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

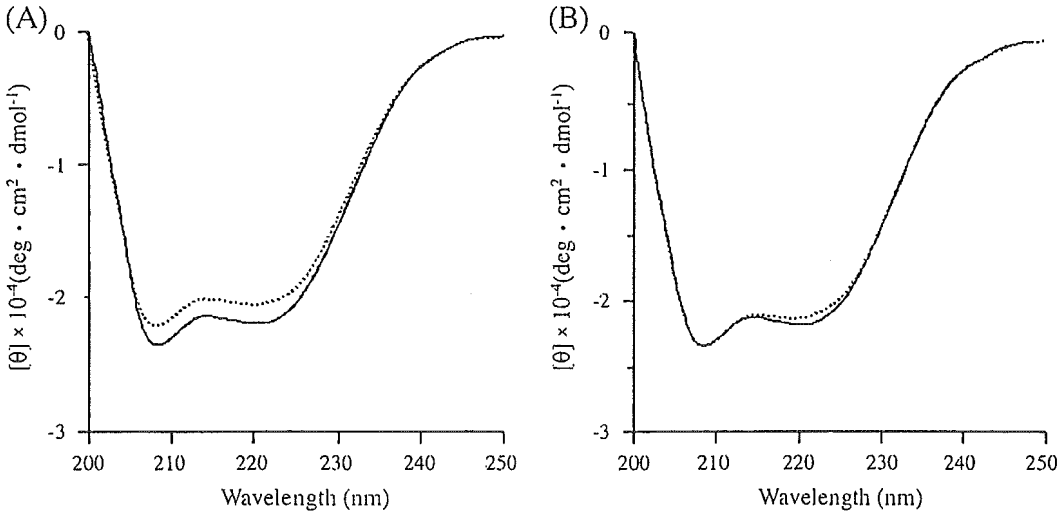

Fig. 2. Far-UV CD spectra of alloalbumins and their normal, endogenous counterparts. (A) Alb Herborn and (B) Alb Brest; the dotted and full curves represent variant and corresponding Alb A, respectively. The curves are averages for three experiments.

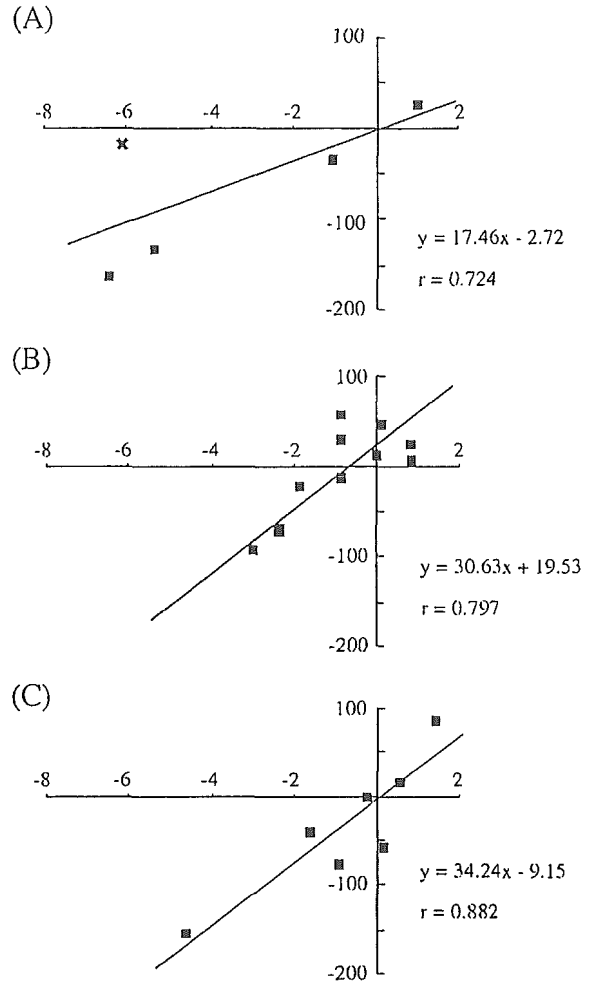

Fig. 3. Abscissas: Changes in percentages of the α-helical content of HSA caused by single amino acid substitutions. Ordinates: Corresponding $\Delta(\Delta H_v)$ given as kJ/mol, taken from Table 1. Results are shown for domain I (A), domain II (B) and domain III (C). In all three cases, the relation 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.

