functional and conformational characterization of new mutants of heart fatty acid-binding protein, Biochem. J. 344 (1999) 495–501.
[2] J. Funahashi, K. Takano, Y. Yamagata, K. Yutani, Role of surface hydrophobic residues in the conformational stability of human lysozyme at three different positions, Biochemistry 39 (2000) 14448–14456.
[3] M.P. Irún, S. Maldonado, J. Sancho, Stabilization of apoflavodoxin by replacing hydrogen-bonded charged Asp or Glu residues by the neutral isosteric Asn or Gln, Protein Eng. 14 (2001) 173–181.
[4] D. Perl, F.X. Schmid, Electrostatic stabilization of a thermophilic cold shock protein, J. Mol. Biol. 313 (2001) 343–357.
[5] Y.H. Kim, D.M. Kapfer, J. Boekhorst, N.H. Lubsen, H.P. Bächinger, T.R. Shearer, L.L. David, J.B. Feix, K.J. Lampi, Deamidation, but not truncation, decreases the urea stability of a lens structural protein, βB1-crystallin, Biochemistry 41 (2002) 14076–14084.
[6] D. Shortle, Staphylococcal nuclease: a showcase of *m*-value effects, Adv. Protein Chem. 46 (1995) 217–247.
[7] T. Peters Jr., All about Albumin: Biochemistry, Genetics and Medical Applications, Academic Press, San Diego, CA, 1996.
[8] D.C. Carter, J.X. Ho, Structure of serum albumin, Adv. Protein Chem. 45 (1994) 153–203.
[9] S. Sugio, A. Kashima, S. Mochizuki, M. Noda, K. Kobayashi, Crystal structure of human serum albumin at 2.5 Å resolution, Protein Eng. 12 (1999) 439–446.
[10] R. Wetzel, M. Becker, J. Behlke, H. Billwitz, S. Böhm, B. Ebert, H. Hamann, J. Krumbiegel, G. Lassman, Temperature behaviour of human serum albumin, Eur. J. Biochem. 104 (1980) 469–478.
[11] K. Takeda, A. Wada, K. Yamamoto, Y. Moriyama, K. Aoki, Conformational change of bovine serum albumin by heat treatment, J. Protein Chem. 8 (1989) 653–659.
[12] B. Farruggia, G.A. Picó, Thermodynamic features of the chemical and thermal denaturations of human serum albumin, Int. J. Biol. Macromol. 26 (1999) 317–323.
[13] T. Arakawa, Y. Kita, Stabilizing effects of caprylate and acetyltryptophanate on heat-induced aggregation of bovine serum albumin, Biochim. Biophys. Acta 1479 (2000) 32–36.
[14] H. Watanabe, U. Kragh-Hansen, S. Tanase, K. Nakajou, M. Mitarai, Y. Iwao, T. Maruyama, M. Otagiri, Conformational stability and warfarin-binding properties of human serum albumin studied by recombinant mutants, Biochem. J. 357 (2001) 269–274.
[15] S. Gumpen, P.O. Hegg, H. Martens, Thermal stability of fatty acid–serum albumin complexes studied by differential scanning calorimetry, Biochim. Biophys. Acta 574 (1979) 189–196.
[16] P.D. Ross, A. Shrake, Decrease in stability of human albumin with increase in protein concentration, J. Biol. Chem. 263 (1988) 11196–11202.
[17] A. Shrake, P.D. Ross, Biphasic denaturation of human albumin due to ligand redistribution during unfolding, J. Biol. Chem. 263 (1988) 15392–15399.
[18] M. Yamasaki, H. Yano, K. Aoki, Differential scanning calorimetric studies on bovine serum albumin: I. Effects of pH and ionic strength, Int. J. Biol. Macromol. 12 (1990) 263–268.
[19] M. Yamasaki, H. Yano, K. Aoki, Differential scanning calorimetric studies on bovine serum albumin: II. Effects of neutral salts and urea, Int. J. Biol. Macromol. 13 (1991) 322–328.

[20] G.A. Picó, Thermodynamic features of the thermal unfolding of human serum albumin, Int. J. Biol. Macromol. 20 (1997) 63–73.
[21] T. Kosa, T. Maruyama, M. Otagiri, Species differences of serum albumins: II. Chemical and thermal stability, Pharm. Res. 15 (1998) 449–454.
[22] U. Kragh-Hansen, A micromethod for delipidation of aqueous proteins, Anal. Biochem. 210 (1993) 318–327.
[23] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[24] N. Budisa, R. Huber, R. Golbik, C. Minks, E. Weyher, L. Moroder, Atomic mutations in annexin V—thermodynamic studies of isomorphous protein variants, Eur. J. Biochem. 253 (1998) 1–9.
[25] Y.-H. Chen, J.T. Yang, H.M. Martinez, Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion, Biochemistry 11 (1972) 4120–4131.
[26] S.O. Brennan, R.J. Peach, D.R. Boswell, Novel human proalbumin variant with intact dibasic sequence facilitates identification of its converting enzyme, Biochim. Biophys. Acta 993 (1989) 48–50.
[27] Y. Sakamoto, K. Kitamura, J. Madison, S. Watkins, C.-B. Laurell, M. Nomura, T. Higashiyama, F.W. Putnam, Structural study of the glycosylated and unglycosylated forms of a genetic variant of human serum albumin (63 Asp→Asn), Biochim. Biophys. Acta 1252 (1995) 209–216.
[28] M. Galliano, L. Minchiotti, F. Porta, A. Rossi, G. Ferri, J. Madison, S. Watkins, F.W. Putnam, Mutations in genetic variants of human serum albumin found in Italy, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 8721–8725.
[29] L. Minchiotti, S. Watkins, J. Madison, F.W. Putnam, U. Kragh-Hansen, A. Amoresano, P. Pucci, R. Cesati, M. Galliano, Structural characterization of four genetic variants of human serum albumin associated with alloalbuminemia in Italy, Eur. J. Biochem. 247 (1997) 476–482.
[30] S.O. Brennan, A.P. Fellowes, Albumin Hawkes Bay; a low level variant caused by loss of a sulphydryl group at position 177, Biochim. Biophys. Acta 1182 (1993) 46–50.
[31] J. Madison, M. Galliano, S. Watkins, L. Minchiotti, F. Porta, A. Rossi, F.W. Putnam, Genetic variants of human serum albumin in Italy: point mutants and a carboxyl-terminal variant, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 6476–6480.
[32] L. Minchiotti, M. Galliano, M.C. Zapponi, R. Tenni, The structural characterization and bilirubin-binding properties of albumin Herborn, a [Lys240→Glu] albumin mutant, Eur. J. Biochem. 214 (1993) 437–444.
[33] O. Sugita, N. Endo, T. Yamada, M. Yakata, S. Odani, The molecular abnormality of albumin Niigata: 269 Asp→Gly, Clin. Chim. Acta 164 (1987) 251–259.
[34] S.O. Brennan, P. Herbert, Albumin Canterbury (313 Lys→Asn). A point mutation in the second domain of serum albumin, Biochim. Biophys. Acta 912 (1987) 191–197.
[35] L. Minchiotti, U. Kragh-Hansen, H. Nielsen, E. Hardy, A.-Y. Mercier, M. Galliano, Structural characterization, stability and fatty acid-binding properties of two French genetic variants of human serum albumin, Biochim. Biophys. Acta 1431 (1999) 223–231.
[36] M. Galliano, L. Minchiotti, P. Iadarola, G. Ferri, M.C. Zapponi, A.A. Castellani, The amino acid substitution in albumin Roma: 321 Glu→Lys, FEBS Lett. 233 (1988) 100–104.
[37] L. Minchiotti, M. Galliano, M. Stoppini, G. Ferri, H. Crespeau, D. Rochu, F. Porta, Two alloalbumins with identical electrophoretic mobility are produced by differently charged amino acid substitutions, Biochim. Biophys. Acta 1119 (1992) 232–238.
[38] M. Galliano, S. Watkins, J. Madison, F.W. Putnam, U. Kragh-Hansen, R. Cesati, L. Minchiotti, Structural characterization of three genetic variants of human serum albumin modified in subdomains IIB and IIIA, Eur. J. Biochem. 251 (1998) 329–334.
[39] S.O. Brennan, The molecular abnormality of albumin Parklands: 365 Asp→His, Biochim. Biophys. Acta 830 (1985) 320–324.
[40] D. Savva, A.L. Tárnoky, M.F. Vickers, Genetic characterization of an alloalbumin, albumin Kashmir, using gene amplification and allele-specific oligonucleotides, Biochem. J. 266 (1990) 615–617.
[41] M. Galliano, L. Minchiotti, P. Iadarola, M. Stoppini, P. Giagnoni, S. Watkins, J. Madison, F.W. Putnam, Protein and DNA sequence analysis of a "private" genetic variant: albumin Ortonovo (Glu-505→Lys), Biochim. Biophys. Acta 1225 (1993) 27–32.
[42] K. Arai, K. Huss, J. Madison, F.W. Putnam, F.M. Salzano, M.H.L.P. Franco, S.E.B. Santos, M.J.M. Freitas, Amino acid substitutions in albumin variants found in Brazil, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 1821–1825.
[43] E.K.M. Chua, S.O. Brennan, P.M. George, Albumin Church Bay: 560 Lys→Glu a new mutation detected by electrospray ionisation mass spectrometry, Biochim. Biophys. Acta 1382 (1998) 305–310.
[44] L. Minchiotti, M. Galliano, P. Iadarola, M. Stoppini, G. Ferri, A.A. Castellani, Structural characterization of two genetic variants of human serum albumin, Biochim. Biophys. Acta 916 (1987) 411–418.
[45] P. Iadarola, L. Minchiotti, M. Galliano, Localization of the amino acid substitution site in a fast migrating variant of human serum albumin, FEBS Lett. 180 (1985) 85–88.
[46] Y. Abdo, J. Rousseaux, M. Dautrevaux, Proalbumin Lille, a new variant of human serum albumin, FEBS Lett. 131 (1981) 286–288.
[47] S.O. Brennan, K. Arai, J. Madison, C.-B. Laurell, M. Galliano, S. Watkins, R. Peach, T. Myles, P. George, F.W. Putnam, Hypermutability of CpG dinucleotides in the propeptide-encoding sequence of the human albumin gene, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 3909–3913.
[48] S. Watkins, J. Madison, E. Davis, Y. Sakamoto, M. Galliano, L. Minchiotti, F.W. Putnam, A donor splice mutation and a single-base deletion produce two carboxylterminal variants of human serum albumin, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 5959–5963.
[49] L. Minchiotti, M. Campagnoli, A. Rossi, M.E. Cosulich, M. Monti, P. Pucci, U. Kragh-Hansen, B. Granel, P. Disdier, P.-J. Weiller, M. Galliano, A nucleotide insertion and frameshift cause albumin Kénitra, an extended and *O*-glycosylated mutant of human serum albumin with two additional disulfide bridges, Eur. J. Biochem. 268 (2001) 344–352.
[50] S.O. Brennan, T. Myles, R.J. Peach, D. Donaldson, P.M. George, Albumin Redhill (−1 Arg, 320 Ala→Thr): a glycoprotein variant of human serum albumin whose precursor has an aberrant signal peptidase cleavage site, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 26–30.
[51] U. Kragh-Hansen, D. Donaldson, P.H. Jensen, The glycan structure of albumin Redhill, a glycosylated variant of human serum albumin, Biochim. Biophys. Acta 1550 (2001) 20–26.
[52] R.J. Peach, S.O. Brennan, Structural characterization of a glycoprotein variant of human serum albumin: albumin Casebrook (494 Asp→Asn), Biochim. Biophys. Acta 1097 (1991) 49–54.
[53] P.A. Haynes, M. Batley, R.J. Peach, S.O. Brennan, J.W. Redmond, Characterization of oligosaccharides from a glycoprotein variant of human serum albumin (albumin Casebrook) using high-performance anion-exchange chromatography and nuclear magnetic resonance spectroscopy, J. Chromatogr. 581 (1992) 187–193.
[54] M. Munson, S. Balasubramanian, K.G. Fleming, A.D. Nagi, R. O'Brien, J.M. Sturtevant, L. Regan, What makes a protein a protein? Hydrophobic core designs that specify stability and structural properties, Protein Sci. 5 (1996) 1584–1593.
[55] F.M. Richards, Protein stability: still an unsolved problem, Cell. Mol. Life Sci. 53 (1997) 790–802.
[56] J. Chen, Z. Lu, J. Sakon, W.E. Stites, Increasing the thermostability of staphylococcal nuclease: implications for the origin of protein thermostability, J. Mol. Biol. 303 (2000) 125–130.
[57] J.M. Sanchez-Ruiz, G.I. Makhatadze, To charge or not to charge? Trends Biotechnol. 19 (2001) 132–135.

