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Monocyte/macrophage response to β_2-microglobulin modified with advanced glycation end products

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Monocyte/macrophage response to β_2-microglobulin modified with advanced glycation end products. We recently found that acidic β_2-microglobulin (β_2m), a major isoform of β_2m in amyloid fibrils of patients with dialysis-related amyloidosis (DRA), contained early Amadori products and advanced glycation end products (AGEs) formed nonenzymatically between sugar and protein. Further analysis revealed that acidic β_2m induces monocyte chemotaxis and macrophage secretion of bone-resorbing cytokines, suggesting the involvement of acidic β_2m in the pathogenesis of DRA. Acidic β_2m, however, is a mixture of heterogeneous molecular adducts due to various types of modification. In the present study, we investigated the modification responsible for the biological activity of acidic β_2m toward monocytes/macrophages. The presence of a fair amount of β_2m species with deamidation was detected in acidic β_2m isolated from urine of non-diabetic long-term hemodialysis patients, but deamidated β_2m had no biological activity. In contrast, normal β_2m acquired the activity upon incubation with glucose *in vitro*. Among the glycated β_2m, the pigmented and fluorescent β_2m that formed after a long incubation period, that is, AGE-modified β_2m, exhibited biological activity, whereas β_2m modified with Amadori products, major Maillard products in acidic β_2m, had no such activity. These findings suggest that AGEs, although only a minor constituent of acidic β_2m, are responsible for monocyte chemotaxis and macrophage secretion of cytokines, implicating the contribution of AGEs to bone and joint destruction in DRA.

Dialysis-related amyloidosis (DRA) is a serious complication recognized with high frequency among long-term hemodialysis patients [1]. Amyloid deposits are mainly located in joint structures especially in periarticular bones, leading to hemodialysis arthropathy [1]. The incidence of DRA increases with the age of the patient and the duration of hemodialysis [2, 3]. β_2-microglobulin (β_2m) is a major constituent forming amyloid fibrils in DRA [4, 5]. Recently, we found [6] that electrophoretically acidic β_2m is an exclusive isoform of β_2m forming amyloid fibrils in patients with DRA. The acidic isoform of β_2m was also shown to exist as a small fraction (~10%) of β_2m in the serum and urine of long-term hemodialysis patients [6]. Physicochemical and immunochemical analyses revealed that acidic β_2m obtained from the serum, urine, and amyloid fibrils contained the β_2m modified with the Maillard products [6].

Maillard reaction occurs nonenzymatically between sugar aldehyde groups and protein amino groups to reversibly form Schiff bases, and upon rearrangement, convert into more stable Amadori products [N-(1-deoxyfructosyl)derivatives] [7, 8]. Then, over months, some Amadori products are further converted into the advanced glycation end products (AGEs), through a series of chemical rearrangement, dehydration, and fragmentation reactions. AGEs constitute a heterogeneous class of structures that are brown in color, fluoresce, and tend to polymerize and cross link [7, 8]. In fact, acidic β_2m purified from urine of long-term hemodialysis patients contained both Amadori products and AGEs [6, 9]. Our further study showed that a significant amount of pentosidine, a fluorescent cross-linked molecule and a glycoxidation marker for AGEs [10, 11], is present in acidic β_2m obtained from the urine and amyloid fibrils at almost equivalent amounts, indicating that pentosidine is one of the native AGE-structures in β_2m in DRA (unpublished data).

Since AGEs can initiate a variety of biological effects mediated by receptors/cell-surface binding proteins on monocytes/macrophages [12–14], we previously investigated the effect of acidic β_2m on monocytes/macrophages to assess the possible involvement of AGE-modified β_2m in bone and joint destruction of DRA. In these studies, because of difficulties of obtaining enough acidic β_2m from amyloid fibrils sufficient for biological analysis, acidic β_2m purified from the patients' urine that exhibited immunochemical and physicochemical characteristics indistinguishable from that in amyloid fibrils was used instead. It was demonstrated that acidic β_2m induces chemotaxis of monocytes and secretion of potent bone-resorbing cytokines such as tumor necrosis factor-α (TNF-α); interleukin-1β (IL-1β), and IL-6 from macrophages [15, 16]. These findings are in good agreement with previous histological observations by others that amyloid deposits of long-term hemodialysis patients with severe DRA were surrounded by a number of monocytes/macrophages [17–19] immunochemically stained positive for TNF-α and IL-1β [19].

Deamidation was detected previously in acidic β_2m obtained from the serum and amyloid deposits of hemodialysis patient with DRA [20, 21]. As described above, we have shown that acidic β_2m contains Amadori products and AGEs, demonstrating that acidic

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β_2m is a mixture of heterogeneous molecular adducts derived from various types of modification. Therefore, the nature of the modification in acidic β_2m that might contribute to the biological activity toward monocytes/macrophages must be elucidated. In the present study, we analyzed chemical structures of modification in acidic β_2m that was obtained from urine of long-term hemodialysis patients. Furthermore, various isoforms of β_2m prepared *in vitro* exhibiting physicochemical characteristics indistinguishable from those present in acidic β_2m were used to assess their biological functions, because of the difficulty of separating each isoform of β_2m present in acidic β_2m. The results indicate that AGEs, presumably other than pentosidine, are responsible for monocyte chemotaxis and macrophage secretion of cytokines, implicating a possible involvement of AGEs in the bone and joint destruction in DRA.

Methods

Purification of normal and acidic β_2m

Normal and acidic isoforms of β_2m were purified from urine of two non-diabetic hemodialysis patients who were free of urinary infection (56-year-old male undergoing hemodialysis for 8 years and 44-year-old female undergoing hemodialysis for 5 years) as described previously [6]. These patients had been on regular hemodialysis using a cuprophane dialyzer and a dialysate containing 30 mEq/liter bicarbonate and 8 mEq/liter acetate. Soon after the urine samples were collected, they were dialyzed against distilled water and used for purification. All reagents and buffers were free of glucose, and care was taken to avoid contamination with glucose during the purification procedure. The acidic β_2m, but not normal β_2m, showed immunoreactivity to anti-AGE antibody [22] as well as anti-Amadori product antibody [6].

For reduction of Amadori products [N-(1-deoxyfructosyl) derivatives] in acidic β_2m into N-(1-deoxyhexitolyl) derivatives, acidic β_2m (200 μg) was treated with 5 mM sodium borohydride ($NaBH_4$; Nakalai Tesque, Kyoto, Japan) in 1 ml of 0.2 M phosphate buffer (pH 8.5) at room temperature for four hours, dialyzed against 50 mM acetic acid to destroy excess $NaBH_4$, and then dialyzed against phosphate-buffered saline (pH 7.4).

In vitro preparation of Maillard-β_2m, deamidated β_2m, fragmented β_2m, and pentosidine-β_2m

To prepare β_2m modified with Maillard products (Maillard-β_2m), normal β_2m (500 μg) was incubated at 37°C for 10 days (β_2m modified with Amadori products: Amadori-β_2m) and 60 days (β_2m modified with AGEs: AGE-β_2m) with 100 mM D-glucose (Wako Pure Chemicals, Osaka, Japan) in 500 μl of 100 mM phosphate buffer (pH 7.4) containing 1.5 mM phenylmethanesulfonyl fluoride (PMSF; Sigma) under sterile conditions. In some experiments, β_2m modified with reduced Amadori products (reduced Amadori-β_2m) was prepared by incubating normal β_2m with glucose for 10 days in the presence of 40 mM sodium cyanoborohydride ($NaBH_3CN$; Aldrich, Milwaukee, WI, USA).

Deamidated β_2m was prepared by incubating normal β_2m (150 μg) under atmospheric oxygen at 65°C for eight hours in 500 μl of 50 mM Tris-HCl buffer (pH 8.0). Deamidation at two susceptible sites (Asn-17 and Asn-42, see **Results**) in this preparation was confirmed by mass spectrometry.

To prepare fragmented β_2m with lysine-specific cleavage, normal β_2m (200 μg) was cleaved into fragments with 10 μg (2.8 AU/mg) of Endoproteinase Lys-C (Endo Lys-C) (Acromobacter Protease I; Wako Pure Chemicals) in 100 μl of 50 μM Tris-HCl buffer (pH 9.0) at 37°C for eight hours. This mixture was then ultrafiltered through a filter with a cut-off value of a molecular weight of more than 10,000 (Ultrafree C3-LGC; Nihon Millipore Ltd., Tokyo, Japan) to remove Endo Lys-C.