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Original Article

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

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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

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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		normal-HSA	non-DM-HSA	DM-HSA
		non-DM ($n=7$)	DM ($n=10$)			
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

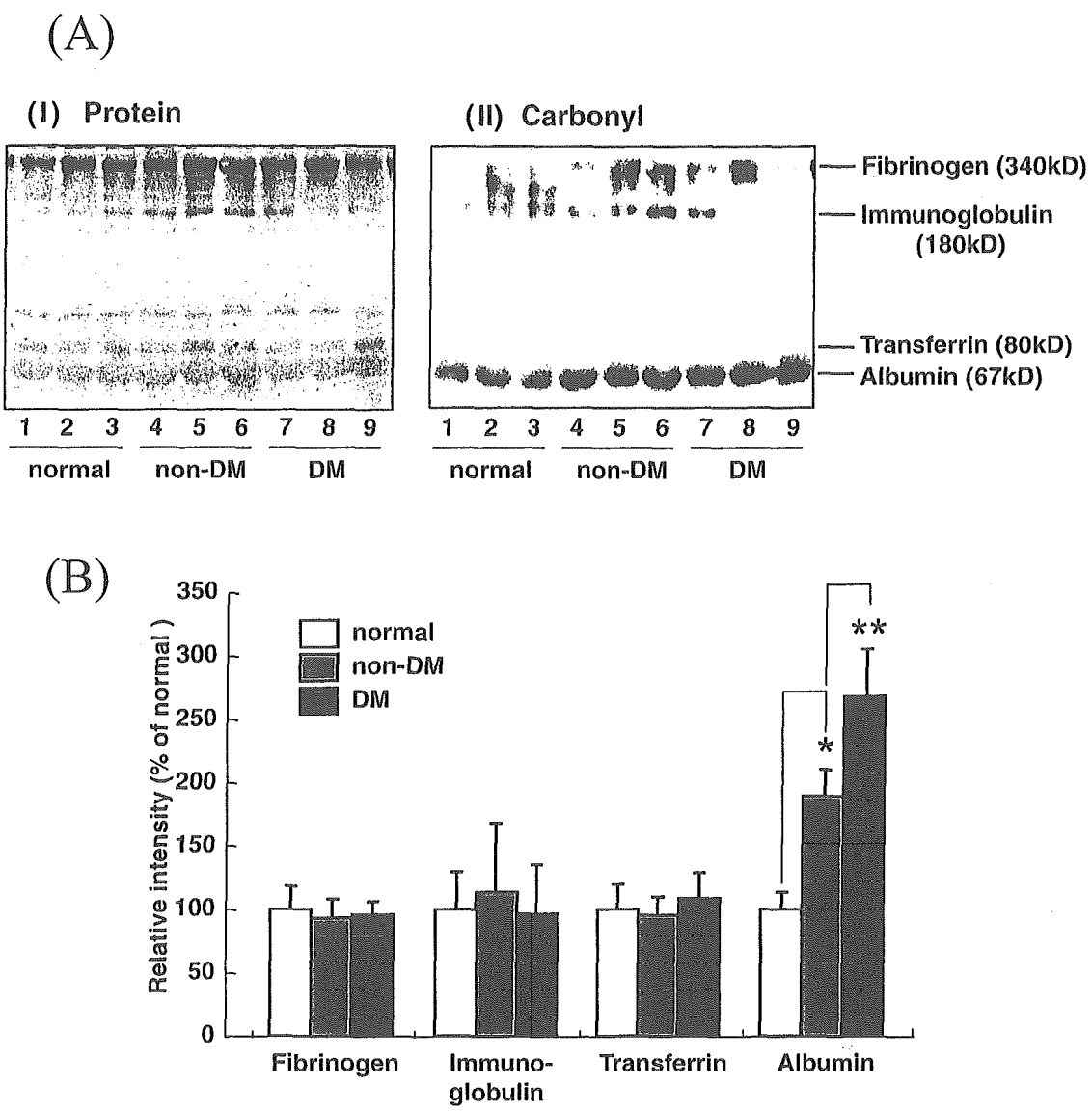

Fig. 1. *Immunochemical carbonyl content of major plasma proteins from normal controls and HD patients with or without diabetes (DM or non-DM). A: Plasma samples from DM or non-DM were derivatized with DNP and subjected to duplicate SDS-PAGE. Following electrotransfer, 1 blot was stained for protein 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-

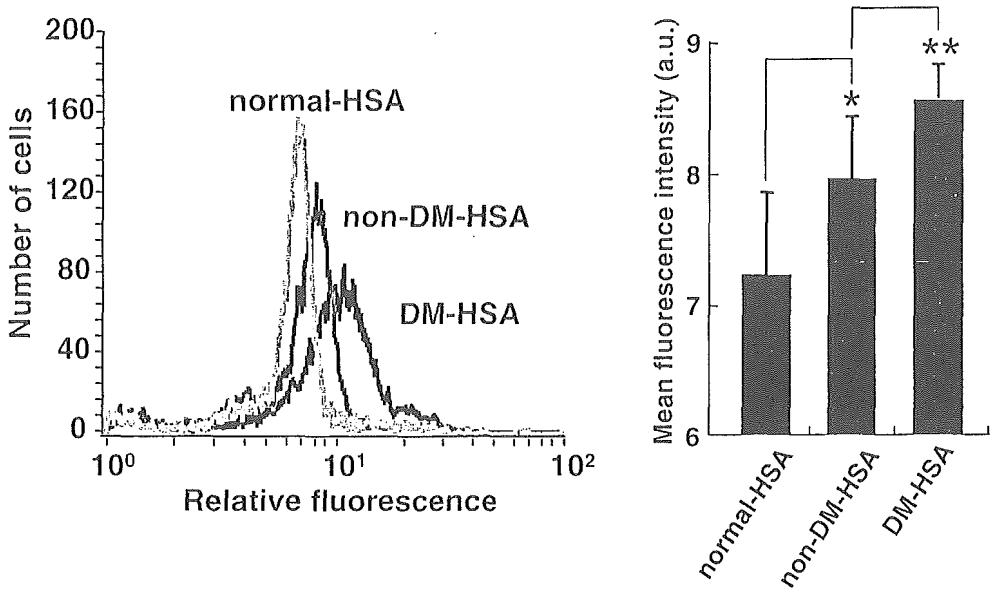

Fig. 2. *ROS production by neutrophils incubated with purified HSA.* *p < *0.05, compared with normal-HSA.* **p < *0.05, compared with non-DM-HSA.*

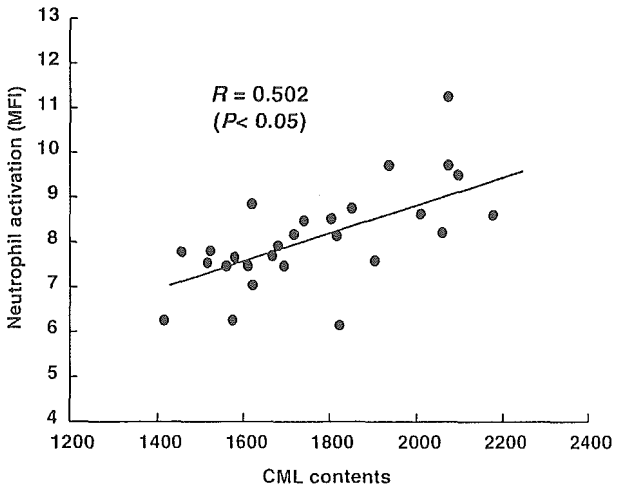

Fig. 3. *Relationship between neutrophil activation and CML-modified HSA (n = 27, r = 0.502, p < 0.05).*

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.