Original Article

Oxidation and Carboxy Methyl Lysine-Modification of Albumin: Possible Involvement in the Progression of Oxidative Stress in Hemodialysis Patients

Katsumi MERA**, Makoto ANRAKU**, Kenichiro KITAMURA*,
Keisuke NAKAJOU, Toru MARUYAMA, Kimio TOMITA*, and Masaki OTAGIRI

Hemodialysis (HD) patients are frequently in a state of increased oxidative stress, and hyperglycemia appears to be a major factor. We recently found that oxidized human serum albumin (HSA) is a reliable marker of oxidative stress in HD patients. However, the issue of whether oxidized HSA is associated with the progression of oxidative stress in HD patients with or without diabetes is not clear. In the present study, we examined the effect of a qualitative modification of HSA in HD patients with or without diabetes. Blood samples from 10 HD patients with diabetes, 7 HD patients without diabetes, and 10 healthy age-matched controls were examined. The increase in plasma protein carbonyl content and advanced glycation endproducts (AGEs) in HD patients was largely due to an increase in the levels of oxidized HSA. Furthermore, these increases were greatest in HD patients with diabetes. Purified HSA from HD patients (non-DM-HSA) was carbonylated and AGE-modified. The amount of modified HSA was the highest in HD patients with diabetes (DM-HSA). Carboxy methyl lysine (CML)-modified HSA triggered a neutrophil respiratory burst, and this activity was closely correlated with the increase in the CML/HSA ratio. These findings indicate that uremia plays an important role in the progression of oxidative stress in HD patients *via* an increase in CML-modified HSA. They also indicate that diabetic complications further exacerbate the progression of oxidative stress by further increasing the amount of these modified HSA molecules. (*Hypertens Res* 2005; 28: 973–980)

Key Words: **hemodialysis patients, oxidative stress, human serum albumin, neutrophil burst**

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in hemodialysis (HD) patients (*1*). Both inflammation (*2*, *3*) and malnutrition increase the risk of death from cardiovascular causes in a synergistic process *via* malnutrition, inflammation, and the atherosclerosis (MIA) syndrome (*4*, *5*). The oxidative modification of proteins and lipids has been implicated in the etiology of numerous disorders and diseases (*6*, *7*), and is generally thought to contribute to inflammation in HD patients (*8*).

The causes of oxidative stress in HD patients are poorly understood. Oxidative stress arises when the normal balance

From the Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan; and *Department of Nephrology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan.

**These two authors contributed equally to this work.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (17590834-0 to K.K., 14370759 and 14657618 to M.O.), and grants from the Salt Science Research Foundation (0529 to K.K.), the Uehara Memorial Foundation (to K.K.), and a Japan Heart Foundation (to K.K.).

Address for Reprints: Masaki Otagiri, Ph.D., Department of Biopharmaceutics, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 5–1 Oe-Honmachi, Kumamoto 862–0973, Japan. E-mail: otagirim@gpo.kumamoto-u.ac.jp

Received August 4, 2005; Accepted in revised form October 17, 2005.

between the production of reactive oxygen species (ROS) and antioxidant activity is tilted in favor of the former. Protein oxidation may be a consequence of the increased production of ROS, a deficiency in antioxidant systems, or both. We previously demonstrated that oxidative stress in HD patients is manifested by an increase in the extent of oxidation of plasma proteins, including thiol oxidation and the formation of carbonyl groups on proteins. We also showed that human serum albumin (HSA) is the major plasma protein target of oxidative stress in uremia, and that increased levels of carbonyl compounds are correlated with the oxidation of HSA in HD patients (*9*). It has been suggested that circulating HSA is a major antioxidant in plasma. It has also been postulated that excess oxidized HSA in plasma increases the production of ROS by stimulating a neutrophil respiratory burst.