To prepare pentosidine-modified β_2m or bovine serum albumin (pentosidine-β_2m or -BSA), pentosidine prepared by the method of Grandhee and Monnier [23] was conjugated with normal β_2m or BSA. Briefly, 700 μg of protein was incubated with 1.5 μmol of pentosidine and 0.01 mmol of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce, Rockford, IL, USA) in the presence of 0.14 mg of N-hydroxysulfosuccinimide (Pierce) in 1 ml of phosphate buffered saline for four hours at room temperature, and then overnight at 4°C. Pentosidine incorporated into these preparations was determined by high-performance liquid chromatographic (HPLC) assay [24] using synthesized pentosidine as a standard.

Mass spectrometry (MS)

An JMS SX102A instrument (JEOL, Akishima, Japan) was used as described previously [9]. For electrospray ionization (ESI)-MS, the source accelerating voltage was 7 kV. Samples were dissolved in a solvent of 49/49/2 methanol/H_2O/acetic acid (vol/vol/vol) at a protein concentration of 10 pmol/ml. An aliquot (20 μl) of the sample solution was introduced into the ESI ion source at a flow-rate of 0.8 μl/min with a syringe pump (Harvard Apparatus Model 22; Woburn, MA, USA). Fast atom bombardment (FAB) ionization mode was employed for analysis of the peptides digested with Endo Lys-C followed by purification by HPLC (model LC-10A; Shimadzu, Kyoto, Japan) on a C18 reverse-phase column (Nakalai Tesque). The FAB gun was operated at a 30 mA current and 3 kV energy, using xenon as a bombarding gas. The source accelerating voltage was 10 kV. Approximately 100 μg of sample was dissolved in 10 μl of glycerol as the sample matrix.

Capillary electrophoresis

Capillary zone electrophoresis was performed using a Quanta 4000CE system (Waters, Tokyo, Japan). The capillary cartridge used was a 60 cm by 75 μm cartridge (Waters). The peptides were dissolved in 50 μM sodium phosphate buffer (pH 2.5). The capillary electrophoresis was run at 12 kV, and peptide elution was monitored at 214 nm.

Assay for the Amadori product

The Amadori product was determined by the colorimetric assay using a kit (Fructosamine Test Roche-II; Nihon Roche Ltd., Tokyo, Japan) according to the manufacturer's instruction. The assay is based upon the property of N-(1-deoxyfructosyl) group to act as a reducing agent in alkaline solution. Poly-L-lysine was used as a standard and the amount of Amadori products in each sample was expressed as mol of N^{ϵ}-(1-deoxyfructosyl)-lysine per 1 mol of β_2m. The limit of detection was 0.05 mol of N^{ϵ}-(1-deoxyfructosyl)-lysine/mol of β_2m.

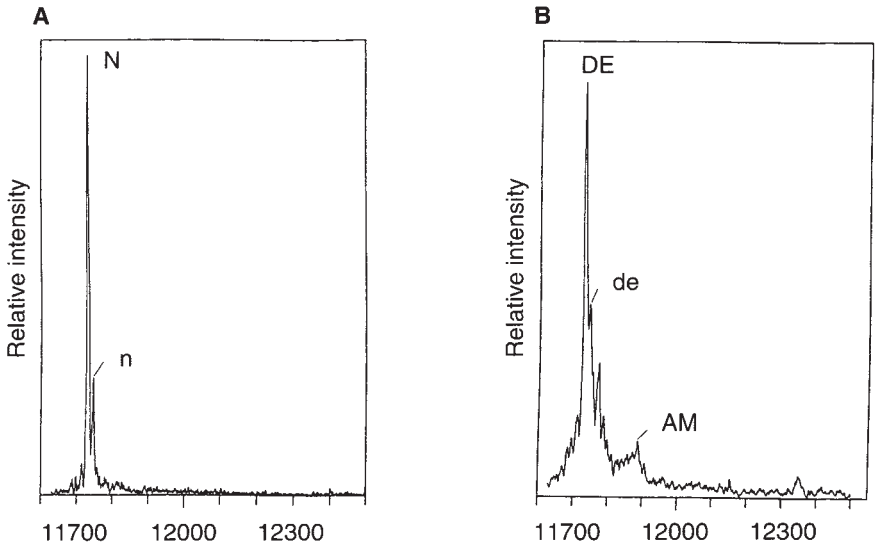


Fig. 1. *Representative transformed ESI mass spectra of normal (A) and acidic β_2m (B).* Abbreviations are: N, intact β_2m; DE, β_2m with deamidation; AM, β_2m with one Amadori product. β_2m oxidized at the carboxyl terminal methionine residue is indicated by the lower cases. The molecular masses are: 11729 Da (peak N), 11745 Da (peak n), 11730 Da (peak DE), 11746 Da (peak de), 11891 Da (peak AM).

Fluorospectrometry

Protein-bound fluorescence was analyzed in a fluorescence spectrophotometer (model RF540; Shimadzu) at a protein concentration of 0.2 mg/ml in 10 mM sodium phosphate buffer (pH 7.2).

Chemotaxis assay

For this experiment, we carefully removed endotoxins from the materials using an endotoxin-adsorbent (Pyro Sep®; Daicel Chemical Industries, Ltd., Tokyo, Japan) as described previously [15]. The monocyte chemotactic activity was assessed by a modification of the Boyden chamber technique in 24-well double chambers separated by a filter (5 μm pore size; Coster Corp., Cambridge, MA, USA) according to our previously reported method [15]. Briefly, a human mononuclear leukocyte suspension (6×10^4 cells) in 0.1 ml of Hanks' balanced salt solution (HBSS) was added to the top compartment of the chamber, and the test material in HBSS was added to the bottom compartment (0.6 ml). The chamber was incubated at 37°C for four hours, and the filter was then removed, fixed in methanol, and stained with Giemsa. *N*-formylmethionylleucylphenylalanine (FMLP; Peninsula Laboratories, Belmont, CA, USA), a synthetic peptide highly chemotactic for monocytes, served as a positive control. Chemotactic activity was defined as the average number of cells that had migrated in response to test substance. The number of cells in four high-power fields (× 400) was counted for each of the triplicate chemotaxis chamber filters. The experiment was repeated three times utilizing mononuclear leukocyte preparations from three healthy males (age range 28 to 33 years old).