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REGULAR ARTICLE

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

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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

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to Thr substitution impairs intracellular protein processing and causes a cross-reacting material -negative FXII deficiency.
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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′-GGTGCGGGCCGGTCCTGCAGGCAGTGAGTGGCCGT-CAGCACCCAGC (underlined T is a mutated base), 5′-AACGG-TACCTGCGCCTGCAGGAAGCTGGC.

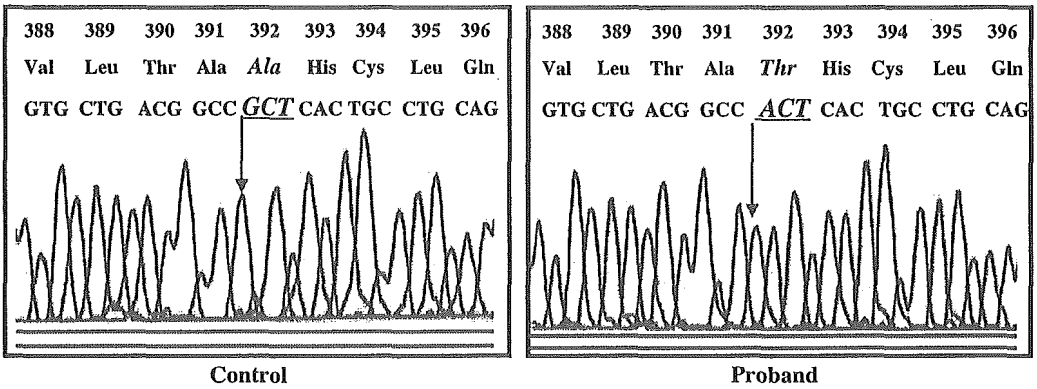

Figure 1 Sequence analysis of the FXII gene mutation in the proband. Samples from healthy subjects were used as controls. Homozygous G to A mutation in exon 10 was detected in the proband, which results in the amino acid substitution Ala392 (GCT) to Thr (ACT).

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

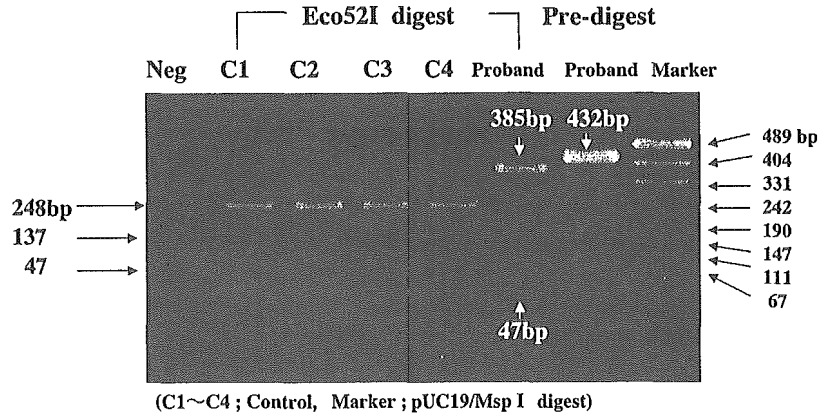

Figure 2 PCR-RFLP. PCR products (432 bp) of the proband and normal subjects (n=95) containing the mutation site in exon 10 were digested with restriction enzyme *Eco*52I. *Eco*52I digested the 432-bp PCR product into 248, 137, and 47 bp in the controls, and 385 and 47 bp in the proband. The electrophoresis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

