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		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

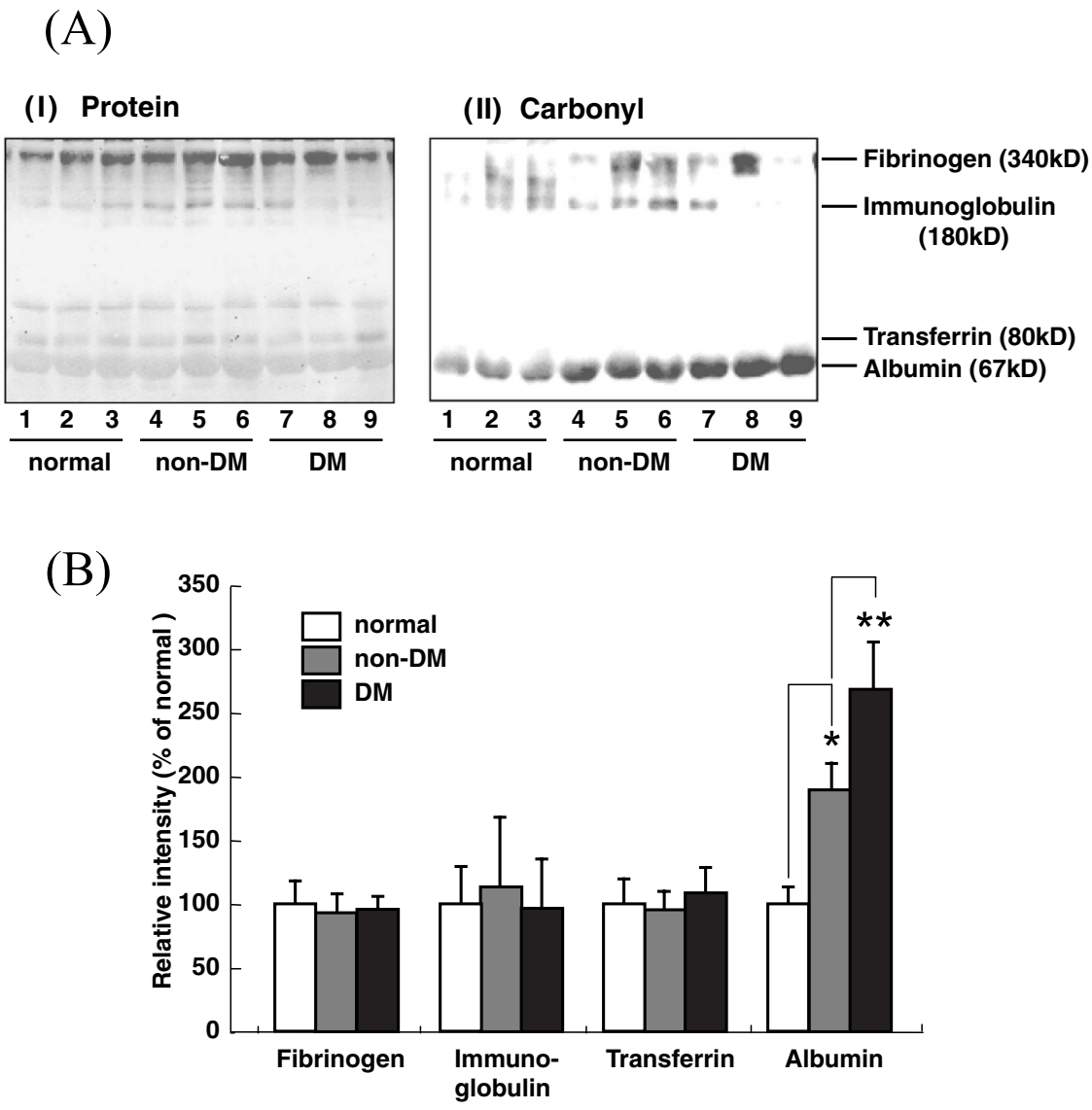

Fig. 1. *Immunochemical carbonyl content of major plasma proteins from normal controls and HD patients with or without diabetes (DM or non-DM). A: Plasma samples from DM or non-DM were derivatized with DNP and subjected to duplicate SDS-PAGE. Following electrotransfer, 1 blot was stained for protein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

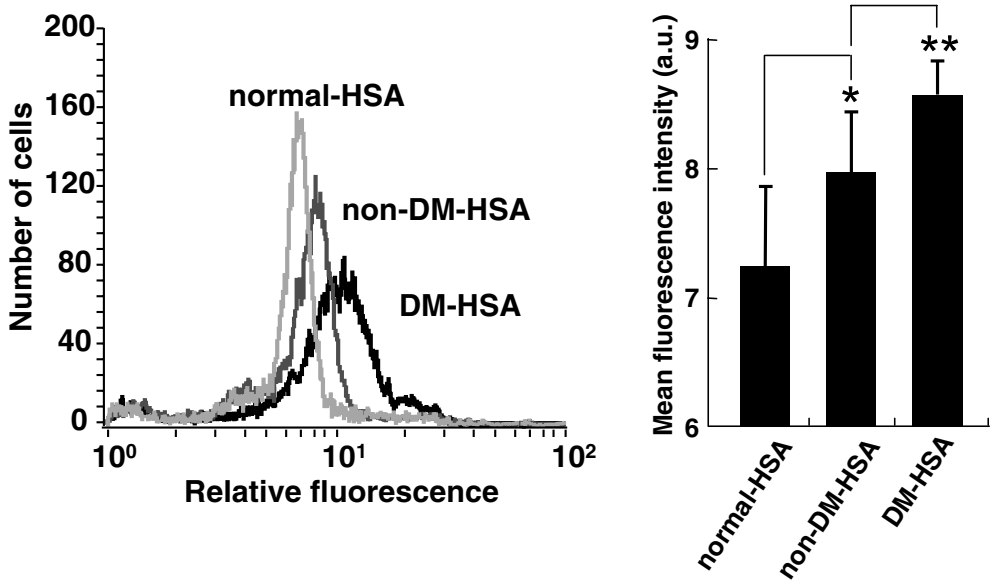

Fig. 2. *ROS production by neutrophils incubated with purified HSA.* *p < *0.05, compared with normal-HSA.* **p < *0.05, compared with non-DM-HSA.*

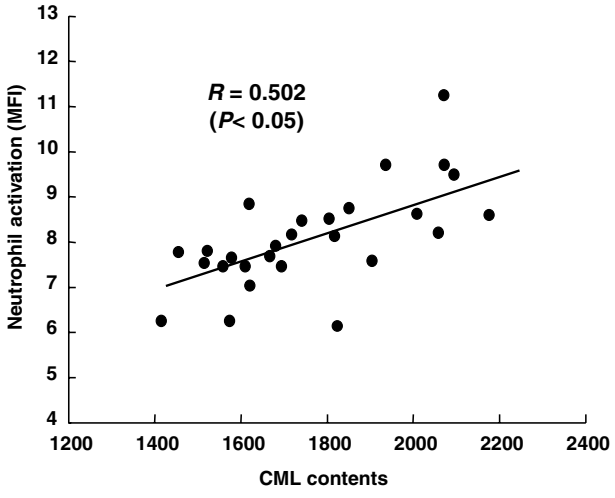

Fig. 3. *Relationship between neutrophil activation and CML-modified HSA (*n = *27,* r = *0.502,* p < *0.05).*

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.

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.