

## **beta 2-Microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis.**

T Miyata, ... , K Maeda, T Kinoshita

*J Clin Invest.* 1993;92(3):1243-1252. <https://doi.org/10.1172/JCI116696>.

### Research Article

beta 2-Microglobulin (beta 2M) is a major constituent of amyloid fibrils in hemodialysis-associated amyloidosis, a complication of long-term hemodialysis patients. Amyloid fibril proteins were isolated from connective tissues forming carpal tunnels in hemodialysis patients with carpal tunnel syndrome. Two-dimensional polyacrylamide gel electrophoresis and Western blotting demonstrated that most of the beta 2M forming amyloid fibrils exhibited a more acidic pI value than normal beta 2M. This acidic beta 2M was also found in a small fraction of beta 2M in sera and urine from these patients, whereas heterogeneity was not observed in healthy individuals. We purified acidic and normal beta 2M from the urine of long-term hemodialysis patients and compared their physicochemical and immunochemical properties. Acidic beta 2M, but not normal beta 2M, was brown in color and fluoresced, both of which are characteristics of advanced glycation end products (AGEs) of the Maillard reaction. Immunochemical studies showed that acidic beta 2M reacted with anti-AGE antibody and also with an antibody against an Amadori product, an early product of the Maillard reaction, but normal beta 2M did not react with either antibody. Incubating normal beta 2M with glucose in vitro resulted in a shift to a more acidic pI, generation of fluorescence, and immunoreactivity to the anti-AGE antibody. The beta 2M forming amyloid fibrils also reacted with anti-AGE antibody. These data [...]

**Find the latest version:**

<https://jci.me/116696/pdf>



# $\beta_2$ -Microglobulin Modified with Advanced Glycation End Products Is a Major Component of Hemodialysis-associated Amyloidosis

Toshio Miyata,\*\* Osamu Oda,<sup>§</sup> Reiko Inagi,<sup>||</sup> Yoshiyasu Iida,\* Norie Araki,\*\* Norio Yamada,\* Seikoh Horiuchi,\*\* Naoyuki Taniguchi,<sup>†</sup> Kenji Maeda,\* and Taroh Kinoshita<sup>‡</sup>

\*Department of Internal Medicine, The Branch Hospital, Nagoya University School of Medicine, Nagoya 461; <sup>‡</sup>Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Osaka 565; <sup>§</sup>The Bio-Dynamics Research Institute, Nagoya Memorial Hospital, Nagoya 468; Departments of <sup>||</sup>Bacteriology and <sup>†</sup>Biochemistry, Osaka University Medical School, Osaka 565; \*\*Department of Biochemistry, Kumamoto University School of Medicine, Kumamoto 860, Japan

## Abstract

$\beta_2$ -Microglobulin ( $\beta_2$ M) is a major constituent of amyloid fibrils in hemodialysis-associated amyloidosis, a complication of long-term hemodialysis patients. Amyloid fibril proteins were isolated from connective tissues forming carpal tunnels in hemodialysis patients with carpal tunnel syndrome. Two-dimensional polyacrylamide gel electrophoresis and Western blotting demonstrated that most of the  $\beta_2$ M forming amyloid fibrils exhibited a more acidic pI value than normal  $\beta_2$ M. This acidic  $\beta_2$ M was also found in a small fraction of  $\beta_2$ M in sera and urine from these patients, whereas heterogeneity was not observed in healthy individuals. We purified acidic and normal  $\beta_2$ M from the urine of long-term hemodialysis patients and compared their physicochemical and immunochemical properties. Acidic  $\beta_2$ M, but not normal  $\beta_2$ M, was brown in color and fluoresced, both of which are characteristics of advanced glycation end products (AGEs) of the Maillard reaction. Immunochemical studies showed that acidic  $\beta_2$ M reacted with anti-AGE antibody and also with an antibody against an Amadori product, an early product of the Maillard reaction, but normal  $\beta_2$ M did not react with either antibody. Incubating normal  $\beta_2$ M with glucose in vitro resulted in a shift to a more acidic pI, generation of fluorescence, and immunoreactivity to the anti-AGE antibody. The  $\beta_2$ M forming amyloid fibrils also reacted with anti-AGE antibody. These data provided evidence that AGE-modified  $\beta_2$ M is a dominant constituent of the amyloid deposits in hemodialysis-associated amyloidosis. (*J. Clin. Invest.* 1993; 92:1243–1252.) **Key words:**  $\beta_2$ -microglobulin • hemodialysis-associated amyloidosis • carpal tunnel syndrome • advanced glycation end products • long-term hemodialysis patient

## Introduction

Hemodialysis-associated amyloidosis (HAA)<sup>1</sup> is a common complication among long-term hemodialysis patients (1). Car-

Address correspondence to Dr. Toshio Miyata, Department of Internal Medicine, The Branch Hospital, Nagoya University School of Medicine, 1-1-20 Daiko-minami Higashi-ku, Nagoya 461, Japan.

Received for publication 25 February 1993 and in revised form 20 April 1993.

1. *Abbreviations used in this paper:* AGEs, advanced glycation end products;  $\beta_2$ M,  $\beta_2$ -microglobulin; CBB, Coomassie brilliant blue; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; CTS, carpal tunnel syndrome; HAA, hemodialysis-associated amyloidosis; PB, sodium phosphate buffer; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

*J. Clin. Invest.*

© The American Society for Clinical Investigation, Inc.

0021-9738/93/09/1243/10 \$2.00

Volume 92, September 1993, 1243–1252

pal tunnel syndrome (CTS), erosive arthropathy, and lytic bone lesions are among the most distinctive clinical features resulting from HAA. Recent biochemical and immunohistological studies have demonstrated that  $\beta_2$ -microglobulin ( $\beta_2$ M) is a major protein constituent of the amyloid fibrils in HAA (2–4). The pathological role of  $\beta_2$ M in HAA, however, remains to be determined. It is well known that the serum  $\beta_2$ M level is markedly higher (usually > 30-fold) in these patients compared with healthy individuals, but there is no statistical correlation between its serum concentration and the occurrence of HAA (5), thereby suggesting that the pathogenesis of HAA is not accounted for merely by an increase in the serum  $\beta_2$ M level. Thus, despite the fact that the prolonged elevation of serum  $\beta_2$ M level is obviously one of the important factors of HAA, it has been suggested that some unknown factor(s) is involved in the pathogenesis (5, 6).

Incubating proteins with glucose leads, through the early products such as Schiff's base and Amadori adducts, to advanced glycation end products (AGEs) (7, 8); this is referred to as the Maillard reaction. Several lines of evidence have emphasized a potential role for AGEs in the pathogenesis of diabetic complications and aging (9–11). AGE proteins are chemotactic for human monocytes (9) and stimulate macrophages in situ via AGE receptors to secrete inflammatory cytokines such as TNF- $\alpha$  and IL-1 (12–14). AGE proteins are also known to stimulate mesangial cells to produce fibronectin (15). Recent studies have demonstrated the in vivo presence of AGEs in human lens crystallin (16) and human hemoglobin (11). Although it is not conclusive that an AGE product(s) is present in serum, one investigator found that serum AGE levels are four times higher in diabetic patients undergoing hemodialysis than in normal individuals (17).

To elucidate the mechanism of HAA, we have been characterizing  $\beta_2$ M obtained from long-term hemodialysis patients with CTS. During these studies, we observed that  $\beta_2$ M from amyloid deposits from patients with CTS migrated upon electrophoresis to a more acidic position (referred to as acidic  $\beta_2$ M) than the normal counterpart, and that this isoform was detectable in sera and urine of these patients, but not in healthy individuals. We then isolated the acidic  $\beta_2$ M from the urine of long-term hemodialysis patients and analyzed it physicochemically and immunochemically. The results indicated that the acidic  $\beta_2$ M is generated by modification with AGEs, implicating a potential link of AGE-modified  $\beta_2$ M to a pathogenesis of HAA.