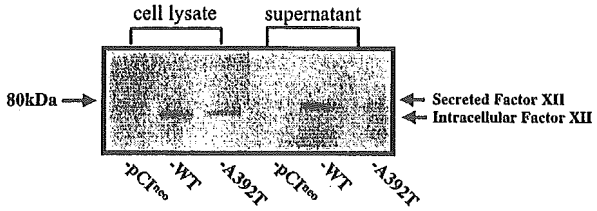

Figure 3 Western blot of recombinant FXII detected in the culture medium and cell lysates. Plasmid constructs (PCI^{neo}, PCI^{neo}/FXII-WT, PCI^{neo}/FXII-A392T) were transiently transfected into CHO cells. Supernatants containing secreted recombinant FXII were immunoprecipitated by anti-FXII antibodies. Transfected cells were solubilized with SDS sample buffer. All samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and probed with anti-FXII antibody. Only FXII-WT antigen was detected in the supernatant. In contrast, both the FXII-WT and A392T antigens were detected in the cell lysates.

antigen levels between WT and A392T. Using three anti-FXII antibodies, FXII antigen secreted into the supernatant of the culture medium was immunoprecipitated with Protein A beads and the transfected cells were simultaneously solubilized. The immunoprecipitates and cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and used for Western blotting (Fig. 3). In the supernatant, the FXII antigen was detected only in WT, and not in A392T, a finding compatible with the CRM-negative phenotype. We observed the FXII antigen in cell lysates of both WT and A392T. The difference in recombinant FXII molecular weights between supernatants and cell lysates is likely due to a posttranslational modification, such as carbohydrate addition.