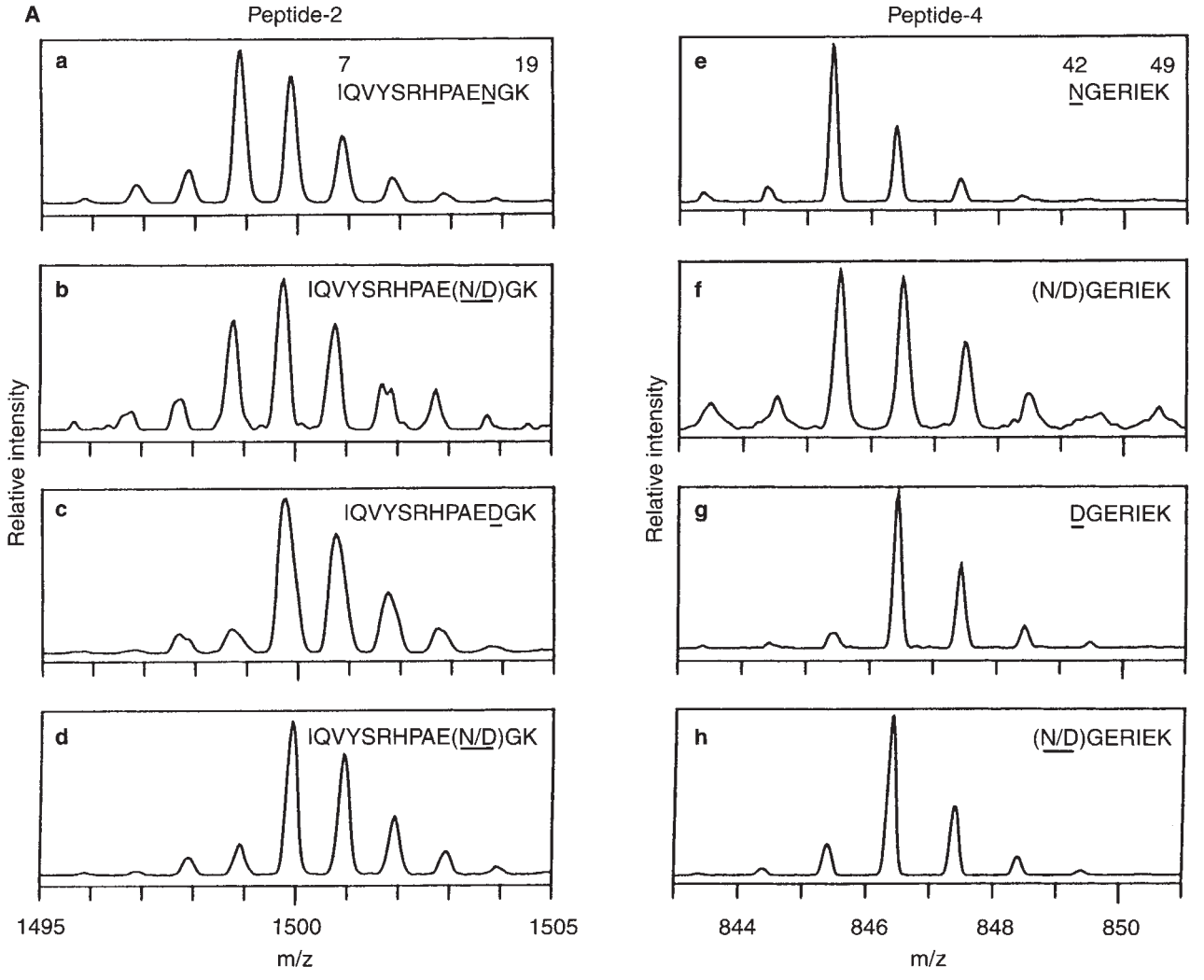
Cytokine assay

Cytokine production by monocyte-derived macrophages was determined by our previously reported method [15]. This experiment was also performed using endotoxin-free materials. Monocytes (2.5×10^5 cells) in 0.5 ml of Dulbecco's modified Eagle's medium (Nacalai Tesque) were incubated with 50 U interferon-γ (IFN-γ; Shionogi Pharmaceutical Co., Osaka, Japan) and test material in triplicate at 37°C for 24 hours. INF-γ was added to transform peripheral monocytes into the equivalent of primed tissue macrophages [13]. *E. coli* 0113:B4 endotoxin (DIFCO, Detroit, MI, USA) was used as a positive control. After incubation in a 48-well culture plate (Sumilon; Sumitomo Bakelite Co. Ltd., Tokyo, Japan), the culture medium was transferred into sterile microfuge tubes and centrifuged at 400 × g for five minutes to remove cells. The cytokine level was then measured in duplicate in each supernatant with an ELISA kit for TNF-α, IL-1β, or IL-6 according to the manufacturer's instruction (Amersham, Buckinghamshire, UK). Limits of detection were 16 pg/ml for TNF-α, 4 pg/ml for IL-1β, and 3 pg/ml for IL-6. The experiment was repeated three times utilizing monocyte preparations from three healthy males.

Statistical analysis

Data are expressed by mean ± SD. Analysis of variance (ANOVA) was used for a statistical evaluation of significant variations. If a significant variation was indicated by the analysis, Scheffe's *t*-test was used to compare the values between different concentrations of the same modification of β_2m, or between normal β_2m and β_2m with modification.

Fig. 2. *Presence of deamidation in acidic β_2m.* **A.** Identification of the deamidation at Asn-17 and Asn-42 in acidic β_2m by FAB-MS. Either normal β_2m, acidic β_2m, or deamidated β_2m was digested with Endo Lys-C and peptides were purified by HPLC on a C18 reverse-phase column. Molecular ion regions for protonated peptides 2 (sequence 7 to 19: a-d) and 4 (sequence 42 to 48: e-h) are presented. Molecular masses for peptides 2 and 4 from normal β_2m are 1497.8 Da and 844.4 Da, respectively, and the mass spectra typically represent their isotopic distributions (a and e). The Asp-containing peptides are larger than those containing Asn by one mass unit (c and g for synthesized peptides). Isotopic distributions of the peptides from acidic β_2m (b and f) and deamidated β_2m (d and h) indicate partial deamidation in these peptides. **B.** Capillary electrophoresis of peptides 2 (a-c) and 4 (d-f). IsoAsp-containing peptides (arrows) are eluted after those containing Asp. IsoAsp is the major product of deamidation in either peptide. a and d, peptides from normal β_2m; b and e, peptides from acidic β_2m; c and f, synthesized peptides containing Asp instead of Asn.


A
Peptide-2
a
7
19
IQVYSRHPAENGK
b
IQVYSRHPAE(N/D)GK
c
IQVYSRHPAEDGK
d
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Relative intensity
1495
1500
1505
m/z
Peptide-4
e
42
49
NGERIEK
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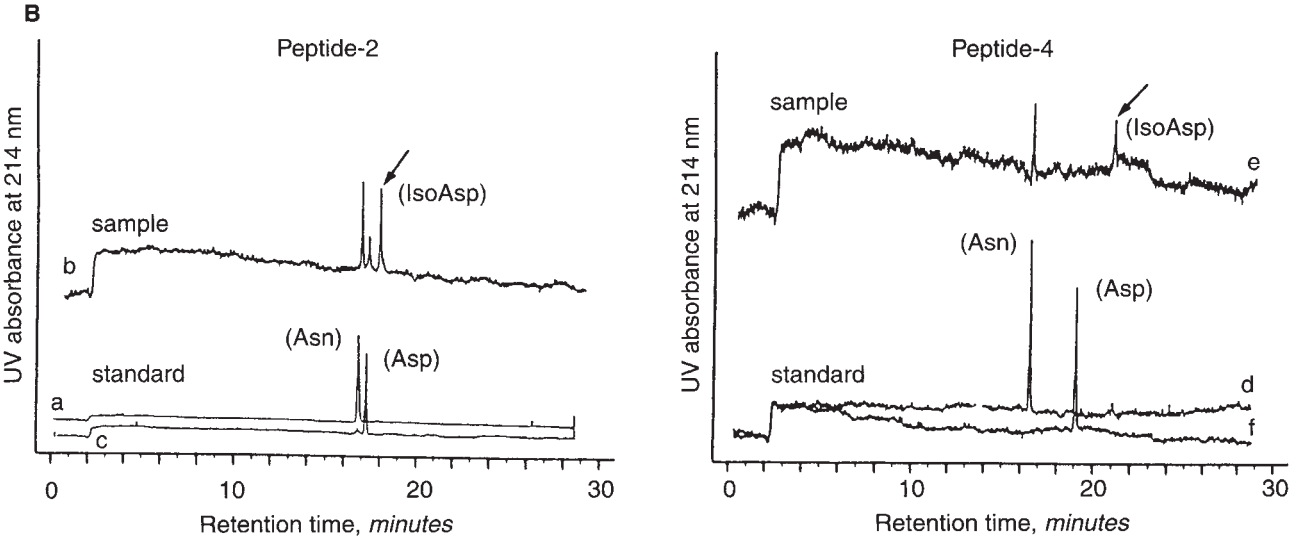
B
Peptide-2
UV absorbance at 214 nm
sample
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Peptide-4
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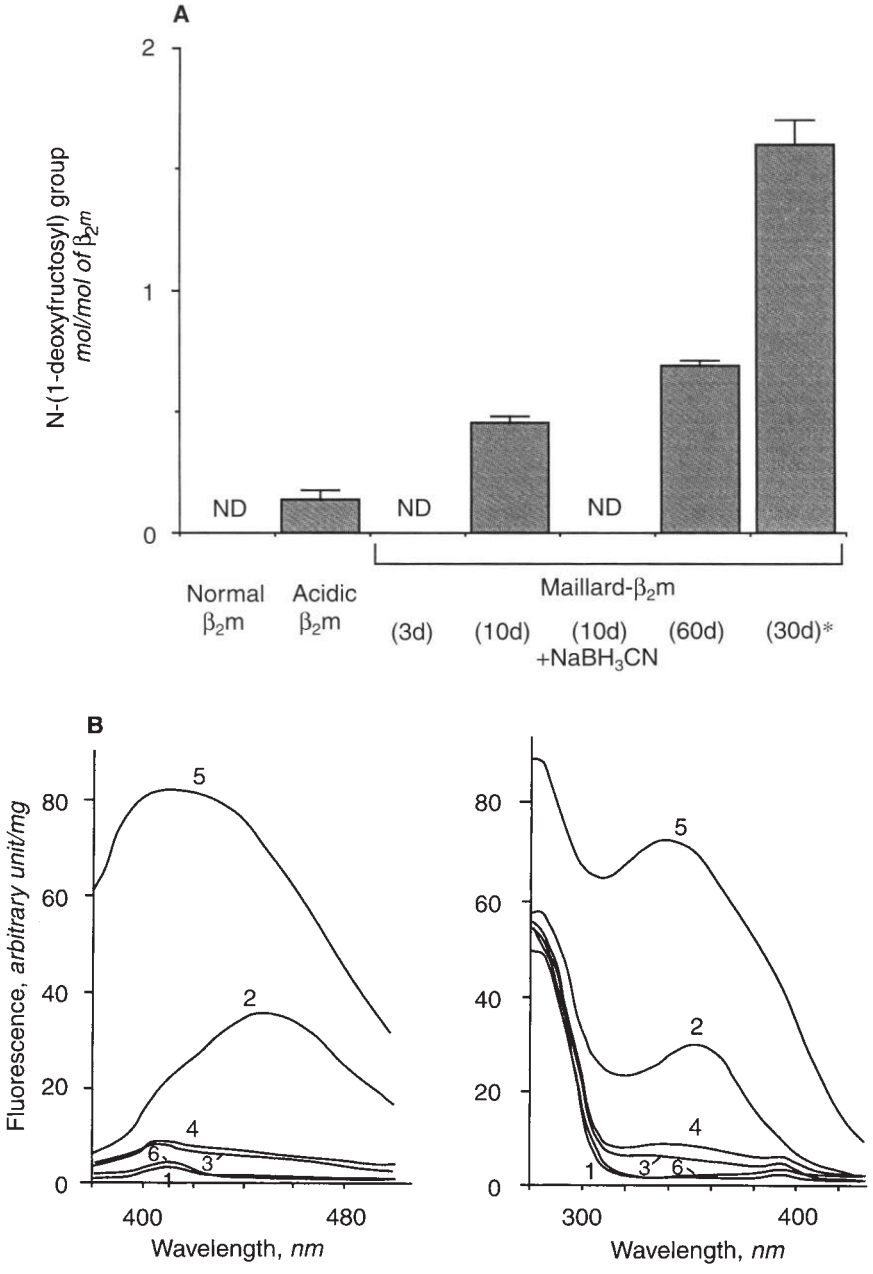

