

β_2 -Microglobulin Modified with Advanced Glycation End Products Is a Major Component of Hemodialysis-associated Amyloidosis

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Abstract

β_2 -Microglobulin (β_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 β_2 M forming amyloid fibrils exhibited a more acidic pI value than normal β_2 M. This acidic β_2 M was also found in a small fraction of β_2 M in sera and urine from these patients, whereas heterogeneity was not observed in healthy individuals. We purified acidic and normal β_2 M from the urine of long-term hemodialysis patients and compared their physicochemical and immunochemical properties. Acidic β_2 M, but not normal β_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 β_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 β_2 M did not react with either antibody. Incubating normal β_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 β_2 M forming amyloid fibrils also reacted with anti-AGE antibody. These data provided evidence that AGE-modified β_2 M is a dominant constituent of the amyloid deposits in hemodialysis-associated amyloidosis. (*J. Clin. Invest.* 1993; 92:1243–1252.) **Key words:** β_2 -microglobulin • hemodialysis-associated amyloidosis • carpal tunnel syndrome • advanced glycation end products • long-term hemodialysis patient

Introduction

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

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 β_2 -microglobulin (β_2 M) is a major protein constituent of the amyloid fibrils in HAA (2–4). The pathological role of β_2 M in HAA, however, remains to be determined. It is well known that the serum β_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 β_2 M level. Thus, despite the fact that the prolonged elevation of serum β_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- α 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 β_2 M obtained from long-term hemodialysis patients with CTS. During these studies, we observed that β_2 M from amyloid deposits from patients with CTS migrated upon electrophoresis to a more acidic position (referred to as acidic β_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 β_2 M from the urine of long-term hemodialysis patients and analyzed it physicochemically and immunochemically. The results indicated that the acidic β_2 M is generated by modification with AGEs, implicating a potential link of AGE-modified β_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

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1. Abbreviations used in this paper: AGEs, advanced glycation end products; β_2 M, β_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.

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Table I. Profile of Subjects Examined in this Study*

Group	Status	Sex		Age yr	Cause of renal failure	Serum creatinine mg/dl	Serum β_2 M μ 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 β_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- β_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- β_2 M rabbit IgG (anti- β_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% H₂O₂. After 2D-PAGE and immunoblotting with anti- β_2 M antibody, the amount of the acidic isoform of β_2 M was determined by densitometry using PDQUEST of The Discovery Series (pdi, Huntington Station, NY).

To detect Amadori products, samples were reduced with NaBH₄ (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, ϵ -(1-deoxyhexitoyl)-lysine, but not with glucose or nonreduced form of the Amadori product. After reducing with NaBH₄, various glycated proteins, including glycated RNase, glycated superoxide dismutase, glycated BSA and HSA, but not nonglycated 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

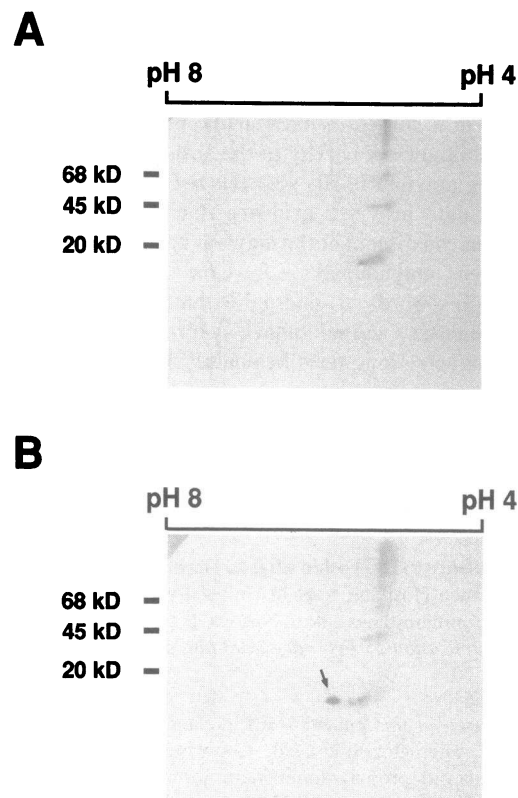


Figure 1. Amyloid fibril proteins from the connective tissues forming carpal tunnels in long-term hemodialysis patients with CTS. After amyloid fibril proteins (1 μ g) were resolved by 2D-PAGE, the gels were immunoblotted with anti- β_2 M antibody (A). Amyloid fibril proteins (1 μ g) were mixed with purified normal β_2 M (2 μ g), followed by 2D-PAGE and immunoblotting with anti- β_2 M antibody (B). The arrow indicates the spot corresponding to normal β_2 M.