It has been demonstrated that the levels of oxidized HSA and the levels of advanced glycation end products (AGEs) in plasma are increased in HD patients (*10*) and that AGEs are a complex and heterogeneous group of compounds that have been implicated in diabetes-related complications (*11*). HSA, a very abundant plasma protein, appears to be involved in the formation of AGEs, suggesting that the oxidation and AGE-modification of HSA play important roles in the progression of oxidative stress in HD patients. Because diabetic nephropathy is now one of the main causes of chronic renal disease, there is a need to separately evaluate the contributions of uremia alone and uremia in conjunction with diabetes to oxidative stress in HD patients (*12*, *13*).

Therefore, in the present study, we investigated the oxidation and AGE-modification of HSA in HD patients with or without diabetes and age- and gender-matched control subjects. The effect of modified HSA on the neutrophil respiratory burst, which has been shown to mediate inflammation *via* the progression of oxidative stress, was also studied.

Methods

Patients

The protocol used in this study was approved by the institutional review board and informed consent was obtained from all subjects. A total of 27 subjects were enrolled: 17 stable HD patients (8 men, 9 women) aged 36 to 87 years, with a duration of dialysis ranging from 1 to 9 years, and 10 age- and gender-matched healthy control subjects. The HD patients were divided into 2 groups: HD patients with diabetes (DM) ($n=10$) and HD patients without diabetes (non-DM) ($n=7$). End-stage renal failure in the HD patients was caused by glomerulonephritis ($n=5$), nephrosclerosis ($n=2$) or diabetic nephropathy ($n=10$). At enrollment, all HD patients were receiving regular bicarbonate hemodialysis therapy (4 to 5 h per session, 3 times per week) using high-flux polysulfone hollow-fiber dialyzers. The profiles of healthy controls and DM or non-DM group are summarized in Table 1.

Purified HSA from Healthy Controls and DM or non-DM Group

HSA samples were isolated by the polyethylene glycol fractionation of blood plasma followed by chromatography on a Blue Sepharose CL-6B column (Amersham Pharmacia Co., Uppsala, Sweden) (*14*). The resulting fraction was then dialyzed against deionized water for 48 h at 4°C, followed by lyophilization. The purity of the HSA samples was at least 95%, and the percentage of dimers did not exceed 7%, as evidenced by SDS-PAGE and native-PAGE, respectively. The long-chain fatty acid contents of isolated HSA samples was determined using the copper triethanolamine method (*15*), and no significant change in long-chain fatty acid content was found in purified HSA from healthy controls and HD patients.

Chromatographic Analysis of HSA in Normal Subjects and DM or non-DM Group

The high-performance liquid chromatography (HPLC) analysis of HSA was performed as described in a previous report (*9*). HSA is a mixture of mercaptalbumin (HMA; reduced form) and nonmercaptalbumin (HNA; oxidized form). HMA contains one highly reactive sulfhydryl group at position 34 (Cys-34), while other serum proteins contain few or no highly reactive sulfhydryl groups. HNA is comprised of at least three types of molecules. The major HNA component is a mixed disulfide with cysteine or glutathione (HNA-1). The other is a more highly oxidized product than the mixed disulfide, in which the thiol group has been oxidized to the sulfenic (SOH), sulfinic (SO_2H) and sulfonic (SO_3H) states (HNA-2), the proportions of which are extremely small in extracellular fluids (*16*, *17*). The method of HPLC analysis of albumin developed by Sogami *et al.* (*16*) and Era *et al.* (*17*) permits the clean separation of HSA into HMA and HNA, and is used for the determination of the redox state for various pathophysiologic conditions. This method was applied as follows. Serum samples were frozen immediately after they were drawn, and were stored at −80°C until used for HPLC. The HPLC was performed using 5 μl aliquots of each serum sample and a Shodex Asahipak ES-502N column (Showa Denko Co., Ltd., Tokyo, Japan; column temperature: 35±0.5°C). The HPLC system consisted of an L-6200 intelligent pump equipped with a gradient programmer and an F-1050 fluorescence detector (Jasco Co., Ltd., Tokyo, Japan). Elution was performed using a linear gradient of ethanol (from 0% to 5%) with the serum dissolved in a mixture of 0.05 mol/l sodium acetate and 0.40 mol/l sodium sulfate (pH 4.85) at a flow rate of 1.0 ml/min. From the HPLC profiles of HSA, the value of each albumin fraction (f(HMA), f(HNA-1), and f(HNA-2)) was estimated by dividing the area of each fraction by the total area corresponding to HSA.

Table 1. Characteristics of the Normal and Patient Groups

	Normal subjects ($n=10$)	HD patients	
		non-DM ($n=7$)	DM ($n=10$)
Age (years)	68.3±1.7	58.7±9.8	65.1±15.6
Creatinine (mg/dl)	0.9±0.2	11.8±1.8*	9.6±3.0*
Gender (M/F)	5/5	3/4	5/5
HbA_{1C} (%)	—	—	7.0±1.1

Values are expressed as mean±SD. HD, hemodialysis; DM, HD patients with diabetes; M, male; F, female. *$p<0.01$ as compared with normal subjects.

Table 2. Determination of Serum Protein and Purified Albumin Oxidation

	Serum protein			Purified HSA		
	Normal subjects ($n=10$)	HD patients				
		non-DM ($n=7$)	DM ($n=10$)	normal-HSA	non-DM-HSA	DM-HSA
Carbonyl content (nmol/mg protein)	2.06±0.33	2.51±0.18[a]	2.96±0.48[a,b]	2.13±0.14	2.66±0.07[c]	2.88±0.22[c,d]
AGE content (fluorescence intensity [a.u.])	232±54	746±58[a]	1,006±116[a,b]	245±25	722±25[c]	956±43[c,d]
f(HMA) (%)	55.7±5.9	45.3±7.3[a]	37.9±6.0[a,b]	50.0±3.9	37.6±4.2[c]	30.3±5.4[c,d]
f(HNA-1) (%)	36.5±5.8	44.8±6.7[a]	52.3±5.7[a,b]	38.7±4.1	49.9±4.6[c]	56.8±5.1[c,d]
f(HNA-2) (%)	7.8±1.0	9.3±0.9[a]	9.8±1.0[a]	11.3±0.3	12.6±0.9[c]	12.9±0.9[c]

Values are expressed as mean±SD. HD, hemodialysis; DM, HD patients with diabetes; HSA, human serum albumin; AGE, advanced glycation endproduct; HMA, human mercaptalbumin; HNA, human nonmercaptalbumin; f(X), fraction of X. [a]$p<0.01$ as compared with Normal subjects. [b]$p<0.01$ as compared with non-DM group. [c]$p<0.01$ as compared with normal-HSA. [d]$p<0.01$ as compared with non-DM-HSA.

Total Plasma Protein and Individual Plasma Carbonyl Contents Measurement

Plasma protein carbonyl content was determined using the method of Climent *et al.* (*18*). In short, the samples were derivatized with fluoresceinamine, and their contents were quantified from the absorbance of the complexes at 490 nm (Jasco Ubest-35 UV/VIS spectrophotometer, Jasco Co.). The oxidation of individual plasma proteins was measured by Western blot analysis, as described by Shacter *et al.* (*19*). Plasma was diluted to 2 mg/ml of total protein with phosphate-buffered saline (PBS) and derivatized with an anti-2,4-dinitrophenylhydrazine (DNP) using an OxyBlot Kit (Serologicals Corp., 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 protein. Bands were visualized with chemiluminescent chemicals and captured on film at 10 min.

Analysis of Blots

Each Western blot included samples from both HD patients and healthy controls. Thus, the HD patient's samples could be compared with equivalent data for healthy controls developed under the same conditions in all experiments. These data were recorded as DNP area/protein area, and were reported as densitometric units. The mean for each subject group was calculated from each blot.

AGE Content of Plasma HSA and Purified HSA

Based on a report by Westwood *et al.* (*20*), we recorded fluorescence emission spectra of plasma HSA and purified HSA in PBS at an excitation wavelength of 350 nm, with an emission scan from 350 to 600 nm (slit width, 5 nm), using a fluorescence spectrometer (Jasco International Co., Ltd., Tokyo, Japan).