Fig. 3. *Presence of Maillard products in acidic β_2m.* **A.** Detection of Amadori products in normal β_2m, acidic β_2m, and Maillard-β_2m. Amadori products in these materials were determined with the colorimetric method. Incubation for Maillard-β_2m was carried out *in vitro* with 0.1 M glucose or 0.4 M glucose (*) in 0.1 M phosphate buffer (pH 7.4) for the incubation period (days) shown in parentheses. Reduced Amadori-β_2m was prepared by incubating normal β_2m with glucose in the presence of NaBH$_3$CN (Maillard-β_2m + NaBH$_3$CN). ND, Not detected. **B.** Protein-bound fluorescence for AGEs in normal β_2m (line 1), acidic β_2m (line 2), deamidated β_2m (line 3), Maillard-β_2m after a 10 day incubation (Amadori-β_2m; line 4), a 60 day incubation (AGE β_2m; line 5), and a 10 day incubation in the presence NaBH$_3$CN (reduced Amadori-β_2m; line 6). The emission fluorescence spectra upon excitation at 350 nm (left) and the excitation spectra for emission at 450 nm (right) were measured at a protein concentration of 0.2 mg/ml.

Results

Presence of heterogeneous molecular adducts in acidic β_2m

The molecular mass of β_2m was analyzed by ESI-MS. Normal β_2m was identified as a single major peak (peak N in Fig. 1A) with a molecular mass of 11729.1 ± 0.1 Da, in good agreement with the theoretical value (11729.18 Da for chemical mass). A minor component (peak n) with a molecular mass of 11745 Da was also identified as the β_2m oxidized at the carboxyl terminal methionine residue. In the molecular mass analysis, acidic β_2m showed two features different from normal species (Fig. 1B). First, the molecular mass for the major peak (peak DE) was larger than that of normal β_2m by about one mass unit. This peak was ascribed to the β_2m with deamidation as demonstrated by FAB-MS and capillary electrophoresis of peptides as follows. Among eight peptides isolated from an Endo Lys-C digest of acidic β_2m on reverse-phase HPLC, only fractions corresponding to peptide 2 (sequence 7 to 19) and peptide 4 (sequence 42 to 48) showed aberrant isotopic distributions, obviously indicating partial deamidation of asparagine residues (Asn-17 and Asn-42; Fig. 2A). Deamidation of β_2m has been reported or suggested at Asn-17 [20] and Asn-42 [21]. At both sites, Asn is followed by Gly and thus must be very susceptible for deamidation [25–27]. Since the deamidation of Asn residues forms isoAsp:Asp peptide in a ratio of about 3:1 and the resulting isoAsp products are resistant to Edman degradation [26–28], we carried out capillary electrophoresis and confirmed deamidation at these sites (Fig. 2B).

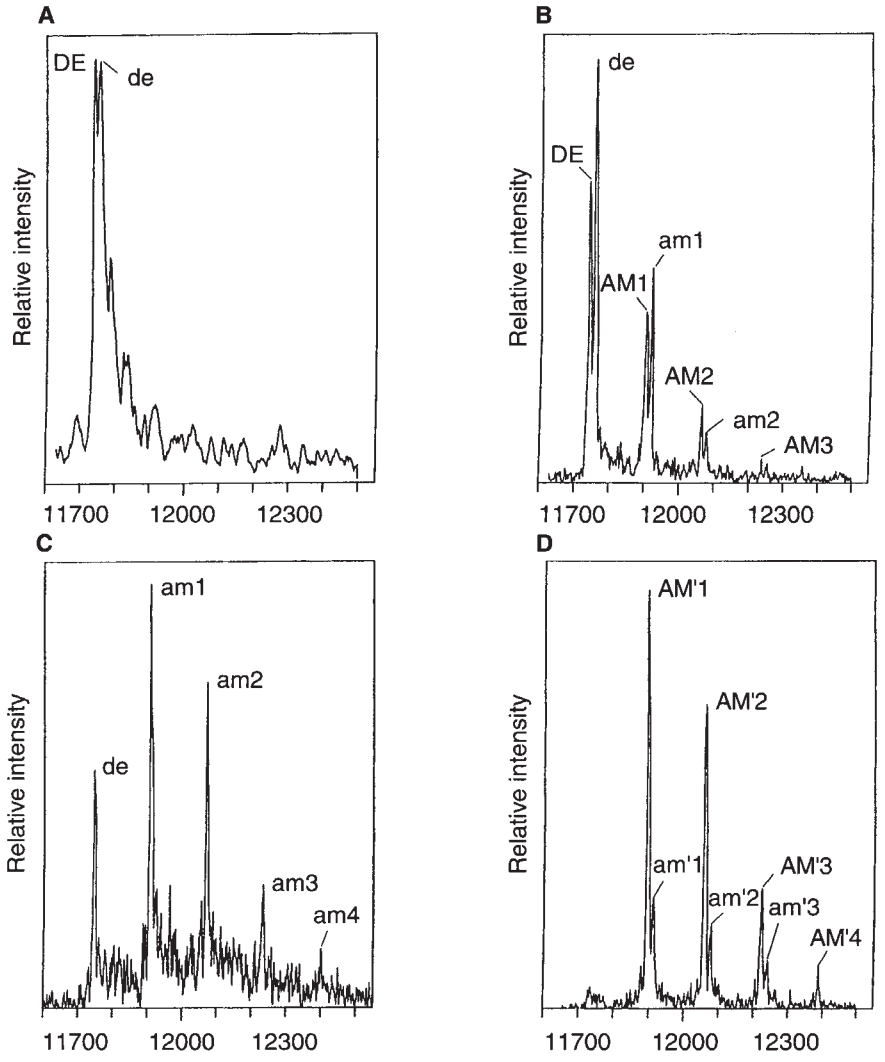

Fig. 4. *Representative transformed ESI mass spectra of deamidated β_2m* (**A**) *and Maillard-β_2m upon incubation for 10 days (Amadori-β_2m;* **B**), *60 days (AGE-β_2m;* **C**), *and 10 days in the presence of $NaBH_3CN$ (reduced Amadori-β_2m;* **D**). Abbreviations are: DE, β_2m with deamidation; AMX, β_2m with Amadori products (X indicates the number of Amadori products contained); AM'X, β_2m with reduced Amadori products (X indicates the number of reduced Amadori products contained). β_2m oxidized at the carboxyl terminal methionine residue is shown by the lower cases. The molecular masses are 11730 Da (peak DE), 11891 Da (peaks AM1), 12053 Da (peak AM2), 12215 Da (peak AM3), 12377 Da (peak AM4), 11893 Da (peak AM'1), 12057 Da (peak AM'2), 12221 Da (peak AM'3), and 12385 Da (peak AM'4).