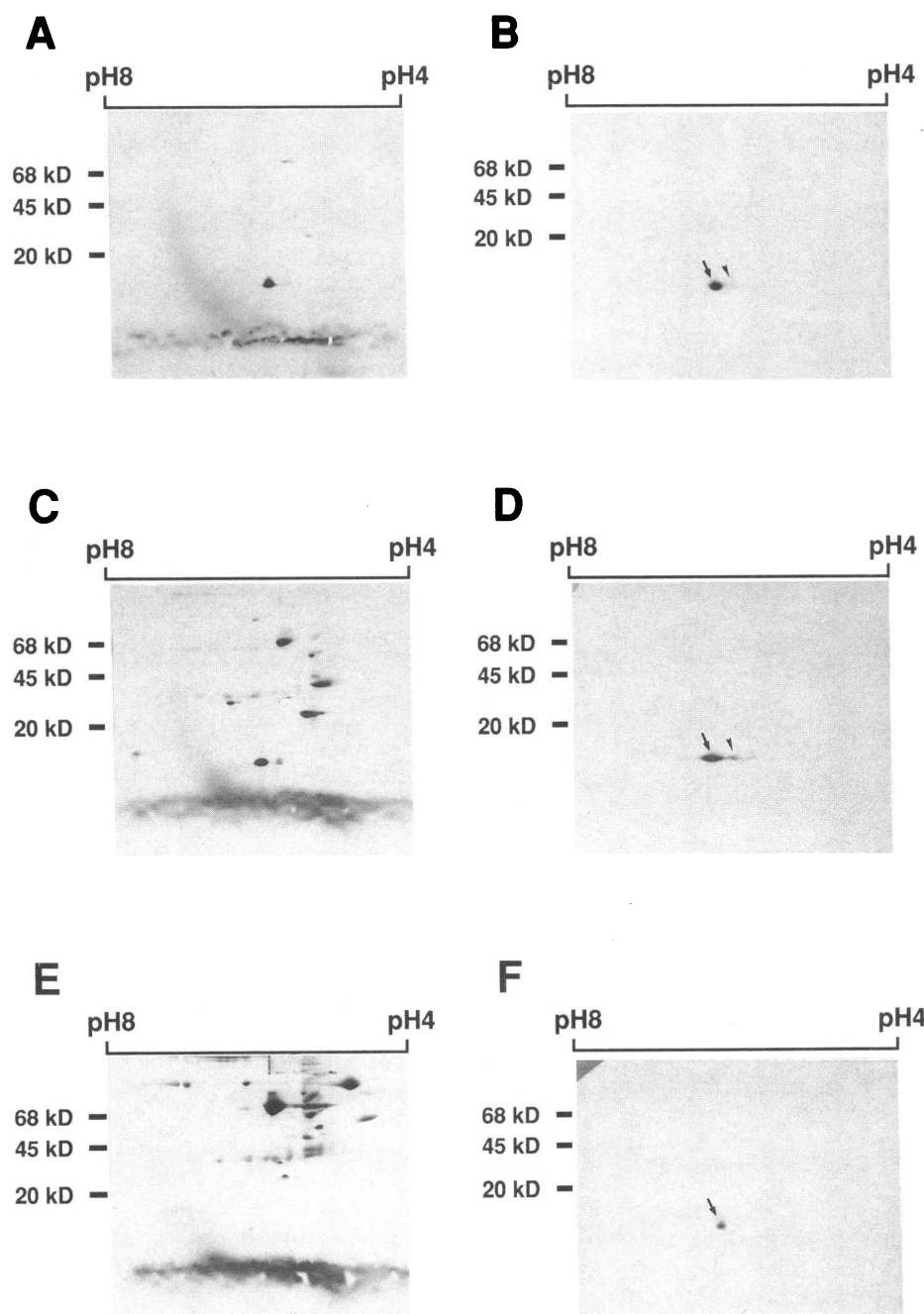


Figure 2. The presence of acidic β_2 M in sera and urine of hemodialysis patients with CTS. Partially purified β_2 M (5 μ g) from serum (*A* and *B*) and urine proteins (30 μ g) (*C* and *D*) of hemodialysis patients with CTS and urine proteins (30 μ g) of normal individuals (*E* and *F*) were resolved by 2D-PAGE. Gels were stained with CBB (*A*, *C*, and *E*) or immunoblotted with anti- β_2 M antibody (*B*, *D*, and *F*). The spots corresponding to acidic β_2 M and normal β_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 β_2 M and acidic β_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- β_2 M antibody. Normal and acidic β_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 β_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 β_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 