Enzyme-Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) was performed as described elsewhere (*21*). Briefly, each well of a

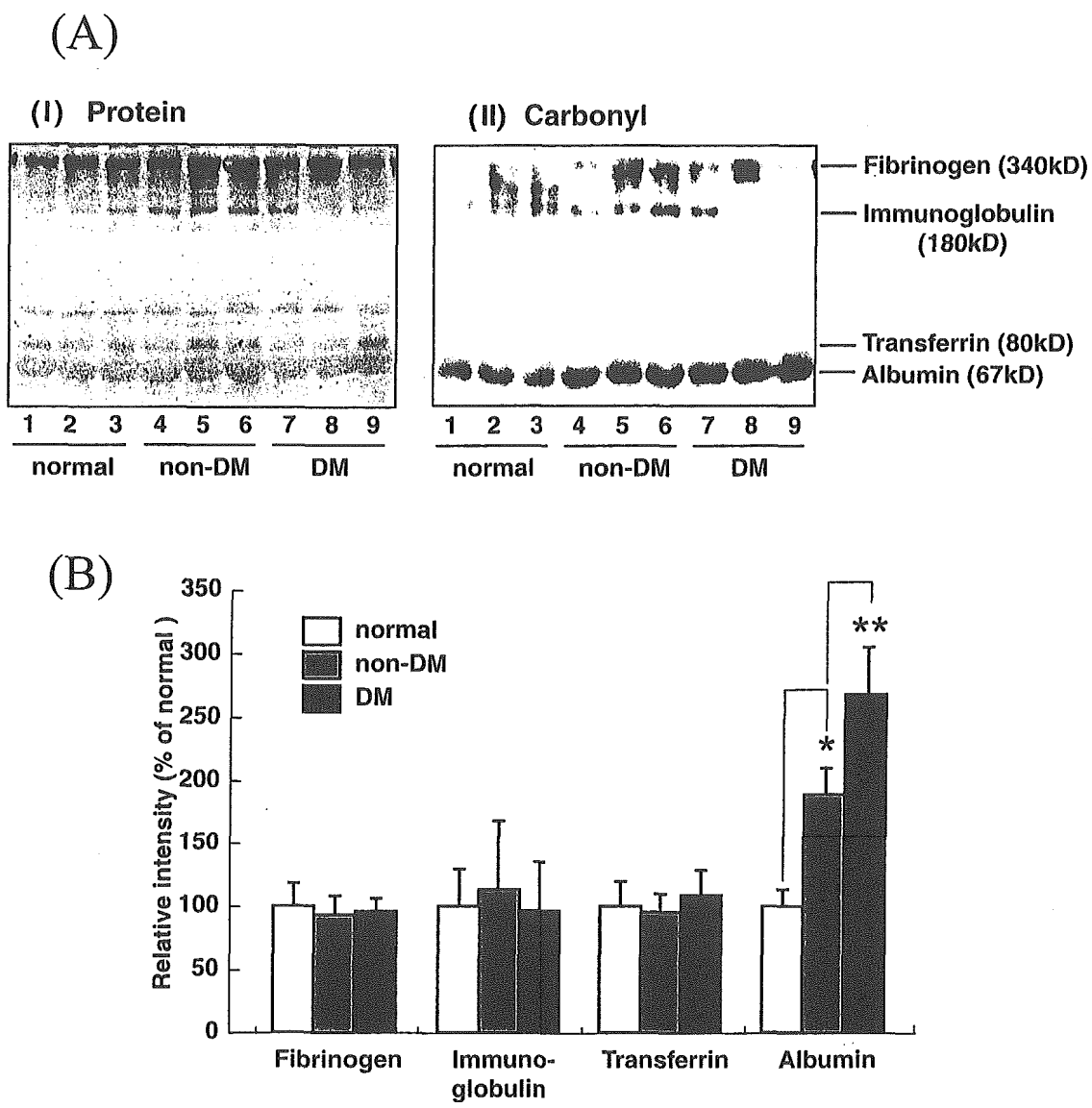

Fig. 1. *Immunochemical carbonyl content of major plasma proteins from normal controls and HD patients with or without diabetes (DM or non-DM). A: Plasma samples from DM or non-DM were derivatized with DNP and subjected to duplicate SDS-PAGE. Following electrotransfer, 1 blot was stained for protein with Coomassie brilliant blue G (I), and the second blot was stained for DNP using OxiBlot kit reagents (II). B: Carbonyl modification of major plasma proteins (albumin, transferrin, immunoglobulin, and fibrinogen) was evaluated as the densitometric ratio of DNP area and protein area, and was recorded in densitometric units. Values are expressed as the mean±SEM;* $n=11$ *for the control group and each patient group.* $^{*}p<0.05$*, compared with plasma from controls.* $^{**}p<0.05$*, compared with plasma from non-DM.*

96-well microtiter plate was coated with 100 µl of the sample to be tested in 50 mmol/l sodium carbonate buffer (pH 9.6). Each well was then blocked with 0.5% gelatin, and washed 3 times with PBS containing 0.05% Tween 20 (washing buffer). The wells were incubated for 1 h with a monoclonal antibody against pentosidine, carboxy methyl lysine (CML), pyrraline or imidazolone dissolved in washing buffer. The wells were then washed with washing buffer 3 times, incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody, and finally incubated with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by the addition of 0.1 ml of 1.0 mol/l sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader.

Measurement of Neutrophil Respiratory Burst

Neutrophils were isolated from heparinized peripheral blood

Table 3. Amount of Pentosidine, CML, Pyrraline or Imidazolone Determined by ELISA

	normal-HSA	non-DM-HSA	DM-HSA
Pentosidine	1,101±60	1,086±42	1,044±42
CML	1,622±115	1,843±144*	1,874±208*
Pyrraline	2,152±180	2,188±158	2,352±246**
Imidazolone	2,399±128	2,529±219*	2,503±152*

Values are expressed as mean±SD. CML, carboxy methyl lisine; ELISA, enzyme-linked immunosorbent assay; HSA, human serum albumin; DM, hemodialysis patients with diabetes. *$p<0.01$, **$p<0.05$ as compared with normal-HSA.

of healthy donors using Polymorphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. The purity of the neutrophil preparations routinely exceeded 95%, and cell viability, as determined by propidium iodide staining, was at least 98%. The accumulation of dihydrorhodamine 123 (DRD) in the neutrophil suspension was measured using a flow cytometer, by monitoring the fluorescence at 526 nm (*22*). Suspensions of neutrophils (1×10^6 cells) were incubated with 5 μmol/l DRD for 15 min at 37°C in serum-free medium. After DRD incubation, the neutrophil suspension was centrifuged and washed to remove unincorporated probe. The cells were treated with several concentrations of HSA medium for 1 h at 37°C, and then placed on ice. The mean fluorescence intensity of rhodamine (RD) in the cells was measured using a flow cytometer (FACS Calibur; Becton Dickinson Biosciences, Franklin Lakes, USA).

Statistics

Statistical significance was evaluated using the two-tailed, unpaired Student's *t*-test for comparisons between 2 means, or ANOVA analysis followed by the Newman-Keuls method for more than 2 means. A value of $p<0.05$ was considered to indicate statistical significance. The results are reported as the mean±SD.

Results

Carbonylation and AGE-Modification of Plasma Protein from Normal Controls and DM or non-DM Group

Protein oxidation is typically associated with an increase in carbonyl and AGE contents. An increase in carbonyl and AGE contents reflects the oxidation of Lys, Arg, or Pro residues in a protein. The plasma protein carbonyl and AGE contents were significantly increased in HD patients, and, in the diabetic group, the carbonylation and AGE-modification of plasma protein was further increased (Table 2).

Figure 1 summarizes the results obtained from Western blot analysis. HSA was the only major plasma protein that was significantly oxidized in HD patients ($p<0.05$) and, in the diabetic group, the oxidation of HSA was further increased. No significant difference in the carbonyl contents of other plasma proteins (transferrin, immunoglobulin, and fibrinogen) was found among the 3 groups. These findings suggest that the increase in plasma protein carbonyl and AGE contents in HD patients is largely due to an increase in oxidized HSA, and that this increase in oxidized HSA was greatest in DM group.

Oxidation of HSA in Normal Controls and DM or non-DM Group

In a previous study, we examined the redox states of HD patients during oxidative stress, especially the oxidation of HSA. In the present study, using HPLC analysis, we determined the oxidation status of the Cys-34 residues in HSA. The ratio of each HSA fraction to the total HSA (f(HMA), f(HNA-1), and f(HNA-2)) was calculated, and these data are summarized in Table 2. The non-DM group had a markedly increased f(HNA-1) and f(HNA-2) ratio, compared with normal controls ($p<0.01$), and f(HNA-1) was further increased in the DM group ($p<0.01$). These results may suggest that diabetes increases the oxidation of HSA *via* the effects of hyperglycemia.