## Methods

*Isolation of amyloid fibril proteins.* The connective tissues forming carpal tunnels were obtained from surgical operation in three hemodialysis patients with CTS (Table I, group I). Amyloid fibril protein was then extracted from the connective tissues according to the method of

Table I. Profile of Subjects Examined in this Study\*

Group	Status	Sex		Age yr	Cause of renal failure	Serum creatinine mg/dl	Serum $\beta_2$ M $\mu$ g/ml	Duration of hemodialysis yr
		Male	Female					
I (n = 3)	Hemodialysis patients with carpal tunnel syndrome	2	1	51.3 $\pm$ 5.7	Chronic glomerulonephritis	13.3 $\pm$ 0.2	40.3 $\pm$ 0.7	17.33 $\pm$ 2.86
II (n = 5)	Hemodialysis patients without CTS	3	2	43.4 $\pm$ 3.8	Chronic glomerulonephritis	11.4 $\pm$ 1.5	33.1 $\pm$ 4.5	8.00 $\pm$ 0.94
III (n = 3)	Healthy individuals	2	1	41.7 $\pm$ 5.7	—	0.8 $\pm$ 0.1	1.3 $\pm$ 0.1	—

\* The results are expressed as the mean $\pm$ SD.

Pras et al. (18) and identified by Congo-red staining. The extracted proteins were lyophilized and solubilized in a minimum volume of water containing 2% 3-([3-cholamidopropyl]-dimethylammonio)-1-propane-sulfonate (CHAPS) and 8 M urea. The protein concentration was determined using protein assay reagents (Bio-Rad Laboratories, Richmond, CA) with HSA as the standard.

**Sera and urine.** Fresh serum and urine samples were obtained from non diabetic hemodialysis patients who had given informed consent (Table I, groups I and II). These patients had been undergoing regular hemodialysis using a cuprophane dialyzer and a dialysate containing 30 mEq/liter of bicarbonate and 8 mEq/liter of acetate. None of them had any complications at the time of sample collection. Fresh serum and urine samples were also obtained from healthy individuals (Table I, group III). Serum levels of  $\beta_2$ M were determined immunologically according to Berggård et al. (19). Urine samples were lyophilized after overnight dialysis against deionized water.

**Two-dimensional PAGE (2D-PAGE) and SDS-PAGE.** 2D-PAGE in the presence of SDS was performed by the method of O'Farrell (20) with some modifications. Briefly, isoelectric focusing in the first dimension was performed in a gel column (0.2  $\times$  6.0 cm) containing 0.4% of pH 3.5–10, and 0.1% of pH 3.5–5.0 ampholines (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden), 2% CHAPS, and 8 M urea. The electrophoresis in the second dimension was performed in a slab gel containing a gradient 8–17% polyacrylamide, 1% SDS, and 8 M urea. SDS electrophoresis in a 15–25% gradient or a 15% polyacrylamide slab gel was performed according to the method of Laemmli (21) under reducing conditions.

**Immunochemical analyses.** Immunoblots were performed using anti- $\beta_2$ M, anti-AGE, and anti-Amadori product antibodies as follows. After 2D- or SDS-PAGE, one gel was stained with Coomassie brilliant blue (CBB), and the other was electrophoretically transblotted to a nitrocellulose membrane (Amersham International, Buckinghamshire, UK). The membrane was incubated then with blocking reagents and reacted with anti- $\beta_2$ M rabbit IgG (anti- $\beta_2$ M) (Dakopatts, Glostrup, Denmark) or anti-AGE antibody (22). After washing, the membrane was further reacted with peroxidase-conjugated goat anti-rabbit IgG (Miles Laboratories Inc., Kankakee, IL), and then with 0.2 mM 3,3'-diaminobenzidine solution containing 0.018% H<sub>2</sub>O<sub>2</sub>. After 2D-PAGE and immunoblotting with anti- $\beta_2$ M antibody, the amount of the acidic isoform of  $\beta_2$ M was determined by densitometry using PDQUEST of The Discovery Series (pdi, Huntington Station, NY).

To detect Amadori products, samples were reduced with NaBH<sub>4</sub> (23) and run on a 15% SDS-PAGE slab gel under reducing conditions. The proteins in the gels were transblotted to a polyvinylidene difluoride membrane (Japan Millipore Ltd., Tokyo, Japan) and reacted with polyclonal anti-Amadori product antibody. The membrane was then processed as described above. The anti-Amadori product antibody used in the present study was raised in rabbits by immunizing with hexitolysine-keyhole limpet hemocyanin in complete adjuvant and purified by ammonium sulfate fractionation, DEAE cellulose chromatography, and affinity chromatography on hexitolysine-ovalbumin conjugated

to cyanogen bromide-activated Sepharose. The antibody reacts with the reduced form of the Amadori product,  $\epsilon$ -(1-deoxyhexitolyl)-lysine, but not with glucose or nonreduced form of the Amadori product. After reducing with NaBH<sub>4</sub>, various glycosylated proteins, including glycosylated RNase, glycosylated superoxide dismutase, glycosylated BSA and HSA, but not nonglycosylated proteins, react with this antibody (Taniguchi N., et al., manuscript in preparation).

An ELISA was performed as described previously (16). In brief, each well of a 96-well microtiter plate was incubated for 1 h with 0.1 ml of sample in 5 mM carbonate buffer (pH 9.6) and washed three times with PBS containing 0.05% Tween 20 (buffer A). Each well was blocked with 0.5% gelatin, washed with buffer A, and reacted with 0.1

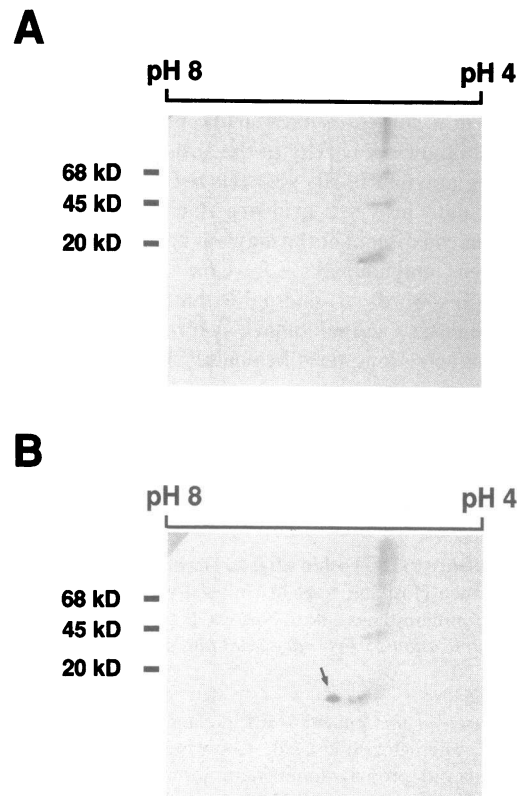
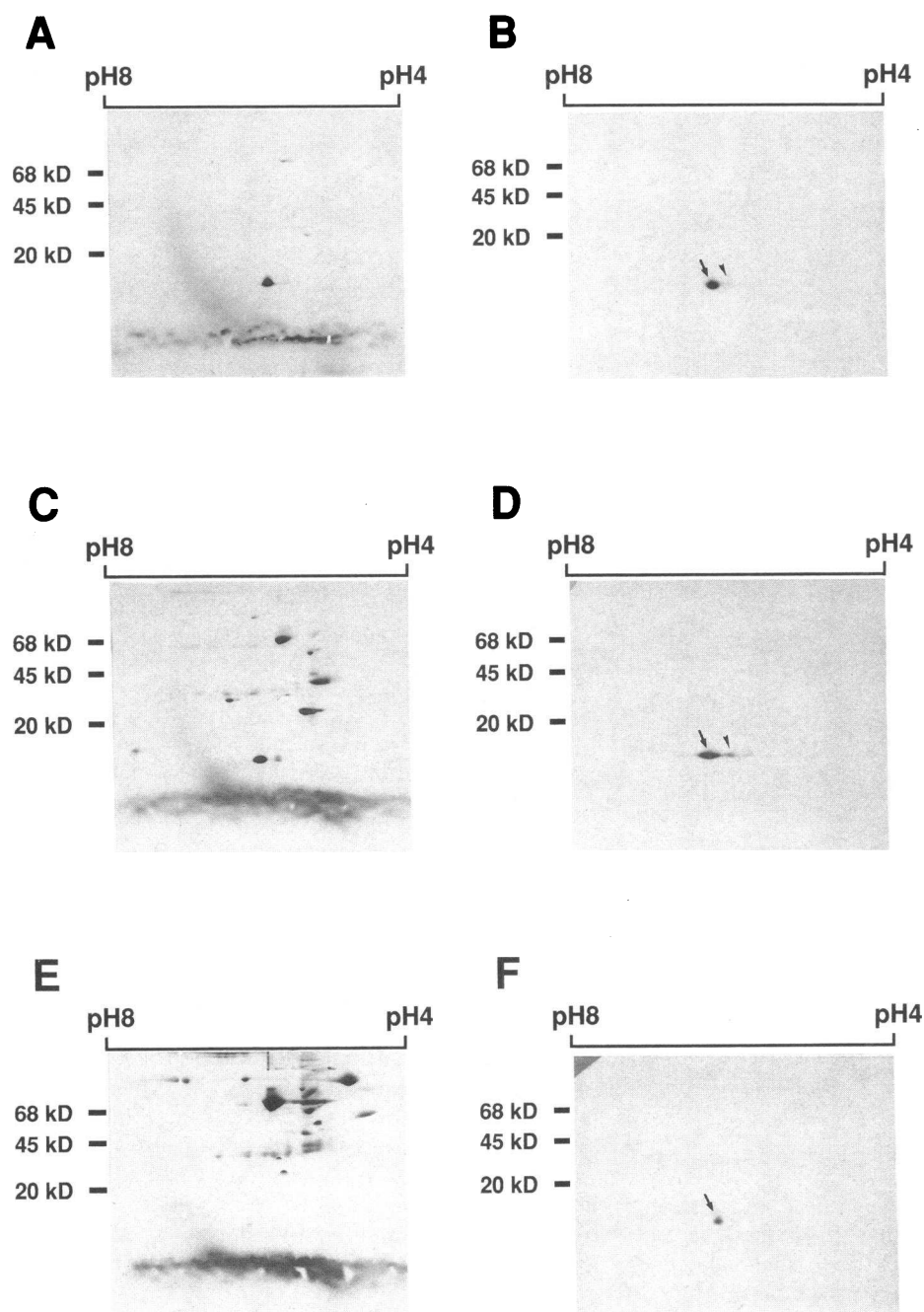