μ g of normal and acidic β_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 β_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 β_2 M was prepared in vitro by the method of Vlassara et al. (12) with some modifications. Briefly, 2 mg/ml of purified normal β_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

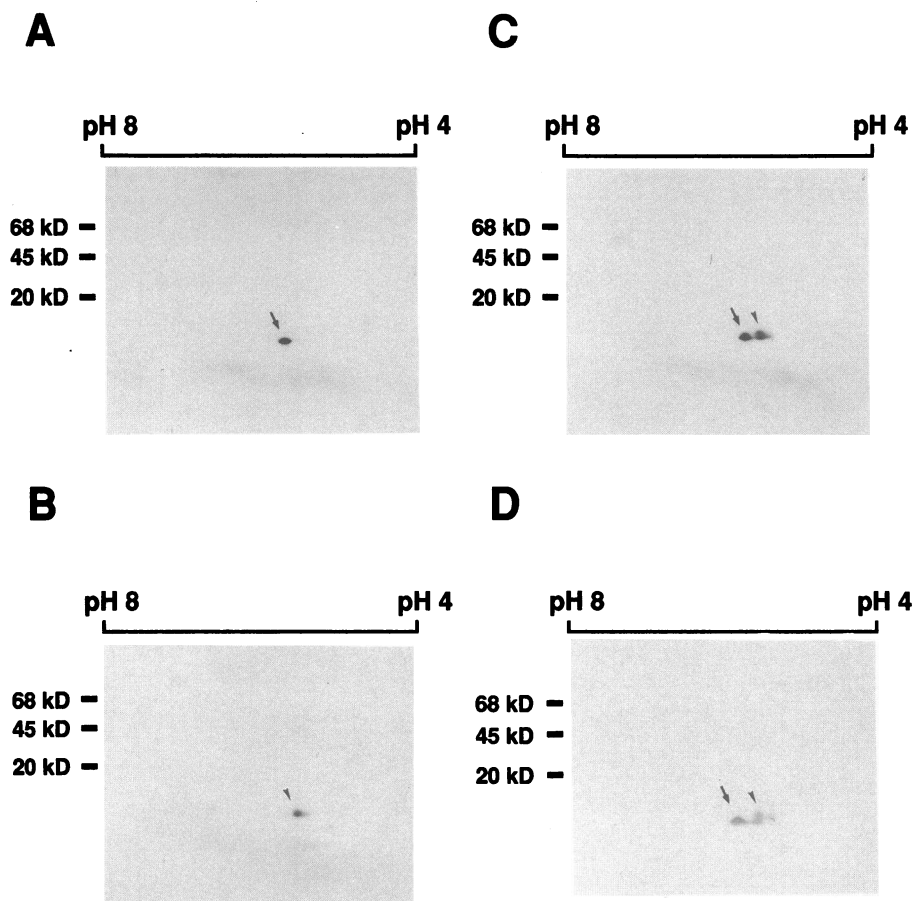


Figure 3. Acidic and normal β_2 M purified from the urine of long-term hemodialysis patients. Purification procedures are described in Methods. 2 μ g of normal β_2 M (A), acidic β_2 M (B), and an equimolar combination of both (C) were resolved by 2D-PAGE and stained with CBB. The gel containing an equimolar combination of both was also immunoblotted with anti- β_2 M antibody (D). The spots corresponding to acidic and normal β_2 M are indicated by arrowheads and arrows, respectively.