The second remarkable feature of acidic β_2m in Figure 1B was the presence of very heterogeneous molecules with masses ranging from −100 to + 400 Da around the major component. Among these heterogeneous components, a distinct peak was identified at 11891 Da (peak AM in Fig. 1B). The difference of 162 Da from the normal β_2m indicated that this component was the β_2m with one Amadori product. Since $NaBH_4$ can reduce Amadori product into N-(1-deoxyhexitolyl) derivative, we first treated acidic β_2m with $NaBH_4$ and then analyzed this by ESI-MS. As expected, a new peak with an adduct of 164 Da, corresponding to N-(1-deoxyhexitolyl) group, was detected instead of 162 Da in the ESI mass spectrum (data not shown). The presence of Amadori product in acidic β_2m was also confirmed by colorimetric method (Fig. 3A): acidic β_2m contained Amadori products (0.14 ± 0.04 mol/mol of β_2m), whereas Amadori product was virtually undetectable in normal β_2m. Our previous studies demonstrating positive immunoreactivity of acidic β_2m but not normal β_2m to anti-Amadori product antibody [6], and intense radioactive incorporation of sodium borotritide into acidic β_2m but not into normal β_2m [9], further support that acidic β_2m contained Amadori products. By combination of peptide sequencing and FAB-MS of Endo Lys-C-digested peptides from acidic β_2m, we recently identified the primary site of Amadori product in acidic β_2m at the α-amino group of the amino terminal isoleucine and the minor sites at the ϵ-amino groups of six lysines [9]. These findings, taken together, indicate that the native structures of the major and minor Amadori products in acidic β_2m are N^{α}-(1-deoxyfructosyl)-isoleucine and N^{ϵ}-(1-deoxyfructosyl)-lysine, respectively.

The present study provides an estimation of the extent of the Maillard modification in acidic β_2m. Based on the ESI mass spectrum of acidic β_2m (Fig. 1B) and the result of colorimetric assay (Fig. 3A), Amadori products accounted for about 10% of the acidic β_2m preparation. This is in good agreement with our recent observation that the radioactive incorporation of sodium borotritide, which reduced Amadori products, was about 15% of acidic β_2m [9]. The proportion of AGEs in acidic β_2m could be less than 1%. However, precise determination was impossible because they did not show distinct peaks on ESI-MS. Since Maillard products contain both Amadori products and AGEs, the

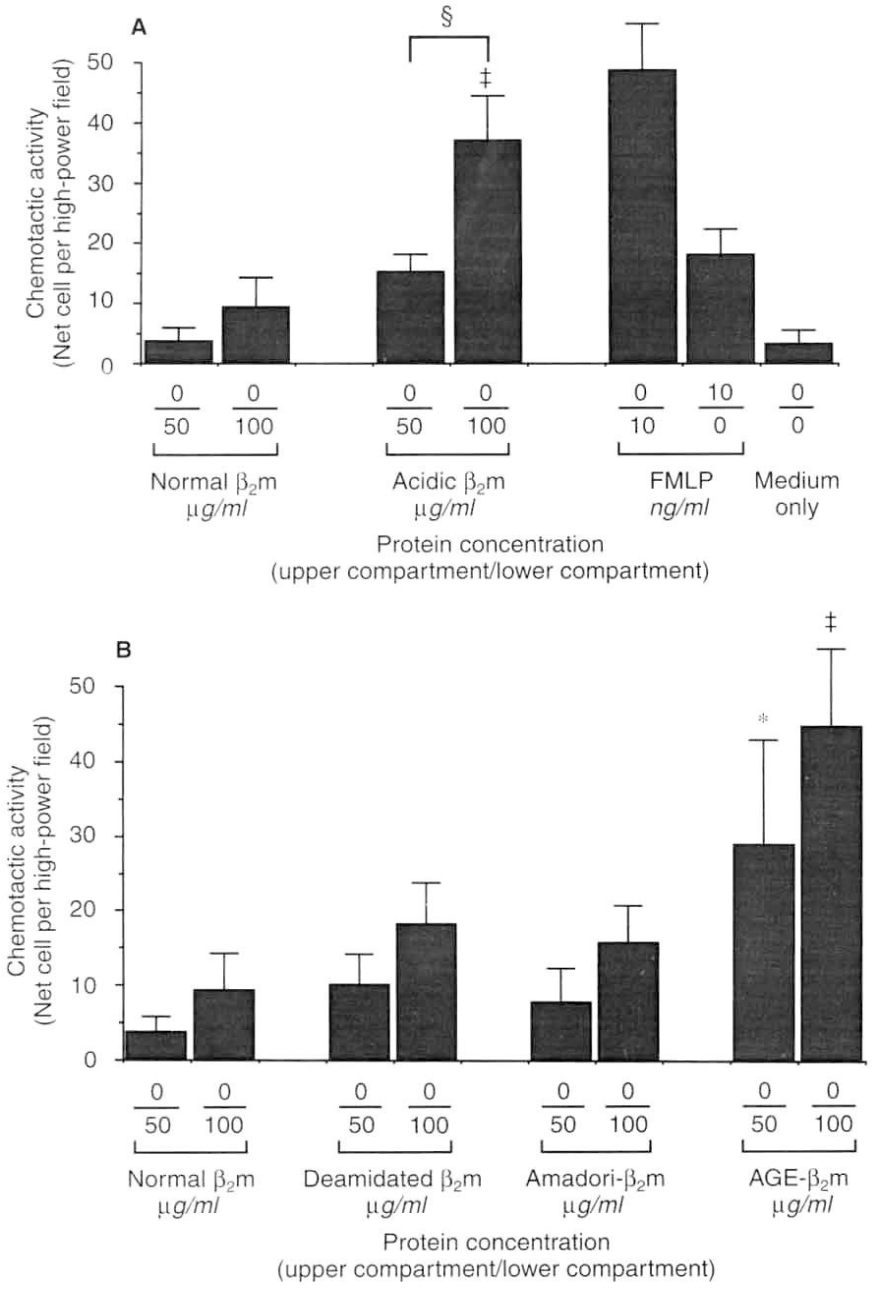

Fig. 5. *Comparison of monocyte chemotactic activity between normal and acidic β_2m* (**A**)*, and among normal β_2m and β_2m with heterogeneous modifications consisting of acidic β_2m* (**B**). Monocyte chemotaxis was examined by a modified Boyden chamber technique with normal and acidic β_2m, using FMLP at 10 ng/ml as a positive control and medium itself as a negative control, or with normal β_2m, deaminated β_2m, and Maillard-β_2m upon incubation for 10 days (Amadori-β_2m) and 60 days (AGE β_2m). ESI mass spectra of these *in vitro* preparations are shown in Figure 4. Representative data from the three experiments are shown. Data are expressed as means ± SD. $N = 3$. $^*P < 0.05$ and $\ddagger P < 0.001$ versus normal β_2m. $\S P < 0.001$ between those of different concentrations of the same modification of β_2m.

latter being a minor constituent, Maillard products may be at most about 10% of acidic β_2m. A large fraction (more than 80%) of acidic β_2m is deamidated as shown above. Our purification procedure of acidic β_2m, depending upon the electrophoretic mobility on ion exchange chromatography, cannot efficiently separate deamidated β_2m from those modified with Maillard products, because both of these modifications result in a potent electrophoretic shift to an acidic position.