Oxidation of Purified HSA from Normal Controls and DM or non-DM Group

Although it has been hypothesized that HSA becomes oxidized in some diseases, this hypothesis has yet to be confirmed. Several physicochemical peculiarities of HSA have been observed in patients with renal diseases, including changes in structural properties (*23*). However, none of these features is indicative of functional changes in HSA isolated from normal controls or HD patients. To determine the roles that oxidized HSA plays in the effects of oxidative stress, such as neutrophil activation, we purified HSA from normal controls (normal-HSA) and HD patients with or without diabetes (DM-HSA or non-DM-HSA). Purified HSA carbonyl and AGE contents were significantly increased in HD patients, and were further increased in DM group (Table 2). Also, the non-DM-HSA had a markedly increased f(HNA-1) and f(HNA-2) ratio, compared with normal-HSA ($p<0.01$), and f(HNA-1) was further increased in the DM group ($p<0.01$). In addition, the HNA/HMA ratio of purified HSA was closely correlated with the HNA/HMA ratio for sera ($r=0.952$, $p<0.01$; $n=27$; data not shown). These results suggest that the state of the purified HSA accurately reflects the redox state of HSA in blood.

AGE Content (Pentosidine, CML, Pirraline, Imidazolone)

The accumulation of AGEs in plasma increases with the pro-

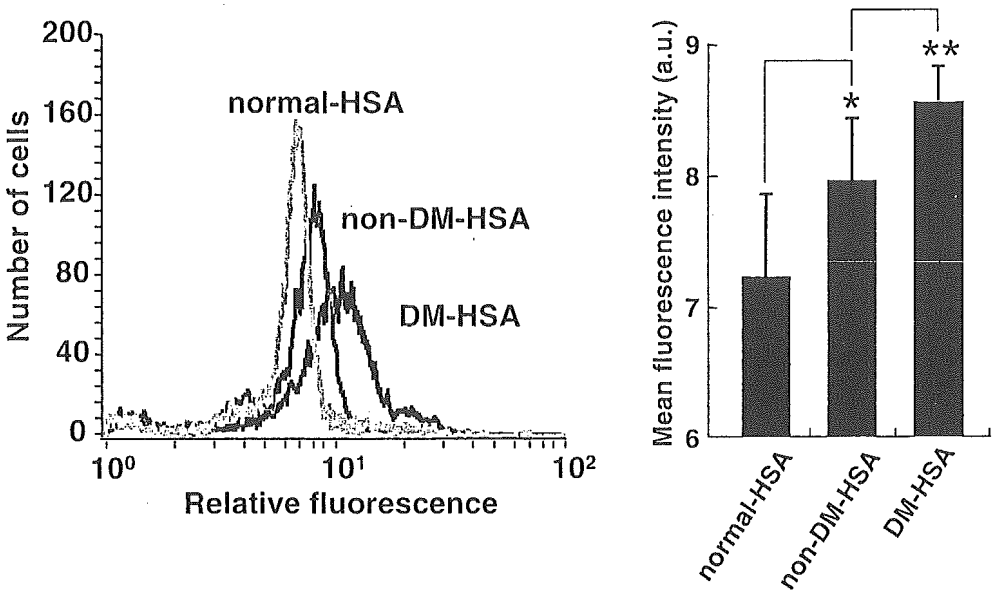

Fig. 2. *ROS production by neutrophils incubated with purified HSA.* *p < *0.05, compared with normal-HSA.* **p < *0.05, compared with non-DM-HSA.*

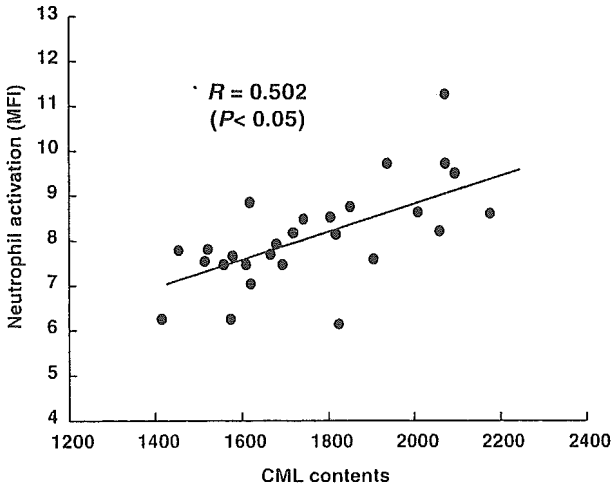

Fig. 3. *Relationship between neutrophil activation and CML-modified HSA (n = 27, r = 0.502, p < 0.05).*

gression of diabetic complications and atherosclerosis. The AGE content of each of the purified HSA samples is shown in Table 3. The findings indicate that uremia can induce the CML- and imidazolone-modification of HSA and that hyperglycemia appears to further increase the extent of those modifications.

Neutrophil Respiratory Burst

It was recently reported that *in vitro*-oxidized albumin up-regulated reactive oxygen species (ROS) generation in neutrophil suspensions. Therefore, in the present study, in order to directly determine whether non-DM-HSA or DM-HSA could induce oxidative stress in neutrophil suspensions, we used the DRD method and a fluorescence-activated cell sorter (FACS) analysis. The levels of ROS were higher for non-DM-HSA than for normal HSA (Fig. 2). Furthermore, the levels of ROS were higher for DM-HSA than for non-DM-HSA. Interestingly, the increase in ROS production was closely correlated with the increase in the CML/HSA contents ratio (r=0.502, p<0.05, Fig. 3), but not correlated with those of other AGEs/HSA contents ratio. These results suggest that the CML-modification of HSA increases the neutrophil burst.

Discussion

The findings herein demonstrated that the oxidation and AGE-modification of HSA are increased in non-DM group, and that these HSA modifications are increased even further in DM group. We also demonstrated that the treatment of neutrophils with purified HSA from HD patients without diabetes induced oxidative stress, as evaluated by neutrophil respiratory burst measurement, and that the diabetic state further stimulated the burst. The stimulation of the neutrophil respiratory burst was closely correlated with an increase in the CML modification of purified HSA. Oxidative stress led to increased levels of circulating oxidized and CML-modified HSA in HD patients. These findings might suggest that an increase in the levels of modified HSA contributes to increased vascular oxidative stress and increased risk of cardiovascular disease in HD patients, especially those with diabetes.

A number of epidemiological studies have demonstrated an

inverse relationship between serum albumin levels and mortality risk (*24–27*). The fact that HSA is a major antioxidant in extracellular fluids suggests that a decrease in HSA levels in HD patients contributes to the high incidence of cardiovascular events that are frequently associated with an increase in oxidative stress. Like the plasma concentration of HSA, the chemical state of HSA may affect its biological properties. Witco-Sarsat *et al.* demonstrated that carotid artery intima-media thickness is associated with the levels of plasma advanced oxidation protein products (AOPP), and with structural and functional alterations of HSA (*11*). Therefore, the chemical state of HSA may be a determinant of the level of oxidative stress in plasma. In fact, Terawaki *et al.* found that oxidative stress, determined the oxidation status of the Cys-34 residues in HSA, was enhanced in correlation with the level of renal dysfunction among HD patients (*28*). We also suggested that telmisartan effectively lowers the blood pressure in addition to reducing aldosterone concentration, brain natriuretic peptide, and oxidative stress, and is safe and well-tolerated by HD patients (*29*). Thus, these reports suggest that the "redox state of HSA" is a good marker to investigate the current status of oxidative stress in HD patients with renal failure.

Cardiovascular diseases continue to be the major cause of morbidity and mortality for patients requiring HD therapy. For HD patients, the annual mortality rate for cardiovascular disorders is approximately 9%, which is 10–20 fold higher than that of the general population, even when adjusted for age, sex, race, and the presence or absence of diabetes (*30*). In diabetes, the binding of glucose to albumin is more frequent, and involves the non-enzymatic covalent attachment of glucose to the ε-amino group of a lysine residue. Approximately 6% to 10% of the albumin in normal human serum is modified by non-enzymatic glycation (*31*). This proportion typically increases two- to three-fold in hyperglycemia (*32*). Moreover, diabetic patients exhibit elevated levels of iron and copper ions that, in the presence of glycated protein, have been shown to generate free radicals *in vitro* (*33*). That diabetic nephropathy has become one of the main causes of end-stage renal disease suggests that the hyperglycemia associated with diabetic nephropathy increases the level of modified HSA, thereby contributing to the increase in vascular oxidative stress. Therefore, a more complete understanding of oxidative mechanisms in HD patients requires the evaluation of modified HSA in HD patients with and without diabetes.