μ g/ml gentamicin, and 1.5 mM PMSF. AGE-modified β_2 M was characterized by fluorospectrometry and 2D-PAGE. AGE-modified BSA was also prepared as described previously (22).

Results

The presence of acidic β_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- β_2 M antibody showed that all these bands were reactive to the antibody, suggesting that β_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 β_2 M was subjected to 2D-PAGE followed by immunoblotting with anti- β_2 M antibody, the β_2 M in amyloid deposits migrated to a more acidic position than normal β_2 M (Fig. 1 B). It was also evident that the acidic β_2 M represents an exclusive isoform of β_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 β_2 M is a major protein in amyloid fibril tissues from patients with CTS.

To determine whether acidic β_2 M is present in serum, the β_2 M fraction was partially purified from sera of the same patients (group I) by gel filtration with Sephacryl S-200. The partially purified β_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- β_2 M antibody revealed two bands: a major band and a more acidic minor band (Fig. 2 B). This minor band corresponded to acidic β_2 M as judged by its electrophoretic mobility and it was not detected in the partially purified β_2 M fraction from healthy individuals (group III) (data not shown). These results indicate that acidic β_2 M is present in the sera of hemodialysis patients with CTS but is totally absent from the sera of healthy individuals. All the β_2 M fractions partially purified from the sera of long-term hemodialysis patients without CTS (group II) also possessed acidic β_2 M (data not shown). Assuming that normal and acidic β_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 β_2 M accounted for $\sim 10\%$ of the serum β_2 M both in groups I and II.

Acidic β_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- β_2 M antibody. As shown in Fig. 2 D, acidic β_2 M was detected next to the major band of normal β_2 M. Densitometry showed that acidic β_2 M constituted $\sim 11\%$ of the total urinary β_2 M. Acidic β_2 M was also found in all the urine from patients in group II (data not shown). As with serum, however, acidic β_2 M was not found in the urine from normal individuals (Fig. 2 F).

Purification of acidic β_2 M from the urine of long-term hemodialysis patients. The above finding, that acidic β_2 M, a major isoform of β_2 M in amyloid fibril tissues, occurred in both the sera and urine of long-term hemodialysis patients, indicates

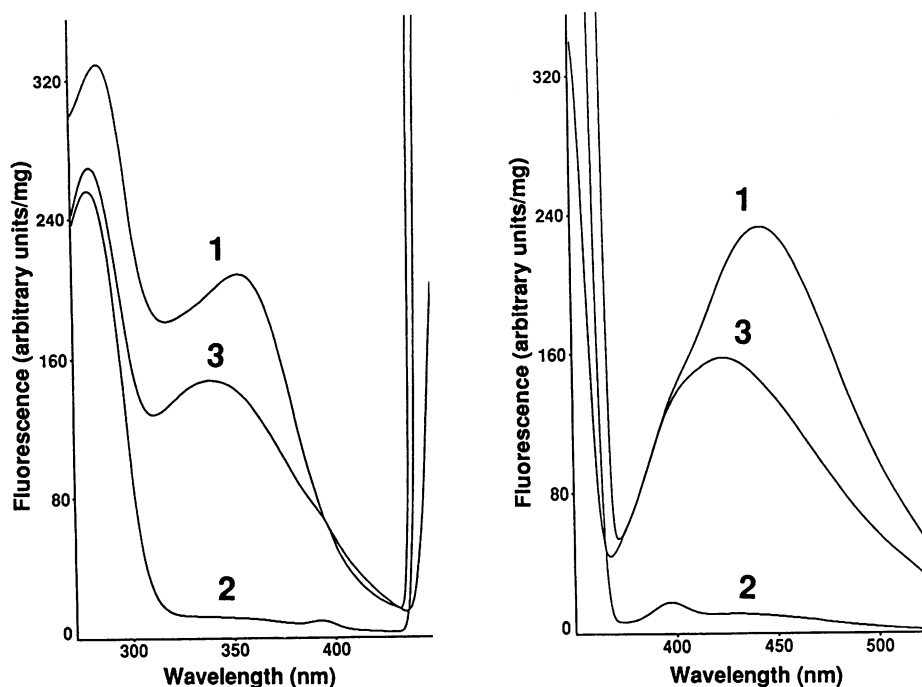
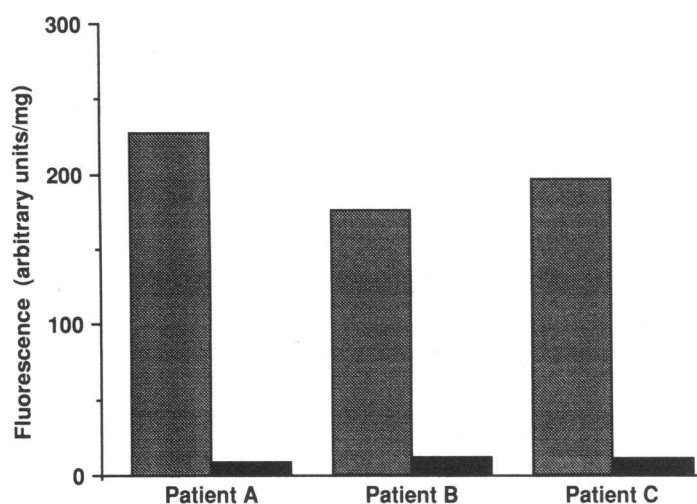
A**B**