Figure 1. Amyloid fibril proteins from the connective tissues forming carpal tunnels in long-term hemodialysis patients with CTS. After amyloid fibril proteins (1  $\mu$ g) were resolved by 2D-PAGE, the gels were immunoblotted with anti- $\beta_2$ M antibody (A). Amyloid fibril proteins (1  $\mu$ g) were mixed with purified normal  $\beta_2$ M (2  $\mu$ g), followed by 2D-PAGE and immunoblotting with anti- $\beta_2$ M antibody (B). The arrow indicates the spot corresponding to normal  $\beta_2$ M.



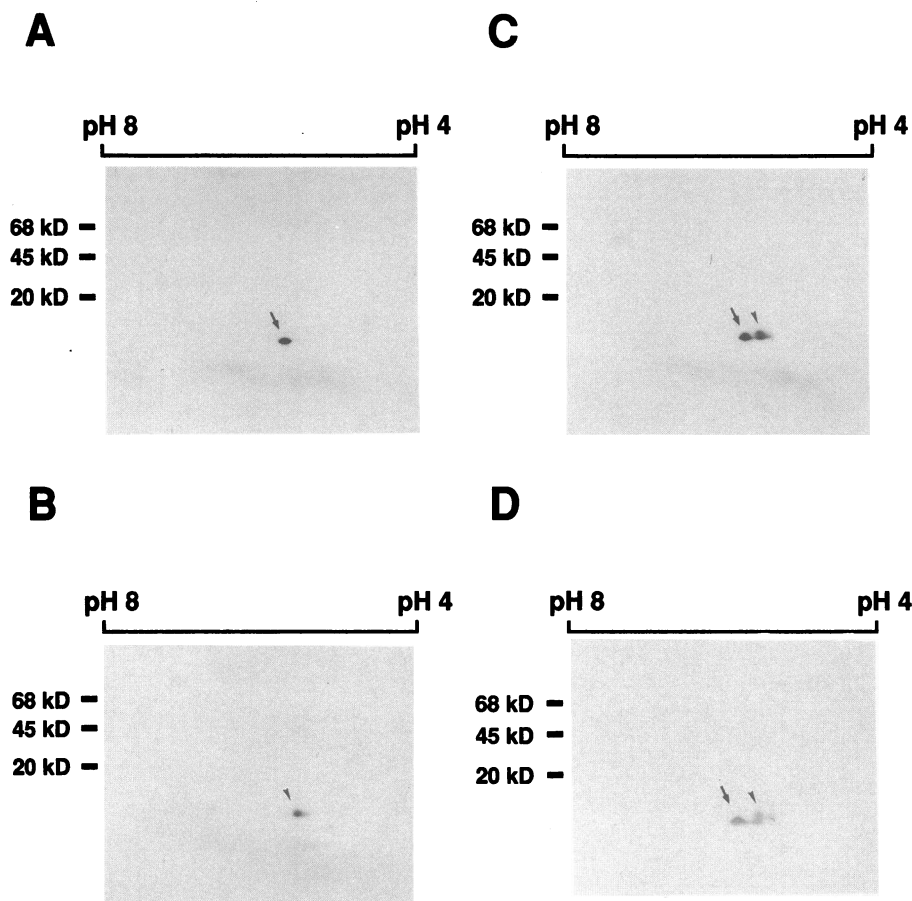
**Figure 2.** The presence of acidic  $\beta_2$ M in sera and urine of hemodialysis patients with CTS. Partially purified  $\beta_2$ M (5  $\mu$ g) from serum (*A* and *B*) and urine proteins (30  $\mu$ g) (*C* and *D*) of hemodialysis patients with CTS and urine proteins (30  $\mu$ g) of normal individuals (*E* and *F*) were resolved by 2D-PAGE. Gels were stained with CBB (*A*, *C*, and *E*) or immunoblotted with anti- $\beta_2$ M antibody (*B*, *D*, and *F*). The spots corresponding to acidic  $\beta_2$ M and normal  $\beta_2$ M are indicated by arrowheads and arrows, respectively.

ml of anti-AGE antibody. The wells were then washed with buffer A, incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA), and reacted with avidin-biotin horseradish peroxidase complexes, and then with 1,2-phenylenediamine dihydrochloride. The reaction was terminated with 1 M sulfuric acid, and the absorbance at 492 nm was measured on a micro-ELISA plate reader.

**Purification of normal  $\beta_2$ M and acidic  $\beta_2$ M.** Lyophilized urine samples from long-term dialysis patients (group II) (615 mg) were dissolved in 40 ml of 10 mM sodium phosphate buffer, pH 7.2 (PB). After removing the insoluble precipitates by centrifugation at 6,000 *g* for 15 min, the supernatants were applied to a DEAE-Sephadex A-50 column (5.0  $\times$  40 cm) (Pharmacia LKB Biotechnology Inc.) equilibrated with 20 mM PB. The column was washed with 1,000 ml of 20 mM PB, and proteins were eluted stepwisely with 100 ml each of the same buffer containing 50 mM NaCl and 75 mM NaCl. The fractions (15 ml) were monitored by 2D-PAGE and immunoblotting with anti- $\beta_2$ M antibody. Normal and acidic  $\beta_2$ M were eluted with 50 and 75 mM NaCl, respectively. The eluates were collected and lyophilized after dialysis against

deionized water and then dissolved in 10 mM PB. The normal  $\beta_2$ M fraction was purified by gel filtration on a Sephacryl S-100 column (2.6  $\times$  90 cm) (Pharmacia LKB Biotechnology Inc.). The acidic  $\beta_2$ M fraction was similarly gel filtrated on Sephacryl S-100 and further fractionated on a DEAE-Sephadex A-50 column (2.0  $\times$  30 cm) equilibrated with 10 mM PB, pH 8.0. The purified form was eluted with a linear gradient of 400 ml of 50–75 mM NaCl in the same buffer. Finally, 55 mg and 980  $\mu$ g of normal and acidic  $\beta_2$ M, respectively, were obtained. The fluorescence spectra of the purified proteins were measured in a fluorescence spectrophotometer (model RF540; Shimadzu, Kyoto, Japan) (24). Acid hydrolysates of purified  $\beta_2$ M were analyzed on a high speed amino acid analyzer (model L-8500; Hitachi Ltd., Tokyo, Japan) using an ion-exchange column.