In the present studies, using a Western blot immunoassay, we demonstrated that the oxidation of HSA accounts for nearly all of the excess plasma protein oxidation in HD patients with uremia alone or with uremia and diabetes, and that diabetic complications further increase the extent of oxidation of HSA (Fig. 1). Plasma AGE levels were also significantly increased in non-DM group, and diabetic complications further increased AGE levels (Table 2). The overall level of modified HSA was increased in DM group. Various amino acids such as Cys, His, Trp, and Lys are thought to play important roles in free radical damage to proteins, and, the thiol group of Cys residues is thought to play a particularly important role in such damage (*34*). This is consistent with the present observation that the number of thiol groups in purified HSA from HD patients was less than 60% of the number of thiol groups in HSA from healthy controls, as indicated by HPLC analysis (Table 2). Dean *et al.* (*34*) proposed that thiol groups function either as a radical sink, thus protecting the protein from complete denaturation, or as agents that transfer damage to other residues such as His, Trp, and Lys. This in turn suggests that excess modified HSA, which acts as a pro-oxidant, increases cardiovascular complications in HD patients with or without diabetes. To test the hypothesis that the increased oxidative stress in the blood of HD patients is caused by the oxidation and AGE-modification of HSA, we evaluated the effects of incubating neutrophils with purified HSA from healthy donors or DM or non-DM group. non-DM-HSA induced oxidative stress, as indicated by the neutrophil respiratory burst, and DM-HSA further stimulated the burst (Fig. 2). In addition, the CML ratio of purified HSA was closely correlated with the strength of the neutrophil burst ($r=0.502$, $p<0.05$, Fig. 3). In previous studies, excess AGE-modified HSA was reported to be associated with a high level of respiratory burst, and this activity was blocked completely by excess soluble antibody to CML-modified albumin (*35*). Taking these results together, it seems likely that CML-modified HSA contributes substantially to the risk of cardiovascular events in HD patients.

Acknowledgements

The authors thank Dr. Shunichi Sakaguchi (Midorigaoka Clinic, Kumamoto, Japan) for collecting blood samples from HD patients.

References

1. De Lemos JA, Hillis LD: Diagnosis and management of coronary artery disease in patients with end-stage renal disease on hemodialysis. *J Am Soc Nephrol* 1996; **7**: 2044–2054.
2. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long term complications in insulin dependent diabetes mellitus. *N Engl J Med* 1993; **329**: 977–986.
3. Nitta K, Akiba T, Uchida K, *et al*: Left ventricular hypertrophy is associated with arterial stiffness and vascular calcification in hemodialysis patients. *Hypertens Res* 2004; **27**: 47–52.
4. Stenvinkel P, Alvestrand A: Inflammation in end-stage renal disease: sources, consequences, and therapy. *Semin Dial* 2002; **15**: 329–337.
5. Stenvinkel P: Inflammatory and atherosclerotic interactions in the depleted uremic patient. *Blood Purif* 2001; **19**: 53–61.
6. Himmelfarb J: Linking oxidative stress and inflammation in kidney disease: which is the chicken and which is the egg?

Semin Dial 2004; **17**: 449–454.
7. Baynes, JW: Perspectives in diabetes: role of oxidative stress in development of complications in diabetes. *Diabetes* 1991; **40**: 405–412.
8. Wang AY, Woo J, Wang M, *et al*: Association of inflammation and malnutrition with cardiac valve calcification in continuous ambulatory peritoneal dialysis patients. *J Am Soc Nephrol* 2001; **12**: 1927–1936.
9. Anraku M, Kitamura K, Shinohara A, *et al*: Intravenous iron administration induces oxidation of serum albumin in hemodialysis patients. *Kidney Int* 2004; **66**: 841–848.
10. Papanastasiou P, Grass L, Rodela H, Patrikarea A, Oreopoulos D, Diamandis EP: Immunological quantification of advanced glycosylation end-products in the serum of patients on hemodialysis. *Kidney Int* 1994; **46**: 216–222.
11. Witko-Sarsat V, Friedlander M, Nguyen Khoa T, *et al*: Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure. *J Immunol* 1998; **161**: 2524–2532.
12. Makita Z, Randoff S, Rayfield EJ, *et al*: Advanced glycosylation end products in patients with diabetic nephropathy. *New Engl J Med* 1991; **19**: 836–842.
13. Makita Z, Bucala R, Rayfield RJ, *et al*: Reactive glycosylation endproducts in diabetic uremia and treatment of renal failure. *Lancet* 1994; **343**: 1519–1522.
14. Watanabe H, Yamasaki K, Kragh-Hansen U, *et al*: *In vitro* and *in vivo* properties of recombinant human serum albumin from *Pichia pastoris* purified by a method of short processing time. *Pharm Res* 2001; **18**: 1775–1781.
15. Duncombe WG: The colorimetric micro-determination of non-esterified fatty acid in plasma. *Clin Chim Acta* 1964; **10**: 122–125.
16. Sogami M, Nagoka S, Era S, Honda M, Noguchi K: Resolution of human mercapt- and nonmercaptalbumin by high-performance liquid chromatography. *Int. J. Pept. Protein Res* 1984; **24**: 96–103.
17. Era S, Hamaguchi T, Sogami M, *et al*: Further studies on the resolution of human mercapt- and nonmercaptalbumin and on human serum albumin in the elderly by high-performance liquid chromatography. *Int. J. Pept. Protein Res* 1985; **3**: 435–442.
18. Climent I, Tsai L, Levine RL: Derivatization of gamma-glutamyl semialdehyde residues in oxidized proteins by fluoresceinamine. *Anal Biochem* 1989; **182**: 226–232.
19. Shacter E, Williams JA, Lim M, Levine RL: Differential susceptibility of plasma proteins to oxidative modification: examination by Western blot immunoassay. *Free Radic Biol Med* 1994; **17**: 429–437.
20. Westwood ME, McLellan AC, Thornalley PJ: Receptor-mediated endocytic uptake of methylglyoxal-modified serum albumin. Competition with advanced glycation end product-modified serum albumin at the advanced glycation end product receptor. *J Biol Chem* 1994; **269**: 32293–32298.
21. Nagai R, Horiuchi S: Application of monoclonal antibody libraries for the measurement of glycation adducts. *Biochem Soc Trans* 2003; **31**: 1438–1440.
22. Nakajima H, Takenaka M, Kaimori JY, *et al*: Activation of the signal transducer and activator of transcription signaling pathway in renal proximal tubular cells by albumin. *J Am Soc Nephrol* 2004; **15**: 276–285.
23. Ivanov AI, Korolenko EA, Korolik EV, *et al*: Chronic liver and renal diseases differently affect structure of human serum albumin. *Arch Biochem Biophys* 2002; **408**: 69–77.
24. Tojo A, Onozato ML, Kurihara H, *et al*: Angiotensin II blockade restores albumin reabsorption in the proximal tubules of diabetic rats. *Hypertens Res* 2003; **26**: 413–419.
25. Goldwasser P, Feldman J: Association of serum albumin and mortality risk. *J Clin Epidemiol* 1997; **50**: 693–703.
26. Ishimitsu T, Murayama N, Meguro T, *et al*: Urinary excretions of albumin and type IV collagen in normotensive and hypertensive subjects. *Hypertens Res* 2000; **23**: 459–466.
27. Kohara K, Tabara Y, Tachibana R, Nakura J, Miki T: Microalbuminuria and arterial stiffness in a general population: the Shimanami Health Promoting Program (J-SHIPP) study. *Hypertens Res* 2004; **27**: 471–477.
28. Terawaki H, Yoshimura K, Hasegawa T, *et al*: Oxidative stress is enhanced in correlation with renal dysfunction: examination with the redox state of albumin. *Kidney Int* 2004; **66**: 1988–1993.
29. Shimada H, Kitamura K, Anraku M, *et al*: Effect of telmisartan on ambulatory blood pressure monitoring, plasma brain natriuretic peptide, and oxidative status of serum albumin in hemodialysis patients. 2005; **28**: 987–994.
30. Foley RN, Parfrey PS, Sarnak MJ: Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am J Kidney Dis* 1998; **32**: S112–S119.
31. Shaklai N, Garlick RL, Bunn HF: Nonenzymatic glycosylation of human serum albumin alters its conformation and function. *J Biol Chem* 1984; **259**: 3812–3817.
32. Woodside JV, Yarnell JW, McMaster D, *et al*: Effect of B-group vitamins and antioxidant vitamins on hyperhomocysteinemia double-blind, randomized, factorial-design, controlled trial. *Am J Clin Nutr* 1998; **67**: 858–866.
33. Dean RT, Hunt JV, Grant AJ, Yamamoto Y, Niki E: Free radical damage to proteins the influence of the relative localization of radical generation, antioxidants, and target proteins. *Free Radic Biol Med* 1991; **11**: 161–168.
34. Dean RT, Fu S, Stocker R, Davies MJ: Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 1997; **324**: 1–18.
35. Collison KS, Parhar RS, Saleh SS, *et al*: RAGE-mediated neutrophil dysfunction is evoked by advanced glycation end products (AGEs). *J Leukoc Biol* 2002; **71**: 433–444.