In vitro preparation of heterogeneous molecular adducts present in acidic β_2m

To investigate the modification responsible for the biological activity of acidic β_2m such as chemotaxis of monocytes and secretion of cytokines from macrophages, β_2m with heterogeneous modification present in acidic β_2m was prepared *in vitro*. Since deamidation has been reported to be generated easily at Asn-Gly sequences in proteins *in vitro* [25–28], we prepared β_2m with deamidation by incubating normal β_2m at 65°C for eight hours. Maillard-β_2m was prepared by incubating normal β_2m with glucose. Apparently, Maillard-β_2m after a long-period incubation (60 days) turned a brown color like acidic β_2m, but both deamidated β_2m and Maillard-β_2m after a short-period of incubation (10 days) were colorless like normal β_2m. When these materials were subjected to two-dimensional PAGE, a major portion of all the materials was electrophoresed to a position similar to that of acidic β_2m (data not shown). Of particular interest was the

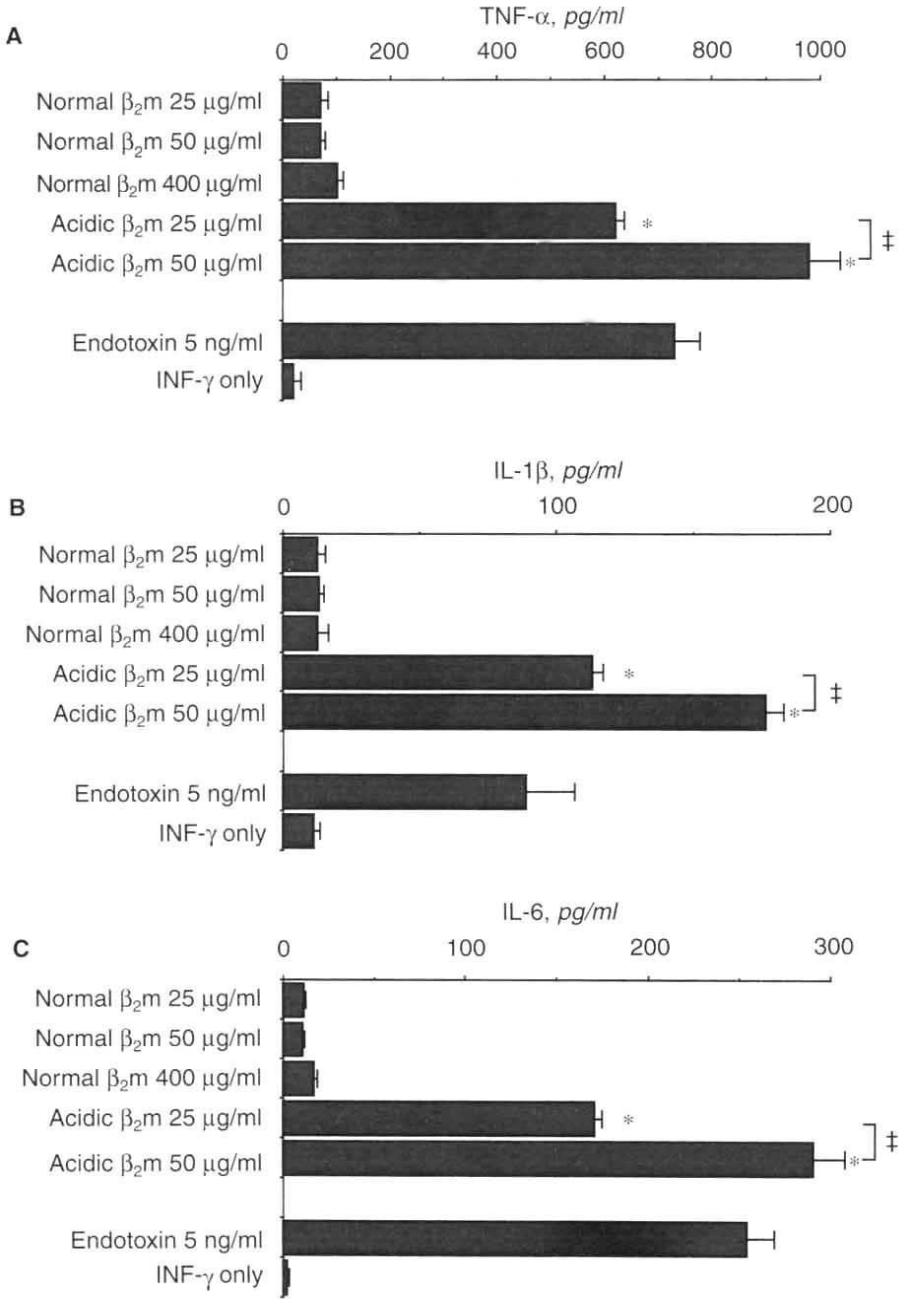

Fig. 6. *Comparison of cytokine inducing activity from human macrophages between normal and acidic β_2m.* Monocytes were incubated for 24 hours at 37°C in medium containing human INF-γ (50 units) in the presence of test substance, using B4 endotoxin from *E. coli* at 5 ng/ml as a positive control and medium containing INF-γ as a negative control, followed by determination of TNF-α (**A**), IL-1β (**B**), and IL-6 (**C**) levels in culture media by ELISAs. Representative data from the three experiments are shown. Data are expressed as means ± SD. $N = 3$. $^*P < 0.0001$ versus normal β_2m. ‡$P < 0.0001$ between those of different concentrations of the same modification of β_2m.

striking polymerization of Maillard-β_2m after a long-period incubation on electrophoresis (data not shown). No such tendency for polymerization of β_2m was observed in deamidated β_2m or Maillard-β_2m upon incubation in the presence of $NaBH_3CN$, which reduces Schiff base, prevents Amadori rearrangement, and inhibits formation of cross linking. When the protein-bound fluorescence was examined (Fig. 3B), Maillard-β_2m after a long-period incubation (line 5) fluoresced markedly with almost the same fluorescence spectra with acidic β_2m (line 2). On the other hand, neither normal β_2m (line 1), deamidated β_2m (line 3), nor Maillard-β_2m after a short-period incubation (line 4) fluoresced at all. Hence, the characteristic triad of AGE-proteins [7, 8], namely, the brown color, fluorescence, and polymerization tendency, was recognized with both Maillard-β_2m after a long-period incubation and acidic β_2m, but not with normal β_2m, deamidated β_2m, nor Maillard-β_2m after a short period of incubation.

ESI-MS of deamidated β_2m (Fig. 4A) gave a mixture of the β_2m with deamidation (peak DE) and its oxidized form (peak de), and FAB-MS of Endo Lys-C-digested peptides from deamidated β_2m (Fig. 2A, d and h) revealed the presence of deamidation at Asn-17 and Asn-42 in about 90% of this preparation. ESI-MS of Maillard-β_2m upon incubation for 10 days (Fig. 4B) gave several major peaks for Amadori products (AM1-AM3 and am1-am2) in addition to the β_2m with deamidation (DE and de). The presence of Amadori products in this preparation was also confirmed by measuring Amadori products with colorimetric assay (Fig. 3A).