**In vitro preparation of AGE-modified proteins.** AGE-modified  $\beta_2$ M was prepared in vitro by the method of Vlassara et al. (12) with some modifications. Briefly, 2 mg/ml of purified normal  $\beta_2$ M was incubated at 37°C for 30 d with or without 0.1 M D-glucose (Wako Pure Chemicals, Tokyo, Japan) in 100 mM PB containing 200 U/ml penicillin, 80



**Figure 3.** Acidic and normal  $\beta_2$ M purified from the urine of long-term hemodialysis patients. Purification procedures are described in Methods. 2  $\mu$ g of normal  $\beta_2$ M (A), acidic  $\beta_2$ M (B), and an equimolar combination of both (C) were resolved by 2D-PAGE and stained with CBB. The gel containing an equimolar combination of both was also immunoblotted with anti- $\beta_2$ M antibody (D). The spots corresponding to acidic and normal  $\beta_2$ M are indicated by arrowheads and arrows, respectively.

$\mu$ g/ml gentamicin, and 1.5 mM PMSF. AGE-modified  $\beta_2$ M was characterized by fluorospectrometry and 2D-PAGE. AGE-modified BSA was also prepared as described previously (22).

## Results

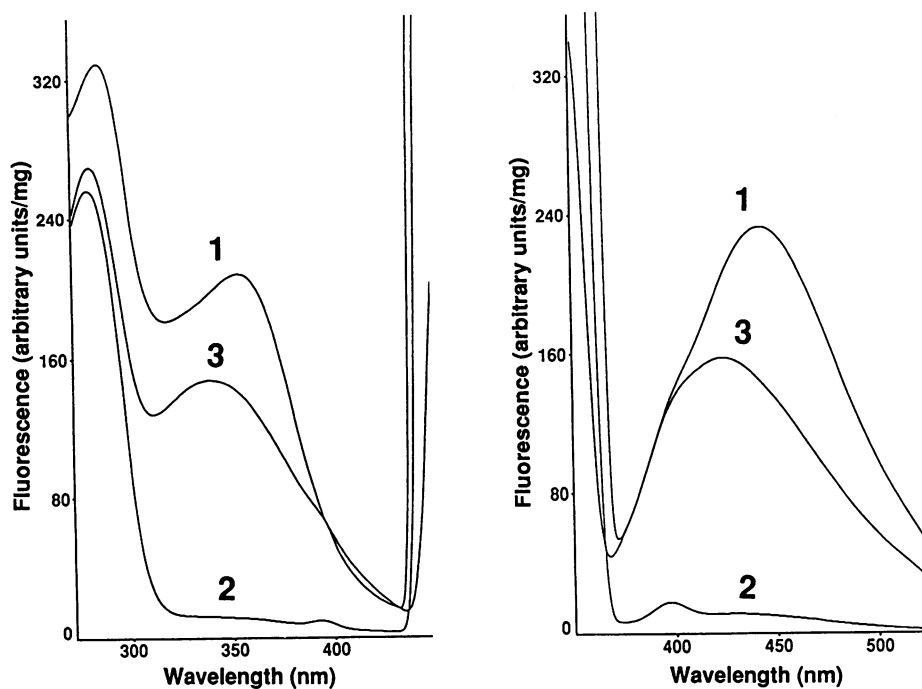
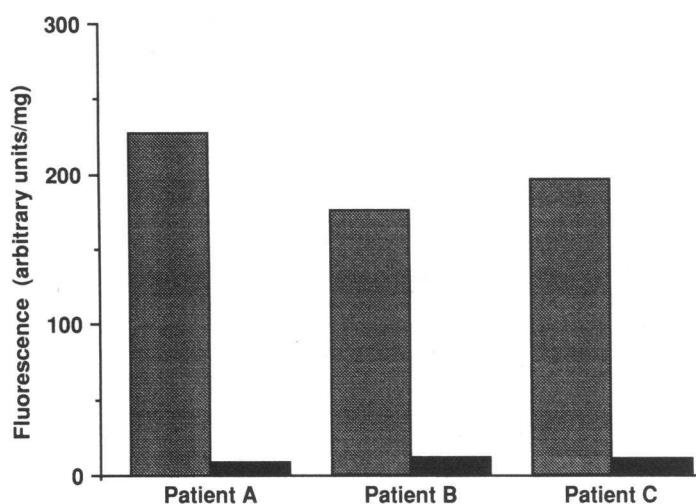
**The presence of acidic  $\beta_2$ M in HAA.** Amyloid fibril proteins were isolated from connective tissues forming carpal tunnels in a hemodialysis patient with CTS. The 2D-PAGE analysis of this material demonstrated three major bands stained with CBB (data not shown). Immunoblots of these proteins with anti- $\beta_2$ M antibody showed that all these bands were reactive to the antibody, suggesting that  $\beta_2$ M is present in tissue amyloid deposits as monomers, dimers, and polymers (Fig. 1 A). When a mixture of the amyloid fibril proteins and purified normal human  $\beta_2$ M was subjected to 2D-PAGE followed by immunoblotting with anti- $\beta_2$ M antibody, the  $\beta_2$ M in amyloid deposits migrated to a more acidic position than normal  $\beta_2$ M (Fig. 1 B). It was also evident that the acidic  $\beta_2$ M represents an exclusive isoform of  $\beta_2$ M in the amyloid fibril tissues. Further analyses of amyloid fibril proteins from two other hemodialysis patients with CTS (group I) revealed the same results, supporting the contention that acidic  $\beta_2$ M is a major protein in amyloid fibril tissues from patients with CTS.

To determine whether acidic  $\beta_2$ M is present in serum, the  $\beta_2$ M fraction was partially purified from sera of the same patients (group I) by gel filtration with Sephacryl S-200. The partially purified  $\beta_2$ M fraction exhibited many bands with a molecular mass ranging from 10 to 40 kD on 2D-PAGE (Fig. 2

A). Immunoblotting with anti- $\beta_2$ M antibody revealed two bands: a major band and a more acidic minor band (Fig. 2 B). This minor band corresponded to acidic  $\beta_2$ M as judged by its electrophoretic mobility and it was not detected in the partially purified  $\beta_2$ M fraction from healthy individuals (group III) (data not shown). These results indicate that acidic  $\beta_2$ M is present in the sera of hemodialysis patients with CTS but is totally absent from the sera of healthy individuals. All the  $\beta_2$ M fractions partially purified from the sera of long-term hemodialysis patients without CTS (group II) also possessed acidic  $\beta_2$ M (data not shown). Assuming that normal and acidic  $\beta_2$ M behaved in a similar fashion throughout the purification and blotting procedures, the densitometric analyses of the two isoforms from these patients showed that acidic  $\beta_2$ M accounted for  $\sim 10\%$  of the serum  $\beta_2$ M both in groups I and II.

Acidic  $\beta_2$ M was also found in urine from long-term hemodialysis patients. The urine collected from group I patients was resolved by 2D-PAGE (Fig. 2 C) and immunoblotting with anti- $\beta_2$ M antibody. As shown in Fig. 2 D, acidic  $\beta_2$ M was detected next to the major band of normal  $\beta_2$ M. Densitometry showed that acidic  $\beta_2$ M constituted  $\sim 11\%$  of the total urinary  $\beta_2$ M. Acidic  $\beta_2$ M was also found in all the urine from patients in group II (data not shown). As with serum, however, acidic  $\beta_2$ M was not found in the urine from normal individuals (Fig. 2 F).

**Purification of acidic  $\beta_2$ M from the urine of long-term hemodialysis patients.** The above finding, that acidic  $\beta_2$ M, a major isoform of  $\beta_2$ M in amyloid fibril tissues, occurred in both the sera and urine of long-term hemodialysis patients, indicates

**A****B**

**Figure 4.** Fluorescence spectra of acidic and normal  $\beta_2M$ . The fluorescence spectra of purified acidic and normal  $\beta_2M$  were measured at a concentration of 0.26 mg/ml (see Methods). (A) Typical excitation fluorescence spectra for emission at 450 nm (left) and emission spectra upon excitation at 360 nm (right). Line 1, acidic  $\beta_2M$ ; line 2, normal  $\beta_2M$ ; line 3, normal  $\beta_2M$  incubated with 0.1 M glucose at 37°C for 30 d. (B) The maximum emission fluorescence intensities at 450 nm upon excitation at 360 nm of acidic (hatched bar) and normal  $\beta_2M$  from three long-term hemodialysis patients (patients A, B, and C in group II).

that acidic  $\beta_2M$  is linked to the pathogenesis of HAA. Monitoring their immunoreactivity to anti- $\beta_2M$  antibody, we purified both acidic and normal  $\beta_2M$  from the urine of three hemodialysis patients (patients A, B, and C in group II) by a combination of DEAE-Sephadex and Sephacryl S-100 chromatography (Fig. 3, A and B). When the purified acidic and normal  $\beta_2M$  were coelectrophoresed, they migrated as two separate bands, demonstrated by both protein staining (Fig. 3 C) and immunoblotting with anti- $\beta_2M$  antibody (Fig. 3 D), confirming that acidic  $\beta_2M$  is distinct from normal  $\beta_2M$ . Protein sequencing demonstrated that the 20 amino-terminal residues of these two  $\beta_2M$  isoforms were identical. The sequences were also identical with that reported for normal serum  $\beta_2M$  (25). The amino acid substitution from asparagine to aspartic acid as reported previ-

ously (26) did not occur in these samples. The pI for the purified acidic  $\beta_2M$  was 5.5–5.7 (Fig. 3). It is thus likely that the acidic form of  $\beta_2M$  is generated by the posttranslational modification of proteins rather than being derived from different amino acid sequences.