Figure 4. Fluorescence spectra of acidic and normal β_2 M. The fluorescence spectra of purified acidic and normal β_2 M 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 β_2 M; line 2, normal β_2 M; line 3, normal β_2 M 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 β_2 M (black bar) from three long-term hemodialysis patients (patients A, B, and C in group II).

that acidic β_2 M is linked to the pathogenesis of HAA. Monitoring their immunoreactivity to anti- β_2 M antibody, we purified both acidic and normal β_2 M 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 β_2 M were coelectrophoresed, they migrated as two separate bands, demonstrated by both protein staining (Fig. 3 C) and immunoblotting with anti- β_2 M antibody (Fig. 3 D), confirming that acidic β_2 M is distinct from normal β_2 M. Protein sequencing demonstrated that the 20 amino-terminal residues of these two β_2 M isoforms were identical. The sequences were also identical with that reported for normal serum β_2 M (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 β_2 M was 5.5–5.7 (Fig. 3). It is thus likely that the acidic form of β_2 M is generated by the posttranslational modification of proteins rather than being derived from different amino acid sequences.

Acidic β_2 M is AGE-modified. One of the most characteristic features of purified acidic β_2 M that we detected was fluorescence. Fig. 4 A shows typical excitation (left) and emission (right) fluorescence spectra of acidic (line 1) and normal β_2 M (line 2) purified from the urine of a long-term hemodialysis patient. The acidic β_2 M 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 β_2 M did not fluoresce.