Thrombosis Research (2005) 115, 191–197

ELSEVIER

THROMBOSIS RESEARCH

intl.elsevierhealth.com/journals/thre

REGULAR ARTICLE

Factor XII Shizuoka, a novel mutation (Ala392Thr) identified and characterized in a patient with congenital coagulation factor XII deficiency☆

Shuji Oguchi[a], Keiko Ishii[a], Takanori Moriki[b], Eiko Takeshita[a], Mitsuru Murata[a,c,*], Yasuo Ikeda[c], Kiyoaki Watanabe[a]

[a]*Department of Laboratory Medicine, School of Medicine, Keio University, Tokyo, Japan*
[b]*Health Center, Keio University, Tokyo, Japan*
[c]*Department of Internal Medicine, Division of Hematology, School of Medicine, Keio University, Tokyo, Japan*

Received 25 June 2004; received in revised form 9 August 2004; accepted 9 August 2004
Available online 28 September 2004

KEYWORDS
Factor XII;
Deficiency;
Mutation;
CRM-negative;
Blood coagulation;
Expression study

Abstract We identified a novel mutation (Ala392Thr) in the factor XII (FXII) gene of a patient with congenital FXII deficiency, designated Factor XII Shizuoka. The proband was an asymptomatic 63-year-old Japanese male with an abnormal coagulation test, discovered by chance during preoperative testing. The FXII activity was under 3% and antigen level was under 10%. Sequence analysis of the proband's FXII gene revealed a homozygous nucleotide substitution G to A in exon 10, resulting in the amino acid substitution Ala392 to Thr in the catalytic domain. We constructed the mutant FXII cDNA in an expression plasmid vector and transfected it into Chinese hamster ovary (CHO) cells. The recombinant wild-type FXII antigen was detected in the culture medium by immunoprecipitation assay, but the mutant FXII (A392T) was not observed. Both the wild-type FXII and A392T cell lysates, however, contained equivalent levels of FXII antigen and FXII mRNA, as estimated by Western blotting and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), respectively. These findings suggest that the Ala392

☆ This work was partially presented at the 45th Annual Meeting of the American Society of Hematology, San Diego, California, December, 2003.

* Corresponding author. Department of Internal Medicine, Division of Hematology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Tel.: +81 3 5363 3785; fax: +81 3 3353 3515.

E-mail addresses: Shuji.Oguchi@adst.keio.ac.jp (S. Oguchi), keiko-ishii@u03.itscom.net (K. Ishii), TakaMoriki@aol.com (T. Moriki), eiko.takeshita@adst.keio.ac.jp (E. Takeshita), murata@sc.itc.keio.ac.jp (M. Murata), yikeda@sc.itc.keio.ac.jp (Y. Ikeda), kiyoakiw@sc.itc.keio.ac.jp (K. Watanabe).

0049-3848/$ - see front matter
doi:10.1016/j.thromres.2004.08.027

to Thr substitution impairs intracellular protein processing and causes a cross-reacting material -negative FXII deficiency.

Introduction

Human blood coagulation factor XII (Hageman factor; FXII) is involved in the coagulation pathway, fibrinolysis, and the complement system in vivo. Congenital FXII deficiency (Hageman trait) is an autosomal recessive trait discovered mostly by chance during preoperative blood coagulation screening tests. In spite of a prolonged activated partial thromboplastin time, patients demonstrate no obvious clinical symptoms such as bleeding tendency, although some reports describe the possibility of a thromboembolic predisposition [1–3].

FXII is a single-chain 596-amino-acid 80-kDa glycoprotein containing 16.8% carbohydrate, primarily produced by hepatocytes as a serine protease precursor. The secreted protein circulates in the plasma as a zymogen. On the negatively charged surface, the attached precursor is cleaved by kallikrein at Arg353–Val354 and converted to activated factor XII (FXIIa), which initiates the rapid intrinsic coagulation pathway by the proteolysis of FXI to FXIa. The protein structure of FXII is composed of a COOH terminal catalytic domain, a kringle domain, two growth factor domains, and fibronectin type I and type II homology domains. The FXII gene is 12 kb, consists of 14 exons, and is located at 5q33-qter. FXII mRNA is approximately 2-kb long, including 150 bases of 3′-noncoding sequences [4].

There are only a few reports describing the molecular basis of congenital FXII deficiency. Several amino acid substitutions have been reported for cross-reacting material (CRM)-negative deficiency, including Tyr34Cys (FXII Tenri) [5], Arg123Pro [6], and Trp486Cys [7,8], which were characterized by in vitro expression studies in homozygous patients, and Gln421Lys in heterozygous patients [6]. Cys571Ser (FXII Washington DC) [9] and Arg353Pro (FXII Locarno) [10] are reported to be CRM-positive deficiencies. A common genetic polymorphism, 46 C to T in exon 1 at the 5′-untranslated region, four bases upstream of initiation ATG codon, is associated with a low translation efficiency and a decrease in plasma FXII level [11].

Here, we present a case of CRM-negative FXII deficiency with a novel FXII amino acid substitution, Ala392 to Thr. In vitro expression studies clarified that the mutation has a molecular etiology.

Materials and methods

Patient characteristics

The proband was a 63-year-old Japanese male living in Shizuoka, who was hospitalized for surgical repair of an inguinal hernia. Prolonged activated partial thromboplastin time (168 s) was identified during preoperative hemostatic tests (normal: 25–35 s). He had no bleeding or thrombotic tendencies and the surgery was successfully performed without complications, including the need for transfusion. Laboratory tests indicated that the FXII antigen level (normal: 50–150%) was less than 10% and FXII clotting activity (normal: 50–150%) was less than 3%, and circulating and lupus anticoagulant were both negative, indicating a diagnosis of CRM-negative FXII deficiency. FXII clotting activity was measured based on the APTT method using FXII deficient plasma. FXII antigen was measured by Laurell's method using rabbit anti-human FXII polyclonal antibody [12]. Other clotting factors were considered to be within the normal range (FVII: 90%, FVIII: 91%, FIX: 92%, FX: 81%, FXI: 71%, FXIII: 166%). The information of other family members was not available except that the parents of the proband were consanguineous.

FXII gene analysis

Peripheral venous blood was collected from the proband after obtaining written informed consent. DNA fragments corresponding to all exons and exon/intron boundaries of the FXII gene were amplified directly from whole blood by polymerase chain reaction (PCR) using Ampdirect (Shimadzu, Kyoto, Japan) [13]. The PCR primers were described previously [8]. In a total volume of 50 μl, 0.5 μl of whole blood, 13 nM sense and antisense primers, 10 μl of Ampdirect, 10 μl of AmpAddition, 0.8 mM dNTPs, and 1.5 U of Taq polymerase were mixed and amplified. Five microliters of 5% DMSO/0.1% Triton was added to exons 8, 9, 10, 11, and 12, and 10 μl of GC-Melt (BD Biosciences Clontech, Palo

Alto, CA) was added to enhance the reaction for exon 10. The annealing temperature was 62 °C, except for exon 9 (64 °C). The PCR products were gel-purified and direct DNA sequence analysis was performed with BigDye Terminator using ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

PCR-restriction fragment length polymorphism (RFLP)

After PCR amplification of the DNA fragments (432 bp) including the site of the one-point mutation in exon 10, the products were digested with restriction enzyme *Eco*52I and electrophoresed in an agarose gel. Normal subjects (n=95) were simultaneously analyzed using the same procedure.