*Acidic  $\beta_2M$  is AGE-modified.* One of the most characteristic features of purified acidic  $\beta_2M$  that we detected was fluorescence. Fig. 4 A shows typical excitation (left) and emission (right) fluorescence spectra of acidic (line 1) and normal  $\beta_2M$  (line 2) purified from the urine of a long-term hemodialysis patient. The acidic  $\beta_2M$  showed intense fluorescence, with a major excitation maximum at 360 nm for emission at 450 nm and a major emission maximum at 450 nm upon excitation at 360 nm. On the other hand, normal  $\beta_2M$  did not fluoresce.

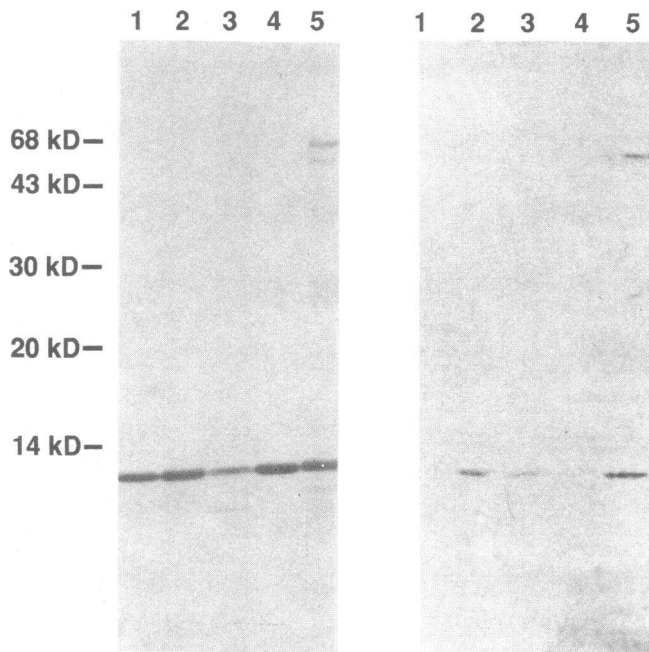
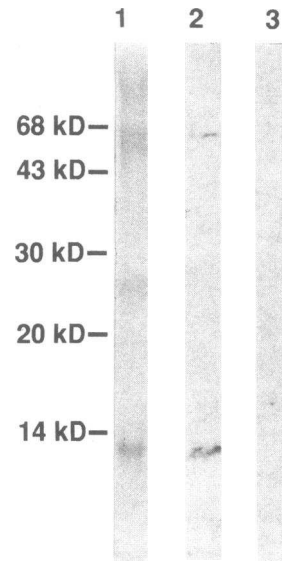
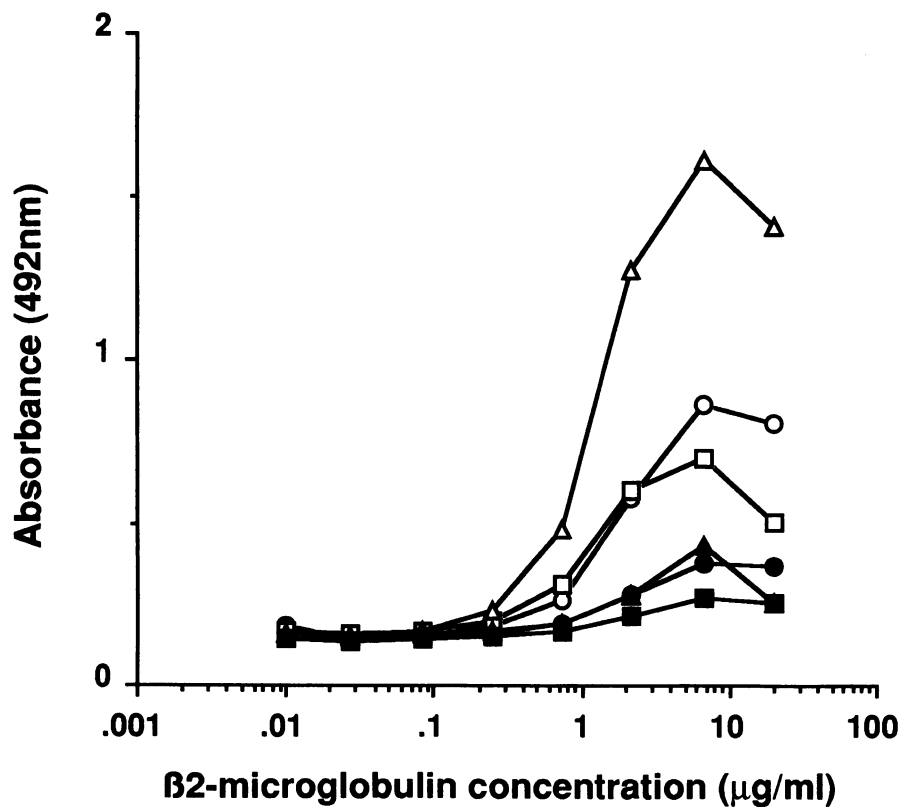
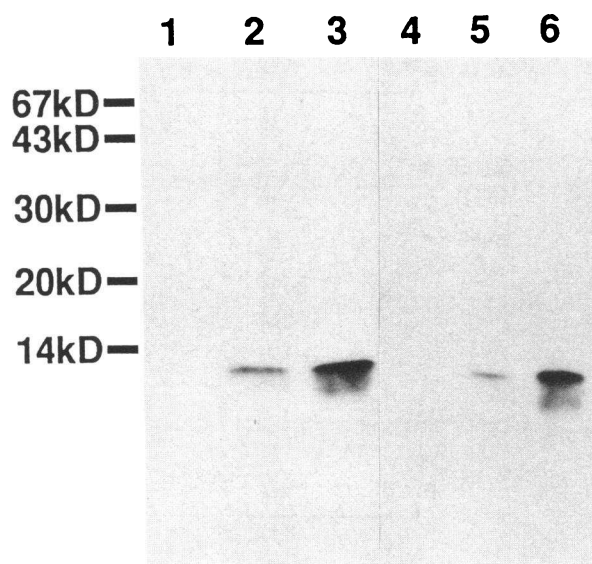
**A****C****B**

Figure 5. Immunochemical reaction of acidic  $\beta_2\text{M}$  to anti-AGE antibody. (A) Acidic and normal  $\beta_2\text{M}$  ( $2 \mu\text{g}$  each) purified from the urine of long-term hemodialysis patients were resolved by 15–25% SDS-PAGE under reducing conditions. The gels were then stained with CBB (*left*) or immunoblotted with anti-AGE antibody (*right*). Lane 1, normal  $\beta_2\text{M}$  from patient A; lane 2, acidic  $\beta_2\text{M}$  from patient A; lane 3, normal  $\beta_2\text{M}$



**Figure 6.** The presence of Amadori products in acidic  $\beta_2$ M. The acidic or normal  $\beta_2$ M purified from the urine of long-term hemodialysis patients (5  $\mu$ g each) were reduced by NaBH<sub>4</sub>, run on 15% SDS-PAGE, and then immunoblotted with anti-Amadori product antibody (see Methods). Lane 1, normal  $\beta_2$ M; lane 2, acidic  $\beta_2$ M from patient A; lane 3, acidic  $\beta_2$ M from patient B; lane 4, normal  $\beta_2$ M after a 10-d incubation without glucose at 37°C; lane 5, normal  $\beta_2$ M after a 3-d incubation with 0.1 M glucose at 37°C; and lane 6, normal  $\beta_2$ M after a 10-d incubation with 0.1 M glucose at 37°C.