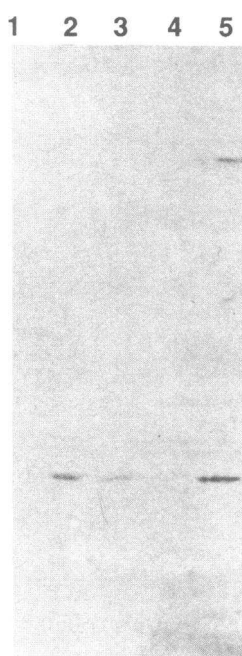
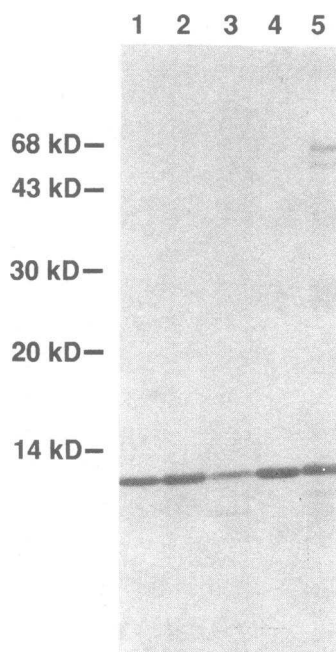
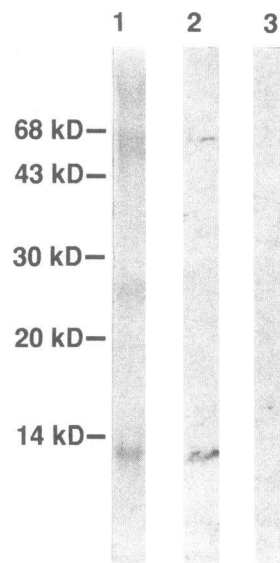
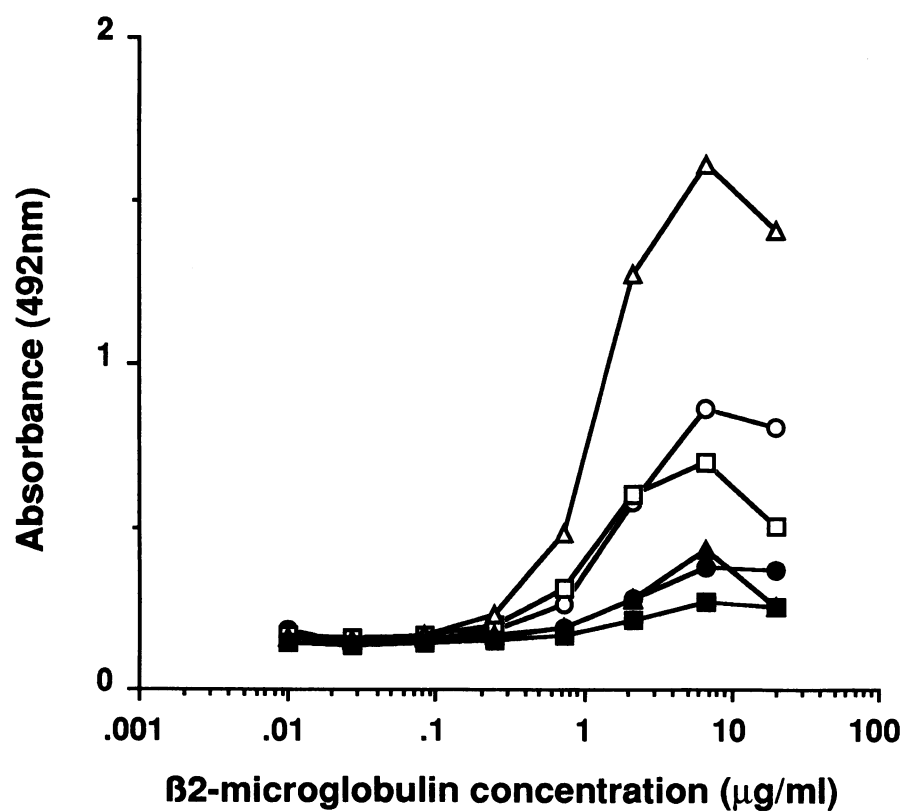
A**C****B**

Figure 5. Immunochemical reaction of acidic β_2 M to anti-AGE antibody. (A) Acidic and normal β_2 M (2 μg 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 β_2 M from patient A; lane 2, acidic β_2 M from patient A; lane 3, normal β_2 M