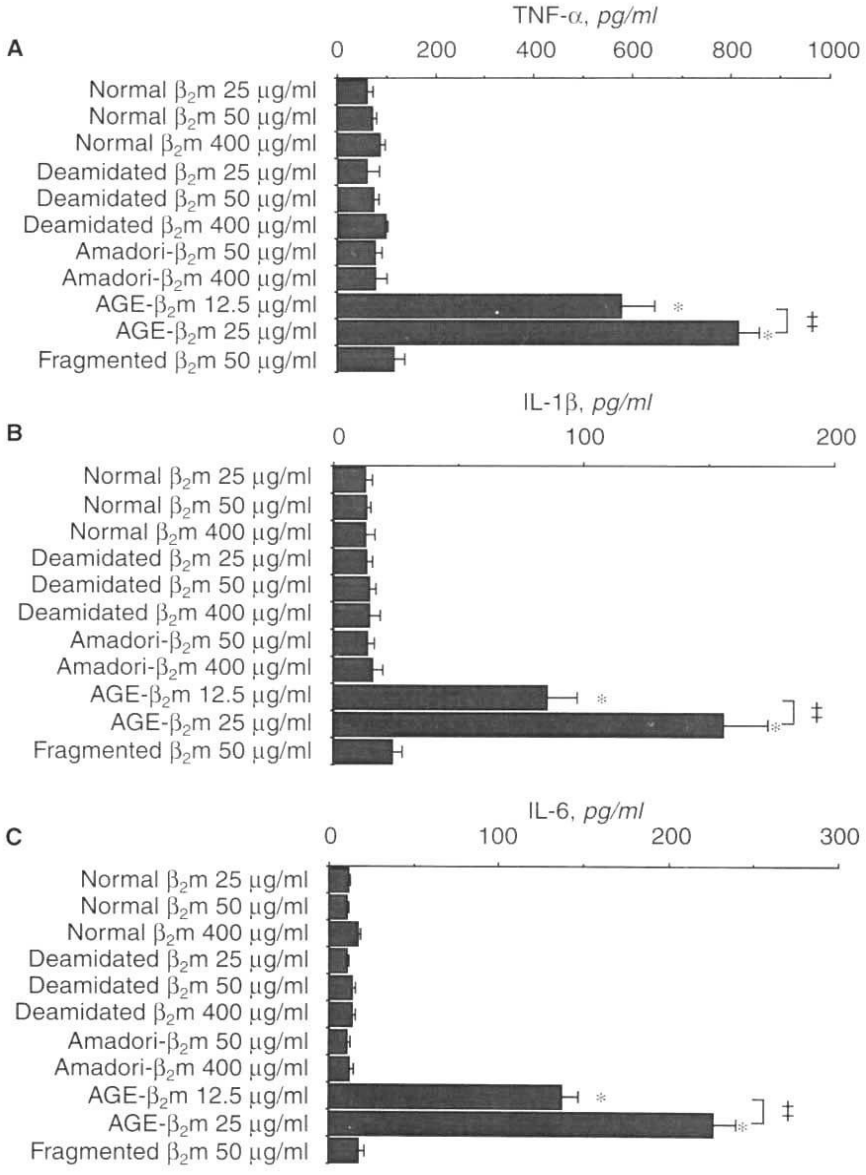

Fig. 7. *Comparison of cytokine inducing activity from macrophages among normal β_2m, β_2m with heterogeneous modifications consisting of acidic β_2m, and fragmented β_2m.* Deamidated β_2m, Maillard-β_2m upon incubation for 10 days (Amadori-β_2m) or 60 days (AGE-β_2m), or fragmented β_2m was prepared *in vitro* and incubated with monocytes for determination of its cytokine inducing activity. **A.** TNF-α. **B.** IL-1β. **C.** IL-6. Representative data from the three experiments are shown. Data are expressed as means ± SD. $N = 3$. $^*P < 0.0001$ versus normal β_2m. ‡$P < 0.0001$ between those of different concentrations of the same modification of β_2m.

Maillard-β_2m upon incubation for 10 days contained a significant amount of Amadori products (0.45 ± 0.03 mol/mol of β_2m) compared to its absence in normal β_2m. The transformed ESI mass spectrum of Maillard-β_2m upon incubation for 60 days (Fig. 4C) was similar to that of Maillard-β_2m upon incubation for 10 days, except for more intense peaks for Amadori products (am1-am4) and the presence of very heterogeneous molecules in the former. Note that Maillard-β_2m upon incubation for 60 days, which possessed the same characteristic properties as the AGE-modified protein, contained mostly Amadori products (0.69 ± 0.02 mol/mol of β_2m, Fig. 3A). The proportion of AGEs in Maillard-β_2m upon incubation for 60 days could be very minor (less than 1%), although precise determination was impossible since ESI-MS did not show distinct peaks for AGEs. These findings indicate that intense Maillard modification occurs even after a shorter incubation period, but these are mainly Amadori products, and that the pigmented and fluorescent adducts (AGEs) are generated only after a longer incubation period.

AGEs are causal for the biological activity of acidic β_2m toward monocytes/macrophages

When migration of peripheral human monocytes toward normal or acidic β_2m was measured in modified Boyden chambers (Fig. 5A), acidic β_2m exhibited direct migratory activity in a dose-dependent manner ($P < 0.001$), whereas normal β_2m elicited only a weak migratory activity of monocytes. Furthermore, addition of acidic β_2m resulted in a dose-dependent increase ($P < 0.0001$) in levels of cytokines such as TNF-α, IL-1β, and IL-6 in the culture medium, released from macrophages (Fig. 6). In contrast, the medium of the cells incubated with normal β_2m, even at a high concentration, contained only a small amount of

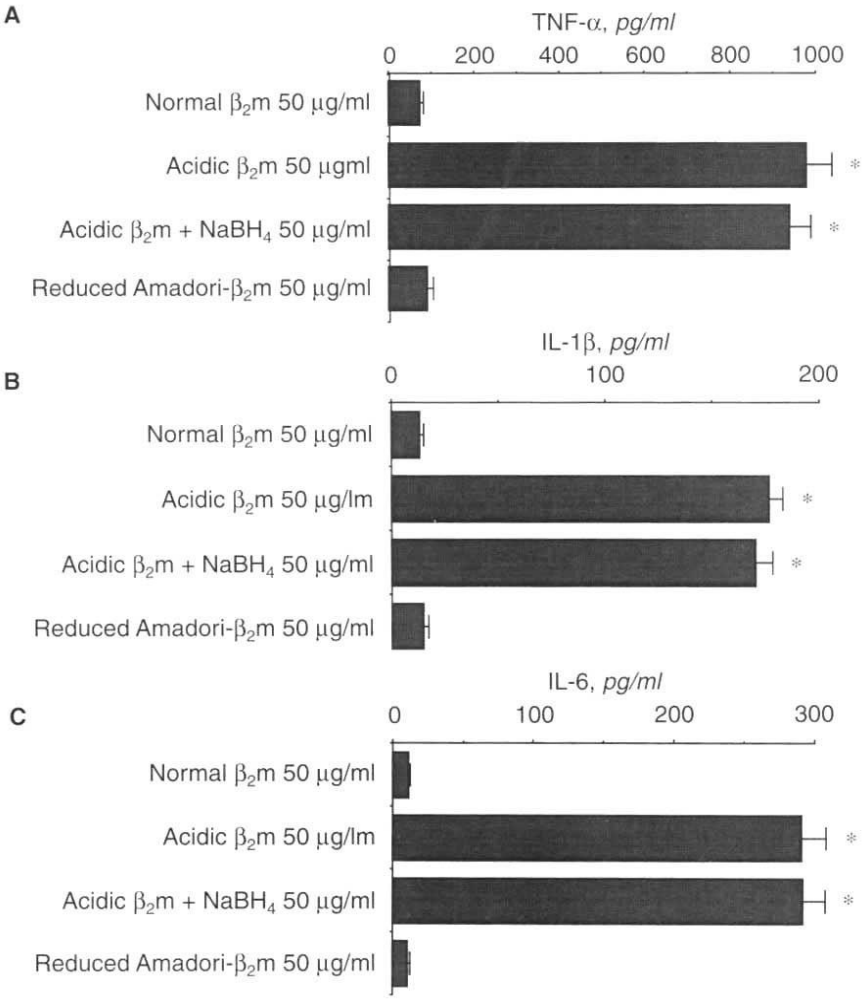

Fig. 8. *Comparison of cytokine inducing activity from macrophages among normal β_2m, acidic β_2m, acidic β_2m treated with $NaBH_4$ (acidic β_2m + $NaBH_4$), and reduced Amadori-β_2m.* Reduced Amadori-β_2m was prepared by incubating normal β_2m with glucose *in vitro* for 10 days in the presence of $NaBH_3CN$. **A.** TNF-α. **B.** IL-1β. **C.** IL-6. Representative data from the three experiments are shown. Data are expressed as means ± SD. $N = 3$. $^*P < 0.0001$ versus normal β_2m.

cytokines. Chemotactic and cytokine inducing activities of acidic β_2m were much higher than those of normal β_2m ($P < 0.001$ and $P < 0.0001$, respectively), in good agreement with our previous results [15, 16].