Plasmid construction

Full-length human FXII cDNA was kindly provided by Dr. Ross T. A. MacGillivray (University of British Columbia, Vancouver, British Columbia, Canada). Wild-type (WT) FXII cDNA was cloned into pBluescript (Stratagene, La Jolla, CA) after removing the *Kpn*I site from the multiple cloning site. PCR-based mutagenesis was performed with mutagenic primers[1] using Platinum Pfx DNA polymerase with enhancer (Gibco BRL, Rockville, MD). The constructed mutant FXII cDNA (A392T) was excised at the *Not*I site and cloned into the expression vector PCI^{neo} (Promega, Madison, WI) and the DNA sequence was confirmed.

Transient expression of FXII WT and A392T in Chinese hamster ovary (CHO) cells

Chinese hamster ovary (CHO) cells were grown in 10% fetal calf serum/Dulbecco's modified Eagle's medium (Gibco BRL) under 5% CO_2, transfected using FuGENE6 (Roche Diagnostics, Basel, Switzerland) and 1 μg of plasmid DNA (PCI^{neo}, PCI^{neo}/FXII-WT, PCI^{neo}/FXII-A392T) in six-well culture dishes. For transient studies, the culture medium was replaced with serum-free Dulbecco's modified Eagle's medium after 24 h of transfection and the supernatant was collected after 24-h incubation. Cell lysates were also collected after solubilization in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl pH6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue).

Immunoprecipitation and Western blotting

Recombinant FXII secreted into serum-free medium was immunoprecipitated using three FXII antibodies (goat anti-FXII CL20055A, Cedarlane Laboratories, Ontario, Canada; GAFXIIIG, Affinity Biology, Ontario, Canada; mouse IgG1 (B7C9) MAB2084, Chemicon International, Temecula, CA) with Immobilized Protein A beads (ImmunoPure, Pierce, Rockford, IL). In a total volume of 1000 μl, collected serum-free medium was mixed with 2.5 μl of each antibody and 100 μl of Immobilized Protein A bead solution, and rotated at 4 °C for 60 min. The combined beads were collected, washed three times with ice-cold washing buffer (1% Triton X-100, 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% SDS), and mixed with 100 μl of SDS sample buffer (with 1% 2-ME). The transfected cells were directly solubilized with SDS sample buffer. After boiling for 5 min, samples were applied onto 5% to 20% gradient SDS polyacrylamide gels and electrophoresed. The separated proteins were transferred to polyvinylidene difluoride membranes (BioRad, Hercules, CA), blocked in 5% skim milk/0.05% Tween-20/TBS (20 mM Tris–HCl, 500 mM NaCl, pH 7.4) and incubated with horseradish peroxidase-conjugated anti-FXII antibody (CL20055HP, Cedarlane Laboratories) in 1:1000 dilution. The membranes were washed three times with Tween-20/TBS and labeled bands were detected using ECL Western Blotting Detection Reagents (Amersham, Buckinghamshire, England).

Quantitative reverse transcriptase (RT)-PCR for FXII mRNA expression

Transfected cells were subjected to FXII mRNA expression level analysis by quantitative RT-PCR with a TaqMan probe. Total RNA was purified from transiently transfected cells using an RNeasy Mini Kit with a QIAshredder spin column and an RNase-Free DNase Set (Qiagen, Hilden, Germany). The RNA purity was confirmed by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). The appropriate PCR primers (FXII-TaqF: 5′-CAGCTGTACCACAAATGTACCCAC; FXII-TaqR: 5′-AAACAGTATCCCCATCGCTGG) and the TaqMan probe (TaqMan-FXII: TGTGCTACCACCCCCAACTTTGATCAG) were designed using the manufacturer's software (Applied Biosystems). Quantitative RT-PCR was performed using a Platinum quantitative RT-PCR ThermoScript one-step system (Invitrogen, Carlsbad, CA) and ABI PRISM 7700 Applied Biosystems

[1] Sense primer: 5′-AGCGCGGAACTGGGGACTGG, antisense primers: 5′-GGTGCGGGCCGGTCCTGCAGGCAGTGAGTGGCCGTCAGCACCCAGC (underlined T is a mutated base), 5′-AACGGTACCTGCGCCTGCAGGAAGCTGGC.

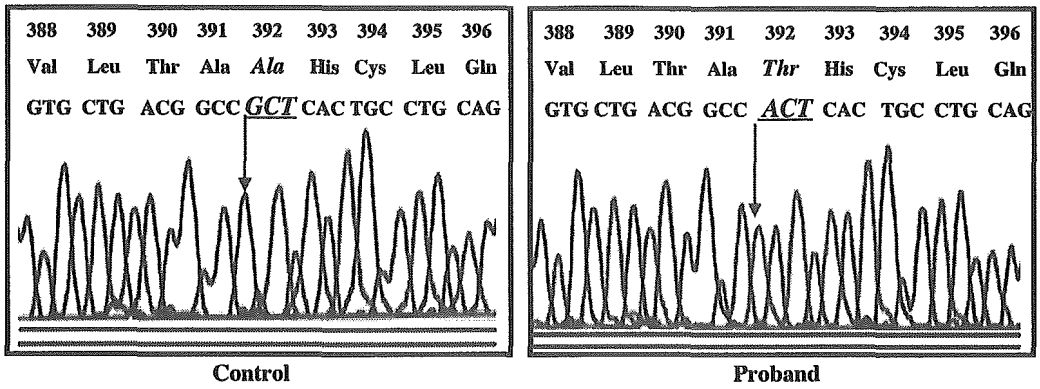

Figure 1 Sequence analysis of the FXII gene mutation in the proband. Samples from healthy subjects were used as controls. Homozygous G to A mutation in exon 10 was detected in the proband, which results in the amino acid substitution Ala392 (GCT) to Thr (ACT).

according to the manufacturer's protocol. Wild-type total RNA was serially diluted and used as a template for quantitative RT-PCR to generate the standard curve. An adequate fluorescent intensity was chosen and the required number of PCR cycles was defined as the threshold cycle. Results were from triplicate RNA samples. The data were statistically analyzed using Student's *t*-test.

Results

Gene analysis

Sequence analysis of all exons and exon/intron boundaries of the FXII gene revealed that the proband was homozygous for a one-base substitution of G to A at nucleotide position 9979 in exon 10 (nucleotide numbering is based on Ref. [13]), which results in the substitution Ala392 (GCT) to Thr (ACT) (Fig. 1). To confirm the detected substitution as a mutation, PCR products including the mutation site from the proband and control subjects (n=95) were digested with restriction enzyme *Eco*52I for RFLP analysis. *Eco*52I digested the 432-bp PCR product into 248, 137, and 47 bp in the controls, and 385 and 47 bp in the proband. The electrophoresis patterns of the digested fragments exhibited the proband's homozygosity for the mutation (Fig. 2). The polymorphism of the FXII gene promoter site, 46C/T, which is considered to affect the plasma FXII antigen level, was 46T/T in the proband. The 46T/T type is common in Japanese, and is associated with a lower plasma FXII antigen level [11].

Immunoprecipitation of the recombinant FXII

We transiently expressed the recombinant FXII protein in CHO cells and performed immunoprecipitation assays to compare the secreted FXII

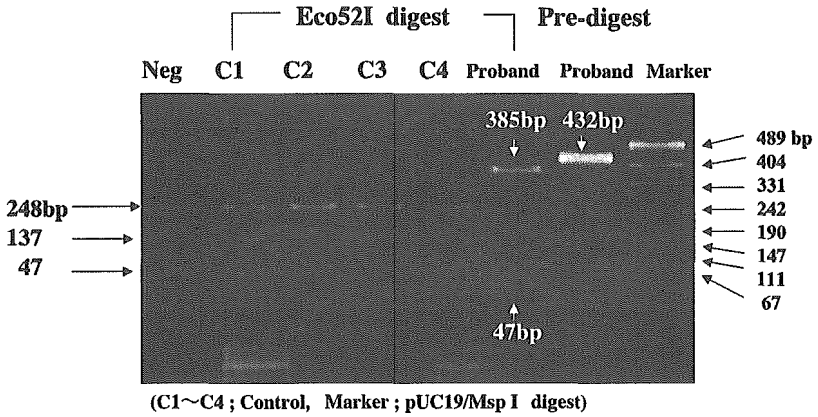

Figure 2 PCR-RFLP. PCR products (432 bp) of the proband and normal subjects (n=95) containing the mutation site in exon 10 were digested with restriction enzyme *Eco*52I. *Eco*52I digested the 432-bp PCR product into 248, 137, and 47 bp in the controls, and 385 and 47 bp in the proband. The electrophoresis patterns of the digested fragments exhibited the proband's homozygosity for the mutation. None of the normal subjects had the mutant pattern.