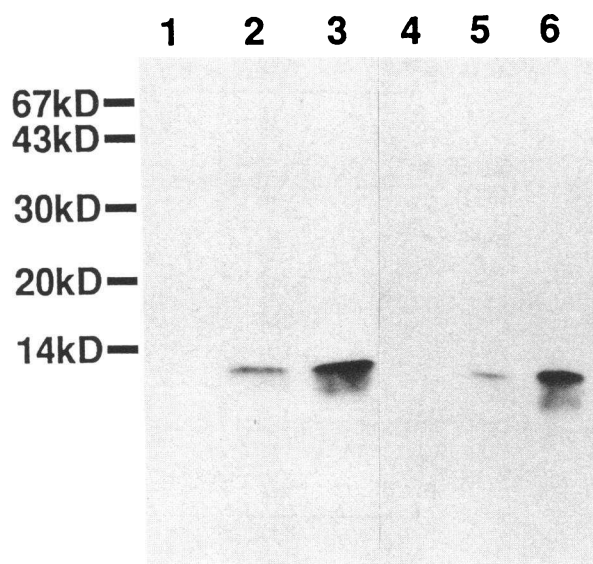


Figure 6. The presence of Amadori products in acidic β_2 M. The acidic or normal β_2 M purified from the urine of long-term hemodialysis patients (5 μ g each) were reduced by NaBH₄, run on 15% SDS-PAGE, and then immunoblotted with anti-Amadori product antibody (see Methods). Lane 1, normal β_2 M; lane 2, acidic β_2 M from patient A; lane 3, acidic β_2 M from patient B; lane 4, normal β_2 M after a 10-d incubation without glucose at 37°C; lane 5, normal β_2 M after a 3-d incubation with 0.1 M glucose at 37°C; and lane 6, normal β_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 β_2 M purified from the urine of the three patients fluoresced significantly. On the other hand, normal β_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 β_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 β_2 M reacted with the anti-AGE antibody whereas the immunoreaction was not observed with normal β_2 M. The binding of acidic β_2 M to the anti-AGE antibody did not reflect the nonspecific binding because, under the present conditions, acidic β_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 β_2 M. This notion was supported by the results of the ELISA (Fig. 5 B). Acidic β_2 M but not normal β_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 β_2 M forming amyloid fibrils contains AGEs (Fig. 5 C).

The presence of early products in these β_2 M preparations was also identified using the polyclonal anti-Amadori product antibody. Immunoblotting showed that acidic β_2 M also reacted with the anti-Amadori antibody (Fig. 6, lanes 2 and 3). No immunoreactivity was observed with normal β_2 M. However, incubating normal β_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 β_2 M was also supported by amino acid analysis. Amino acid analysis of acidic and normal β_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 β_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 β_2 M.

This notion was confirmed by the fact that normal β_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 β_2 M (Fig. 4 A, line 3). Furthermore, normal β_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 β_2 M (> 74% by densitometric quantification) shifted its electrophoretic mobility in 2D-PAGE to a position similar to that of acidic β_2 M after a 30-d incubation with glucose (Fig. 7 B, *top*). In contrast, upon parallel incubation without glucose, acidic β_2 M isoforms were virtually undetectable (Fig. 7 B, *bottom*).

Discussion

This study provided evidence that β_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- β_2 M antibody demonstrated that a major portion of β_2 M forming amyloid fibrils exhibited unusual electrophoretic mobility. The β_2 M forming amyloid fibrils had a more acidic pI value than normal β_2 M. A small but significant fraction of β_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 β_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 β_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 β_2 M from patient B; and lane 5, acidic β_2 M from patient B. (B) The presence of AGEs in acidic β_2 M was also examined by ELISA using anti-AGE antibody. Acidic β_2 M from patients A (○) and B (Δ), normal β_2 from patients A (●) and B (▲), normal β_2 M after a 30-d incubation with 0.1 M glucose at 37°C (□), and normal BSA (■). (C) Amyloid fibril proteins (1.5 μ 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).