When the maximum emission fluorescence intensities at 450 nm upon excitation at 360 nm were compared, acidic  $\beta_2$ M purified from the urine of the three patients fluoresced significantly. On the other hand, normal  $\beta_2$ M from the same patients did not exhibit fluorescence (Fig. 4 B). Since chemical modification of proteins by AGEs of the Maillard reaction results in fluorescent activity (24, 27), we examined whether acidic  $\beta_2$ M possesses AGEs, by means of an immunochemical approach using an anti-AGE antibody. A previous study has demonstrated that this antibody specifically recognizes AGE structures but not the early products such as Schiff's base and Amadori rearrangement products (22). As shown in Fig. 5 A, acidic  $\beta_2$ M reacted with the anti-AGE antibody whereas the immunoreaction was not observed with normal  $\beta_2$ M. The binding of acidic  $\beta_2$ M to the anti-AGE antibody did not reflect the nonspecific binding because, under the present conditions, acidic  $\beta_2$ M did not react with other antibodies, such as anti-HSA antibody and anti-complement factor D antibody (data not shown). Thus, the result shown in Fig. 5 A indicates the presence of AGEs in acidic  $\beta_2$ M. This notion was supported by the results of the ELISA (Fig. 5 B). Acidic  $\beta_2$ M but not normal  $\beta_2$ M significantly reacted with the antibody. This immunoreaction was completely inhibited by an excess of AGE-modified BSA (data not shown). Amyloid fibril proteins extracted from the

connective tissues forming carpal tunnels in long-term hemodialysis patients also reacted with anti-AGE antibody. All the bands stained with CBB reacted with anti-AGE antibody but disappeared when an excess of AGE-BSA was present during the immunoreaction, indicating that  $\beta_2$ M forming amyloid fibrils contains AGEs (Fig. 5 C).

The presence of early products in these  $\beta_2$ M preparations was also identified using the polyclonal anti-Amadori product antibody. Immunoblotting showed that acidic  $\beta_2$ M also reacted with the anti-Amadori antibody (Fig. 6, lanes 2 and 3). No immunoreactivity was observed with normal  $\beta_2$ M. However, incubating normal  $\beta_2$ M with glucose resulted in a time-dependent increase in the reactivity to the antibody (Fig. 6, lanes 4–6). The presence of Amadori products in acidic  $\beta_2$ M was also supported by amino acid analysis. Amino acid analysis of acidic and normal  $\beta_2$ M purified from the urine of two long-term hemodialysis patients revealed a hexitollysine peak in the former, but not in the latter (data not shown). These results indicated that  $\beta_2$ M in long-term hemodialysis patients reacts with glucose to form an Amadori product, which, upon further reaction *in vivo*, gives rise to AGE-modified  $\beta_2$ M.

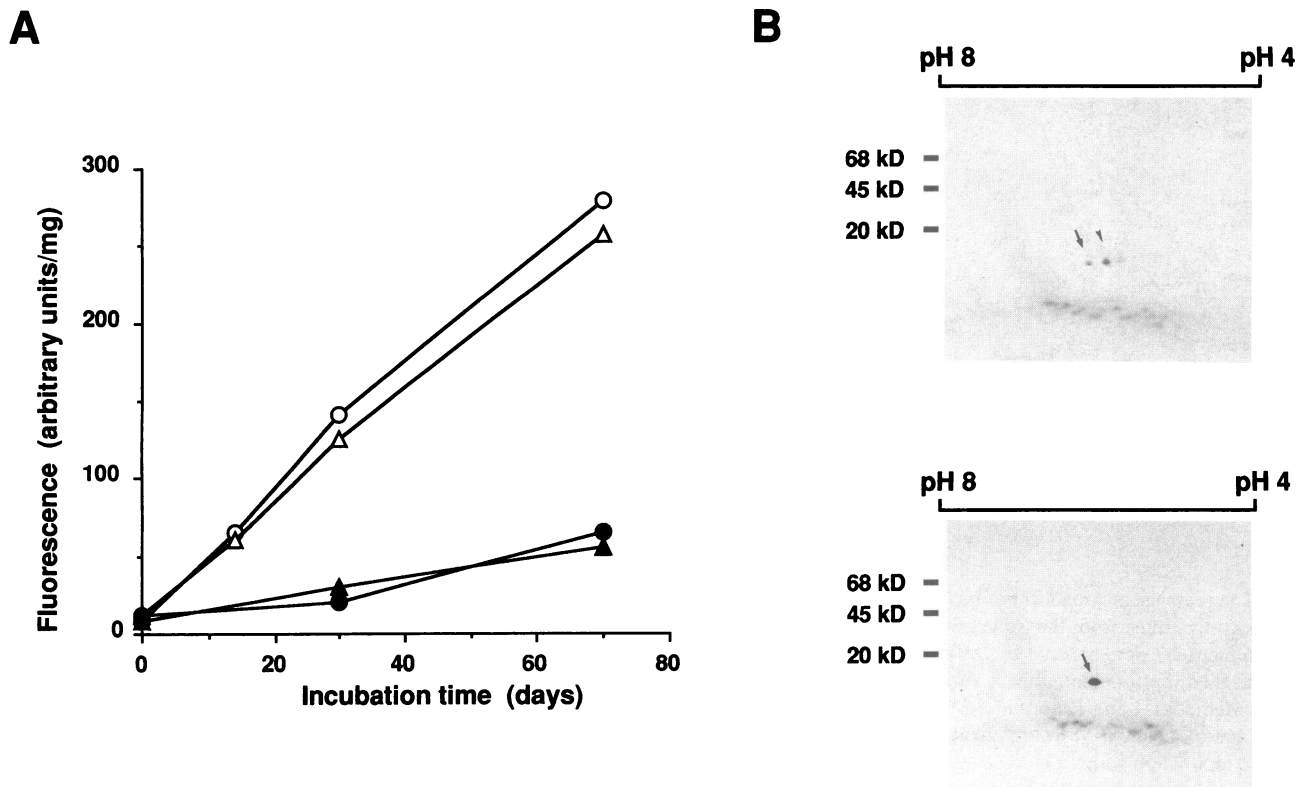
This notion was confirmed by the fact that normal  $\beta_2$ M incubated *in vitro* with glucose turned brown (data not shown) and the fluorescence increased with time (Fig. 7 A). The fluorescence spectra were similar to those of the purified acidic  $\beta_2$ M (Fig. 4 A, line 3). Furthermore, normal  $\beta_2$ M incubated with glucose reacted with anti-AGE antibody (Fig. 5 B). These properties are common among other AGE-proteins prepared *in vitro* such as AGE-BSA, AGE-HSA, and AGE-human hemoglobin (22). A major portion of  $\beta_2$ M (> 74% by densitometric quantification) shifted its electrophoretic mobility in 2D-PAGE to a position similar to that of acidic  $\beta_2$ M after a 30-d incubation with glucose (Fig. 7 B, *top*). In contrast, upon parallel incubation without glucose, acidic  $\beta_2$ M isoforms were virtually undetectable (Fig. 7 B, *bottom*).

## Discussion

This study provided evidence that  $\beta_2$ M forming amyloid fibrils in HAA is AGE modified. The two-dimensional electrophoresis of amyloid fibril proteins from patients with CTS and subsequent immunoblotting with anti- $\beta_2$ M antibody demonstrated that a major portion of  $\beta_2$ M forming amyloid fibrils exhibited unusual electrophoretic mobility. The  $\beta_2$ M forming amyloid fibrils had a more acidic pI value than normal  $\beta_2$ M. A small but significant fraction of  $\beta_2$ M in the serum and urine from the same long-term hemodialysis patients was also acidic whereas such heterogeneity was not observed in normal individuals. We purified  $\beta_2$ M from the urine of long-term hemodialysis patients to homogeneity and demonstrated that this acidic isoform resulted from AGE modification. First, the acidic  $\beta_2$ M purified from the urine of the patients was brown in color (data not shown) and fluoresced. Second, the purified acidic, but not the

(patient A) after a 30-d incubation with 0.1 M glucose at 37°C; lane 4, normal  $\beta_2$ M from patient B; and lane 5, acidic  $\beta_2$ M from patient B. (B) The presence of AGEs in acidic  $\beta_2$ M was also examined by ELISA using anti-AGE antibody. Acidic  $\beta_2$ M from patients A (○) and B (△), normal  $\beta_2$  from patients A (●) and B (▲), normal  $\beta_2$ M after a 30-d incubation with 0.1 M glucose at 37°C (□), and normal BSA (■). (C) Amyloid fibril proteins (1.5  $\mu$ g) from the connective tissues forming carpal tunnels in a long-term hemodialysis patient with CTS (15–25% SDS-PAGE). Lane 1, a CBB staining; lane 2, an immunoblotting with anti-AGE antibody; lane 3, an immunoblotting with anti-AGE antibody in the presence of excess of AGE-BSA (0.2 mg/ml).