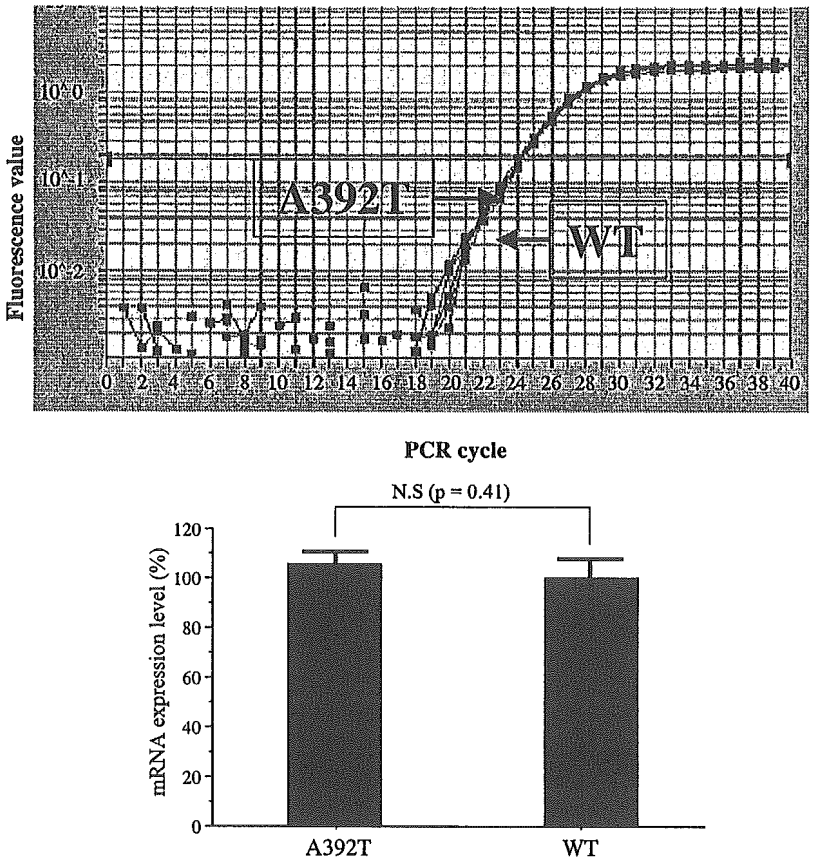

Figure 4 Comparison of mRNA expression levels between A392T and WT. Total RNA was purified from CHO cells transfected transiently by plasmid constructs (PCI^{neo}, PCI^{neo}/FXII-WT, PCI^{neo}/FXII-A392T). Quantitative RT-PCR was performed using a TaqMan probe and appropriate PCR primers using Platinum quantitative RT-PCR ThermoScript one-step system (Invitrogen) and ABI PRISM 7700 (Applied Biosystems). Total RNA from WT was serially diluted and used as a template of quantitative RT-PCR to generate the standard curve. An adequate fluorescent intensity was chosen and the number of required PCR cycles to reach the point was determined as the threshold cycle. The quantitative mRNA expression level of A392T was calculated from the standard curve and depicted as a percentage of WT. The result is from three independent RNA samples. There was no difference in the mRNA expression level between A392T and WT in CHO cells (p=0.41, Student's t-test).

Evaluation of FXII mRNA expression

We investigated the mRNA expression levels of WT and A392T in transient transfectants by quantitative RT-PCR. There was no significant difference in the threshold cycle between WT and A392T (p=0.41, Student's t-test; Fig. 4).

Discussion

We investigated a patient with CRM-negative FXII deficiency and discovered a homozygous mutation in exon 10 of FXII gene. This is a novel mutation, not yet reported, resulting in the amino acid substitution Ala to Thr at position 392 (A392T) in the catalytic domain, and was designated FXII Shizuoka. Transient expression study revealed the absence of A392T protein secretion to the culture medium, whereas the mutant protein and mRNA were present inside of the cells at an equivalent level compared to WT, suggesting that the A392T protein is normally transcribed and synthesized at the endoplasmic reticulum.

Only a few gene mutations related to CRM-negative FXII deficiency have been reported. Screening of mutations in the human FXII gene of 31 unrelated FXII-deficient patients identified six mutations associated with CRM-negative FXII deficiency [14], although expression analysis was not performed. Four CRM-negative mutations were determined by expression analysis. FXII Tenri [5] exhibited a Tyr34Cys substitution. The additional Cys in the NH_2-terminal type II domain is related mostly to proteasome-mediated degradation, and a small amount of residual FXII Tenri was secreted to the plasma combined with α_1-microglobulin, which was connected to the mutated Cys34. Two other CRM-negative FXII mutations, Gln421Lys and Arg123Pro, were examined by in vitro expression analysis [6]. Gln421Lys is likely to accumulate in the endoplasmic reticulum due to an impaired transport system, whereas Arg123Pro is susceptible to proteasome degradation.

In the present case, the amount of intracellular FXII Shizuoka protein detected by Western blot was no more than that of WT; that is, there was no accumulation of FXII Shizuoka in the cells (Fig. 3). Moreover, the mRNA expression level of FXII Shizuoka was equivalent to that of WT (Fig. 4). We and another group previously reported another CRM-negative FXII deficiency, Trp486Cys [7,8]. The recombinant Trp486Cys protein accumulated in the cells more than in WT, with a slight increase in mRNA expression. In the endoplasmic reticulum, the signal of aberrant protein accumulation is transduced to the nucleus by transcription factors, the control of which is related to protein expression for long-term adaptation or apoptosis of the cells [15]. The lack of recombinant FXII Shizuoka protein accumulation and constant mRNA expression in the cells suggest that the molecular etiology of FXII deficiency induced by Ala392Thr is an increased susceptibility to proteasome rather than endoplasmic reticulum retention due to impaired transportation.