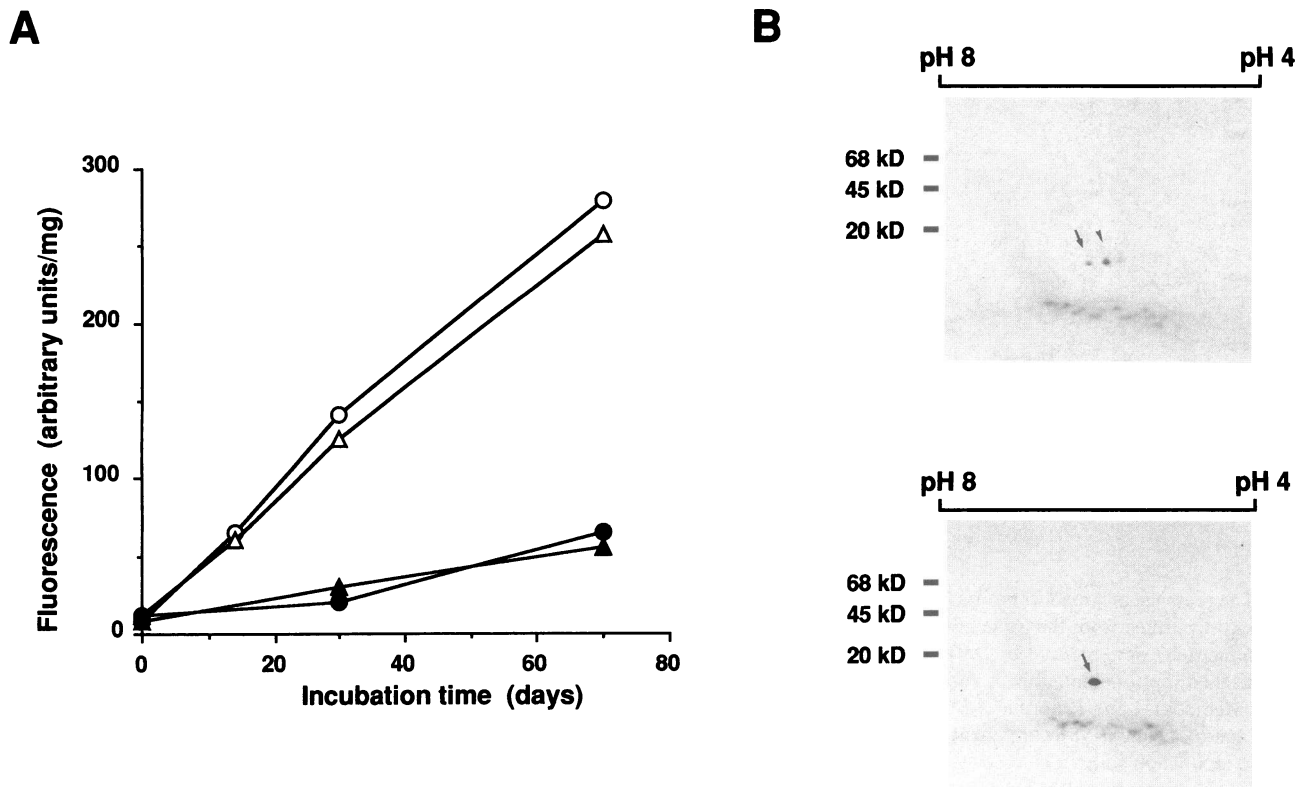


Figure 7. In vitro preparation of AGE-modified β_2 M from normal β_2 M. Purified normal β_2 M (2 mg/ml) was incubated with 0.1 M D-glucose at 37°C (see Methods). (A) Time-dependent changes in the fluorescence of normal β_2 M from patients A (○) and B (△) when incubated with glucose and normal β_2 M from patients A (●) and B (▲) incubated without glucose. The maximum emission fluorescence at 450 nm was measured upon excitation at 360 nm. (B) Typical 2D-PAGE profiles of normal β_2 M (2 μ g) after a 30-d incubation with (top) or without glucose (bottom). Acidic and normal β_2 M are indicated by arrowheads and arrows, respectively.

normal, β_2 M reacted with anti-AGE antibody. Third, β_2 M forming amyloid fibrils in hemodialysis patients with CTS also reacted with this antibody. Finally, incubating normal β_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 β_2 M with AGEs generates acidic β_2 M, the dominant isoform of β_2 M in amyloid deposits of HAA.

Deamination has been proposed as a potential mechanism for the electrophoretic heterogeneity observed with β_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 β_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- α and IL-1 (14). This indicates that once AGE-modified β_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 β_2 M concentration (33), proteolytic modification of β_2 M (34), and complex formation of circulating β_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 β_2 M into the amyloid fibrils. However, the present finding that AGE-modified β_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 β_2 M leads to the polymerization of β_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 β_2 M (12 kD), the appearance of AGE-modified β_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 β_2 M in these patients (> 30-fold) compared with healthy individuals may be also related to a high level of AGE-modified β_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 β_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 β_2 M, but not in normal β_2 M, suggesting the presence of an AGE structure in acidic β_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- β_2 M levels and HAA.

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