**Figure 7.** In vitro preparation of AGE-modified  $\beta_2$ M from normal  $\beta_2$ M. Purified normal  $\beta_2$ M (2 mg/ml) was incubated with 0.1 M D-glucose at 37°C (see Methods). (A) Time-dependent changes in the fluorescence of normal  $\beta_2$ M from patients A ( $\circ$ ) and B ( $\Delta$ ) when incubated with glucose and normal  $\beta_2$ M from patients A ( $\bullet$ ) and B ( $\blacktriangle$ ) incubated without glucose. The maximum emission fluorescence at 450 nm was measured upon excitation at 360 nm. (B) Typical 2D-PAGE profiles of normal  $\beta_2$ M (2  $\mu$ g) after a 30-d incubation with (top) or without glucose (bottom). Acidic and normal  $\beta_2$ M are indicated by arrowheads and arrows, respectively.

normal,  $\beta_2$ M reacted with anti-AGE antibody. Third,  $\beta_2$ M forming amyloid fibrils in hemodialysis patients with CTS also reacted with this antibody. Finally, incubating normal  $\beta_2$ M with glucose in vitro resulted in: (a) a shift to a more acidic pI, (b) generation of fluorescence, and (c) immunoreactivity to the anti-AGE antibody. These results indicate that the chemical modification of normal  $\beta_2$ M with AGEs generates acidic  $\beta_2$ M, the dominant isoform of  $\beta_2$ M in amyloid deposits of HAA.

Deamination has been proposed as a potential mechanism for the electrophoretic heterogeneity observed with  $\beta_2$ M in amyloid deposits (3) and serum (28). From the positive reactivity to anti-AGE antibody, however, it is more likely that the molecular basis for this heterogeneity is AGE modification. Through a series of chemical reactions of protein amino groups with a sugar aldehyde, AGE modification yields an increase in the net negative charge, leading to the electrophoretic heterogeneity. This was exemplified by human IgG, which migrated to a more acidic position on electrophoresis after a prolonged incubation with glucose (29). An acidic shift in the pI also occurred when BSA and HSA were modified by AGEs (Oda, O., et al., unpublished observations).

Previous histological studies have demonstrated that amyloid deposits are surrounded by macrophages and other inflammatory cells (6, 30). Since AGE proteins are chemotactic for human monocytes (9) and macrophages have receptors responsible for endocytic uptake of AGE-modified proteins (12, 13), AGE modification of  $\beta_2$ M forming amyloid deposits could ex-

plain the preferential localization of macrophages and other inflammatory cells to these deposits. In addition, AGE-modified HSA reportedly induces macrophages to synthesize and release TNF- $\alpha$  and IL-1 (14). This indicates that once AGE-modified  $\beta_2$ M in amyloid deposits is recognized by macrophages via AGE receptors, it would then stimulate the cells to secrete these inflammatory cytokines in situ. Those cytokines stimulate the release of collagenase from human synovial cells and fibroblasts (31) and bone resorption (32), both of which are involved in the pathogenesis of HAA. It is also possible that macrophage phagocytic activity in the vicinity of amyloid deposits may accelerate the formation of AGE products.

Several hypotheses have been postulated for the amyloidogenic process. They include a high serum  $\beta_2$ M concentration (33), proteolytic modification of  $\beta_2$ M (34), and complex formation of circulating  $\beta_2$ M with immunoglobulins (35). It is not known to what extent AGE modification correlates with amyloid fibril formation. Also unknown is the mechanism for recruiting AGE-modified  $\beta_2$ M into the amyloid fibrils. However, the present finding that AGE-modified  $\beta_2$ M is a major constituent of amyloid deposits suggests that AGE modification plays an active role in amyloidogenesis. Of particular interest from a pathological perspective, is the tendency of AGE proteins to cross-link and polymerize (7, 8, 36), implicating that AGE modification of  $\beta_2$ M leads to the polymerization of  $\beta_2$ M in amyloid deposits. The close association of AGE-modified proteins with matrix proteins such as collagen has also been emphasized (10, 15, 37).

Through a series of chemical rearrangements and dehydration reactions, some Amadori products are converted to AGEs, which eventually accumulate in long-lived proteins. The accumulation of AGE-modified proteins is either due to increased production during diseases such as diabetes mellitus or to a decreased removal rate in chronic renal failure (17). A previous study has indicated that the serum AGE levels among the small-molecular mass proteins (< 10 kD) are significantly higher than normal in non-diabetic patients undergoing hemodialysis (17). Considering the molecular mass of  $\beta_2$ M (12 kD), the appearance of AGE-modified  $\beta_2$ M in the sera and urine of long-term hemodialysis patients, but not in those of normal individuals, seems to be accounted for by the second mechanism (a decreased removal rate). A high serum concentration of  $\beta_2$ M in these patients (> 30-fold) compared with healthy individuals may be also related to a high level of AGE-modified  $\beta_2$ M in these patients. Further studies will be needed to clarify this speculation.

The reaction process of the early stages of the Maillard reaction (Schiff's base and Amadori products) is well understood. However, less is known about AGEs largely because of a lack of information about the chemical structure of AGEs. Determination of their chemical structures is essential to further elucidate the pathological significance of AGE-modified proteins. The following AGE moieties have been postulated: 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (38), pyrrole aldehyde (39), pentosidine (40), and crosslines (41). A previous immunochemical study has indicated the presence of a common AGE structure, because both polyclonal and monoclonal antibodies reacted with several AGEs from several proteins, lysine derivatives, and monoaminocarboxylic acids (22). That acidic  $\beta_2$ M purified from urine was reactive to the anti-AGE antibody suggests that AGE-modified proteins formed in vivo share an epitope in common with those modified in vitro. At present, no information is available about the chemical structure of the AGE moieties. Our preliminary mass spectrometric analysis revealed that a peak different from that of an Amadori adduct occurs in acidic  $\beta_2$ M, but not in normal  $\beta_2$ M, suggesting the presence of an AGE structure in acidic  $\beta_2$ M (Miyata T., et al., unpublished observation). Determination of this structure will shed light on the mechanism of AGE modification in vivo.

In conclusion, this is the first report, to our knowledge, that highlights the pathological role of AGE modification in arthropathic complications. A further study is needed to establish a correlation between serum AGE- $\beta_2$ M levels and HAA.

## Acknowledgments

We are indebted to Drs. Hisao Seo and Satoshi Ogawa for helpful discussion and Yuko Izuhara for excellent technical assistance.

## References

1. Drücke, T. B. 1991. Beta-2-microglobulin amyloidosis and renal bone disease. *Miner. Electrolyte Metab.* 17:261-272.
2. Gejyo, F., T. Yamada, S. Odani, Y. Nakagawa, M. Arakawa, T. Kunitomo, H. Kataoka, M. Suzuki, Y. Hirasawa, T. Shirahama, et al. 1985. A new form of amyloid protein associated with chronic hemodialysis was identified as  $\beta_2$ -microglobulin. *Biochem. Biophys. Res. Commun.* 129:701-706.
3. Gorevic, P. D., P. C. Munoz, T. T. Casey, C. R. DiRaimondo, W. J. Stone, F. C. Prelli, M. M. Rodrigues, M. D. Poulik, and B. Frangione. 1986. Polymerization of intact  $\beta_2$ -microglobulin in tissue causes amyloidosis in patients on chronic hemodialysis. *Proc. Natl. Acad. Sci. USA.* 83:7908-7912.
4. Shirahama, T., M. Skinner, A. S. Cohen, F. Gejyo, M. Arakawa, M. Suzuki, and Y. Hirasawa. 1985. Histochemical and immunohistochemical characterisa-

tion of amyloid associated with chronic hemodialysis as  $\beta_2$ -microglobulin. *Lab. Invest.* 53:705-709.

5. Gejyo, F., S. Odani, T. Yamada, N. Honma, H. Saito, Y. Suzuki, Y. Nakagawa, H. Kobayashi, Y. Maruyama, Y. Hirasawa, et al. 1986.  $\beta_2$ -microglobulin: a new form of amyloid protein associated with chronic hemodialysis. *Kidney Int.* 30:385-390.