As a member of serine proteases, the structure formed by the catalytic triad His393–Asp442–Ser544 is critical for the FXII enzyme activity [14]. It is noteworthy that the present mutation Ala392Thr is located close to the active site His393. Threonine has a hydroxyl group in its side chain. It is possible that the hydroxyl group at residue 392 makes the new hydrogen bond with other amino acid residues and affects the geometry of the active site. It is reported that the missense mutations Leu395Met and Arg398Gln close to His393 also cause CRM-negative FXII deficiency [14]. All of these Ala392, Leu395 and Arg398 residues are conserved for human, bovine and guinea pig FXII [16,17].

It is interesting that FXII mutations in the catalytic domain could cause both CRM-negative and -positive phenotypes. FXII Washington DC (Cys571Ser) [9], FXII Locarno (Arg353Pro) [10], FXII Asp442Asn, and Gly570Arg are CRM-positive phenotypes [14]. Whether the mutants were degraded or secreted would depend on the misfolded conformation induced by the mutation, which would be recognized by the protein quality control system in the endoplasmic reticulum [18–20].

In conclusion, we identified a novel homozygous FXII mutation, Ala392Thr, and clarified that the mutation was an independent molecular basis of the CRM-negative phenotype. Elucidation of the protein degradation mechanism requires further investigation of the intracellular protein quality control system.

Acknowledgements

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T280M and V249I polymorphisms of fractalkine receptor CX3CR1 and ischemic cerebrovascular disease

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Abstract

The contribution to atherosclerosis of two CX3CR1 single nucleotide polymorphisms, V249I and T280M has been recently reported. The atherosclerosis of intracranial vessels is thought to be the major pathological mechanism of ischemic stroke. In this study, we investigated the risk of ischemic stroke associated with fractalkine receptor CX3CR1 polymorphisms.

We investigated the T280M and V249I mutations in the CX3CR1 gene in 235 Japanese patients with ischemic cerebrovascular disease (CVD) and 306 age- and sex-matched healthy controls. Polymerase chain reaction and restriction fragment length polymorphism were used for genotyping.

There was no significant difference in both polymorphisms between patients with ischemic CVD and controls (VV versus II + VI, $p = 0.83$; TT versus MM + TM, $p = 0.66$). The I and M allele frequencies were not significantly different between CVD patients and controls: odds ratio (OR) = 0.89 (95% confidence interval (CI) = 0.50–1.60, $p = 0.70$) and OR = 1.19 (95% CI = 0.71–2.00, $p = 0.51$), respectively. We found eight of nine possible combined genotypes, including a new haplotype V249-M280, in Japanese.

Our results show that these CX3CR1 gene polymorphisms are not associated with an increased risk for ischemic CVD in the Japanese population.
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Keywords: Ischemic stroke; Atherosclerosis; Polymorphism; CX3CR1; Fractalkine

In 2001, Moatti et al. [12] reported that the I249 allele of the CX3CR1 gene had the ability to reduce the risk of coronary artery disease. Two CX3CR1 single nucleotide polymorphisms, V249I and T280M, are located in the coding sequence, and a recent study demonstrated that I249-M280 homozygotes have lower fractalkine binding affinity on primary peripheral blood mononuclear cells (PBMC) compared with V249-T280 homozygotes [3]. These results prompted researchers to examine the correlation between these alleles and the pathogenesis of atherosclerotic disease.

Imai et al. [7] identified a novel seven-transmembrane receptor for fractalkine, which is expressed mainly on NK cells and monocytes, and partly on T cells, and named it CX3CR1. Fractalkine, a CX3C motif protein, is a transmembrane, mucin/chemokine hybrid molecule, which is induced on activated primary endothelial cells [1]. Fractalkine is captured on the cell surface of inflammatory factor-activated leukocytes, so interaction between fractalkine and CX3CR1 may contribute to atherogenesis, which consists of inflammation, adhesion between leukocytes and the endothelium, and leukocyte migration into the endothelium [4].

Some studies have examined the correlation between these polymorphisms and atherosclerotic disease, but no study has focused on the association between these polymorphisms

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