6. Depierreux, M., M. Goldman, I. Fayt, C. Richard, J. Quintin, M. Dhaene, and J.-L. Van Herweghem. 1988. Osteoarticular amyloidosis associated with haemodialysis: an immunultrastructural study. *J. Clin. Pathol.* 41:158-162.

7. Brownlee, M., A. Cerami, and H. Vlassara. 1988. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* 318:1315-1321.

8. Baynes, J. W., and V. M. Monnier. 1989. The Maillard reaction in aging, diabetes and nutrition. *Prog. Clin. Biol. Res.* 304:1-410.

9. Kirshtein, M., J. Brett, S. Radoff, S. Ogawa, D. Stern, and H. Vlassara. 1990. Advanced protein glycosylation induces transendothelial human monocyte chemotaxis and secretion of platelet-derived growth factor: role in vascular disease of diabetes and aging. *Proc. Natl. Acad. Sci. USA.* 87:9010-9014.

10. Miyata, S., and V. M. Monnier. 1992. Immunohistochemical detection of advanced glycosylation end products in diabetic tissues using monoclonal antibody to pyrraline. *J. Clin. Invest.* 89:1102-1112.

11. Makita, Z., H. Vlassara, E. Rayfield, K. Cartwright, E. Friedman, R. Rodby, A. Cerami, and R. Bucala. 1992. Hemoglobin-AGE: a circulating marker of advanced glycosylation. *Science (Wash. DC).* 258:651-653.

12. Vlassara, H., M. Brownlee, and A. Cerami. 1985. High-affinity-receptor-mediated uptake and degradation of glucose-modified proteins: a potential mechanism for the removal of senescent macromolecules. *Proc. Natl. Acad. Sci. USA.* 82:5588-5592.

13. Vlassara, H., L. Moldawer, and B. Chan. 1989. Macrophage/monocyte receptor for nonenzymatically glycosylated proteins is upregulated by cachectin/tumor necrosis factor. *J. Clin. Invest.* 84:1813-1820.

14. Vlassara, H., M. Brownlee, K. R. Manogue, C. A. Dinarello, and A. Pasagian. 1988. Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science (Wash. DC).* 240:1546-1548.

15. Skolnik, E. Y., Z. Yang, Z. Makita, S. Radoff, M. Kirshtein, and H. Vlassara. 1991. Human and rat mesangial cell receptors for glucose-modified proteins: potential role in kidney tissue remodeling and diabetic nephropathy. *J. Exp. Med.* 174:931-939.

16. Araki, N., N. Ueno, B. Chakrabarti, Y. Morino, and S. Horiuchi. 1992. Immunohistochemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. *J. Biol. Chem.* 267:10211-10214.

17. Makita, Z., S. Radoff, E. J. Rayfield, Z. Yang, E. Skolnik, V. Delaney, E. A. Friedman, A. Cerami, and H. Vlassara. 1991. Advanced glycosylation end products in patients with diabetic nephropathy. *N. Engl. J. Med.* 325:836-842.

18. Pras, M., M. Schubert, D. Zucker-Franklin, A. Rimon, and E. C. Franklin. 1968. The characterization of soluble amyloid prepared in water. *J. Clin. Invest.* 47:924-933.

19. Berggård, I., and A. G. Bearn. 1968. Isolation and properties of a low molecular weight  $\beta_2$ -globulin occurring in human biological fluids. *J. Biol. Chem.* 243:4095-4103.

20. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of protein. *J. Biol. Chem.* 250:4007-4021.

21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.

22. Horiuchi, S., N. Araki, and Y. Morino. 1991. Immunohistochemical approach to characterize advanced glycation end products of the Maillard reaction. *J. Biol. Chem.* 266:7329-7332.

23. Cohen, M. P. 1986. Measurement: borohydride reduction. In *Diabetes and Protein Glycosylation-Measurement and Biologic Reductance*. M. P. Cohen, editor. Springer-Verlag, New York. 30-31.

24. Yang, Z., Z. Makita, Y. Hori, S. Brunelle, A. Cerami, P. Sehajpal, M. Suthanthiran, and H. Vlassara. 1991. Two novel rat liver membrane proteins that bind advanced glycosylation endproducts: relationship to macrophage receptor for glucose-modified proteins. *J. Exp. Med.* 174:515-524.

25. Güssow, D., R. Rein, I. Ginjaar, F. Hochstenbach, G. Seemann, A. Kottman, and H. L. Ploegh. 1987. The human  $\beta_2$ -microglobulin gene: primary structure and definition of the transcriptional unit. *J. Immunol.* 139:3132-3138.

26. Odani, H., R. Oyama, K. Titani, H. Ogawa, and A. Saito. 1990. Purification and complete amino acid sequence of novel  $\beta_2$ -microglobulin. *Biochem. Biophys. Res. Commun.* 168:1223-1229.

27. Monnier, V. M., and A. Cerami. 1981. Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. *Science (Wash. DC).* 211:491-493.

28. Ogawa, H., A. Saito, O. Oda, M. Nakajima, and T. G. Chung. 1988. Detection of novel  $\beta_2$ -microglobulin in the serum of hemodialysis patients and its amyloidogenic predisposition. *Clin. Nephrol.* 30:158-163.

29. Boekel, M. V., and H. Hoenders. 1990. In vitro and in vivo glycation of human IgG. In *The Maillard Reaction Advances in Life Science*. P. A. Finot,

- H. U. Aeschbacher, R. F. Hurrel, R. Liardon, editors. Birkhäuser Verlag, Basel, Switzerland. 431–436.
30. Theaker, J. M., A. E. G. Raine, A. J. Rainey, A. Heryet, A. Clark, and D. O. Oliver. 1987. Systemic amyloidosis of  $\beta_2$ -microglobulin type: a complication of long term haemodialysis. *J. Clin. Pathol.* 40:1247–1251.
31. Dayer, J.-M., B. Beutler, and A. Cerami. 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin  $E_2$  production by human synovial cells and dermal fibroblasts. *J. Exp. Med.* 162:2163–2168.
32. Saklatvala, J. 1986. Tumour necrosis factor  $\alpha$  stimulates resorption and inhibits synthesis of proteoglycan in cartilage. *Nature (Lond.)*. 322:547–549.
33. Connors, L. H., T. Shirahama, M. Skinner, A. Fenves, and A. S. Cohen. 1985. In vitro formation of amyloid fibrils from intact  $\beta_2$ -microglobulin. *Biochem. Biophys. Res. Commun.* 131:1063–1068.
34. Linke, R. P., H. Hampl, H. Lobeck, E. Ritz, J. Bommer, R. Waldherr, and M. Eulitz. 1989. Lysine-specific cleavage of  $\beta_2$ -microglobulin in amyloid deposits associated with hemodialysis. *Kidney Int.* 36:675–681.
35. Falus, A., J. M. Rasmussen, and S. E. Svehag. 1983. Partial characterisation of circulating macromolecular  $\beta_2$ -microglobulin complexes by density gradient ultracentrifugation and PAGE-blotting. *Immunol. Lett.* 7:61–64.
36. Shin, D. B., F. Hayase, and H. Kato. 1988. Polymerization of proteins caused by the reaction with sugars and the formation of 3-deoxyglucosone under physiological conditions. *Agric. Biol. Chem.* 52:1451–1458.
37. Sell, D. R., and V. M. Monnier. 1990. End-stage renal disease and diabetes catalyze the formation of a pentose-derived crosslink from aging human collagen. *J. Clin. Invest.* 85:380–384.
38. Pongor, S., P. C. Ulrich, F. A. Bencsath, and A. Cerami. 1984. Aging of proteins: isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. *Proc. Natl. Acad. Sci. USA.* 81:2684–2688.
39. Njoroge, F. G., L. M. Sayre, and V. M. Monnier. 1987. Detection of glucose-derived pyrrole compounds during Maillard reaction under physiological conditions. *Carbohydr. Res.* 167:211–220.
40. Sell, D. R., and V. M. Monnier. 1989. Structure elucidation of a senescence cross-link from human extracellular matrix. *J. Biol. Chem.* 264:21597–21602.
41. Nakamura, K., T. Hasegawa, Y. Fukunaga, and K. Ienaga. 1992. Cross-lines A and B as candidates for the fluorophores in age- and diabetes-related cross-linked proteins, and their diacetates produced by Maillard reaction of  $\alpha$ -N-acetyl-L-lysine with D-glucose. *J. Chem. Soc., Chem. Commun.* 14:992–994.