We then examined β_2m with heterogeneous modification present in acidic β_2m together with normal β_2m. The pigmented and fluorescent AGE-β_2m exhibited direct migratory activity of monocytes (Fig. 5B). However, deamidated β_2m or Amadori-β_2m elicited only a weak migratory activity. The chemotactic activity of AGE-β_2m was significantly higher than that of normal β_2m ($P < 0.05$), but there was no statistically significant difference between deamidated β_2m or Amadori-β_2m, and normal β_2m.

Furthermore, AGE-β_2m remarkably increased TNF-α, IL-1β, and IL-6 in a dose-dependent manner ($P < 0.0001$) in the culture medium of macrophages (Fig. 7). On the other hand, deamidated β_2m or Amadori-β_2m induced only a small increase of cytokines even at a high concentration. The cytokine inducing activity of AGE-β_2m was remarkably higher than that of normal β_2m ($P < 0.0001$), but there was no significant difference in the activity between deamidated β_2m or Amadori-β_2m, and normal β_2m.

The fragmented β_2m induced only a small amount of cytokine release from macrophages (Fig. 7), and there was no significant difference in the activity between fragmented β_2m and normal β_2m, implicating that fragmentation by itself is not a modification responsible for the cytokine inducing activity from macrophages.

Pretreatment of acidic β_2m with $NaBH_4$ that reduces Amadori products into N-(1-deoxyhexitolyl) derivatives did not alter the cytokine inducing activity of acidic β_2m (Fig. 8). This finding, together with the observation that N-(1-deoxyhexitolyl) derivatives prepared *in vitro* (Fig. 4D) could induce only a small amount of cytokine release from macrophages (Fig. 8), indicates that Amadori products in acidic β_2m do not contribute in a significant manner to the cytokine inducing activity.

Synthesized pentosidine as well as *in vitro*-prepared pentosidine-β_2m and -BSA induced only a small amount of cytokine release from macrophages (Fig. 9), and there was no significant difference in the activity between pentosidine-β_2m and normal β_2m. Thus, it is likely that pentosidine is inert with respect to cytokine inducing activity. However, because the chemical binding of pentosidine to β_2m obtained by the present preparative method was quite different from the native one that involves a lysine and an arginine residue combined in an imidazo-(4, 5b)-pyridinium ring [10], the possibility remains that pentosidine-modified β_2m in the native form *in vivo* may exert a cytokine-inducing activity.

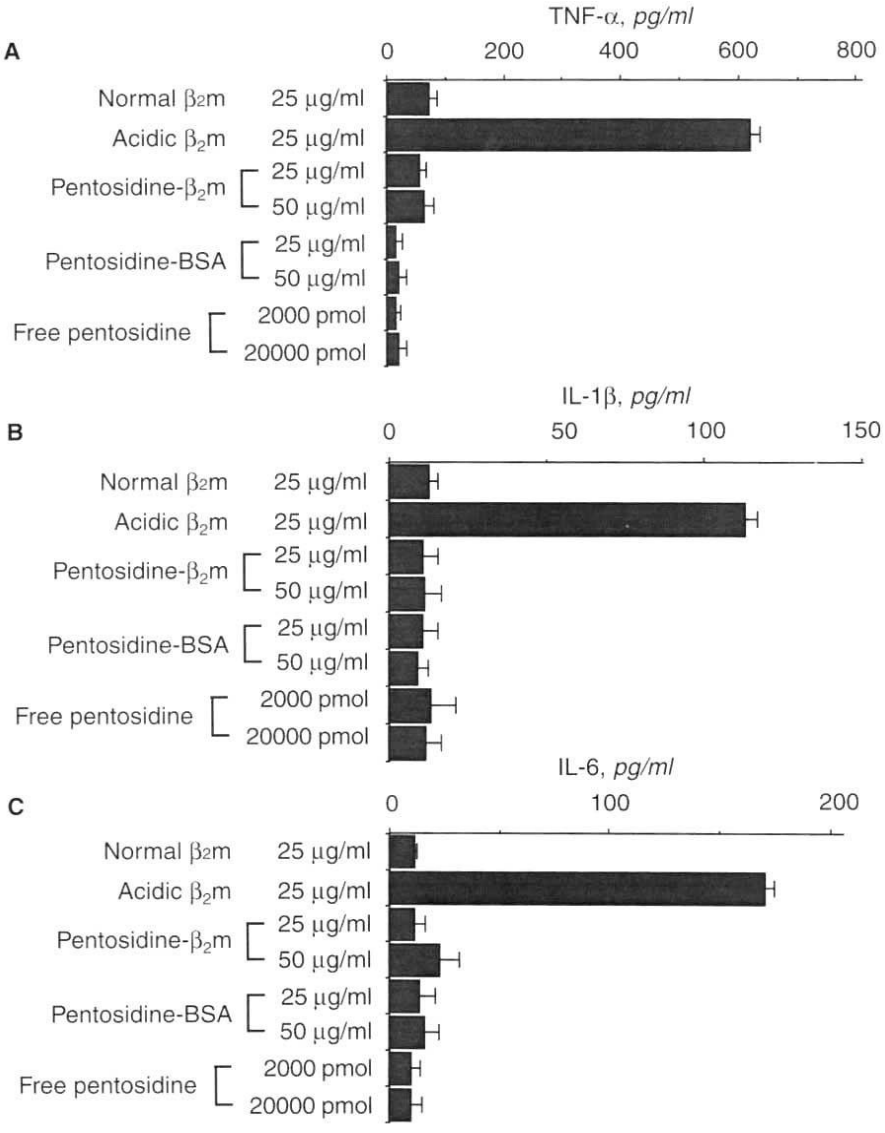

Fig. 9. *Cytokine inducing activity of pentosidine.* **A.** TNF-α. **B.** IL-1β. **C.** IL-6. The pentosidine levels in normal β_2m, acidic β_2m, and pentosidine-β_2m and -BSA were < 0.002 pmol/mg, 0.144 pmol/mg, 21.0 pmol/mg, and 29.1 pmol/mg, respectively, as estimated by HPLC assay [24]. Representative data from the three experiments are shown. Data are expressed as means ± SD. $N = 3$. $^*P < 0.0001$ versus normal β_2m.

Discussion

We hypothesized that AGEs formed nonenzymatically between β_2m and sugar play a pathological role in DRA. This contention was based on two recent findings. First, acidic β_2m is a major constituent of amyloid fibrils in DRA, and acidic β_2m in the serum, urine, and amyloid fibrils contains AGEs that have been implicated in tissue damage associated with diabetic complications and aging [6]. Secondly, acidic β_2m purified from urine of long-term hemodialysis patients exhibits various biological activities toward monocytes/macrophages that are characteristic for AGE-proteins [15, 16]. However, we found that acidic β_2m is a mixture of heterogeneous molecular adducts due to various types of modification. Among these, AGEs are present in only a small fraction of acidic β_2m, as shown previously in serum proteins [24] and skin collagen [29]. Several previous studies have demonstrated the biological effects of AGE-proteins on monocytes/macrophages [12–14], prepared *in vitro* by incubating proteins with glucose. However, these preparations also consisted of heterogeneously modified proteins such as deamidation, Amadori products and AGEs, among which AGEs are minor constituents. To our knowledge, the biological activity of deamidation and Amadori products has not been examined. Thus, we cannot simply conclude that AGEs are the causal modification for chemotactic activity and cytokine inducing activity of acidic β_2m. To further confirm our initial hypothesis, we analyzed the chemical modifications in acidic β_2m purified from urine of long-term hemodialysis patients and investigated the biological activity of each modification. The results indicated that neither deamidated β_2m nor Amadori β_2m, both of which were major modifications present in acidic β_2m, exhibited the biological activity. In contrast, AGE-β_2m, although only a minor constituent in acidic β_2m, induced monocyte chemotaxis and macrophage secretion of potent bone-resorbing cytokines such as TNF-α, IL-1β, and IL-6. These findings suggest that AGEs are responsible for the chemotactic activity and the cytokine inducing activity of acidic β_2m, conferring a pathological significance to AGEs in bone and joint destruction in DRA.

The present study revealed that deamidation occurred at the

two Asn-Gly sequences in β_2m via a succinimide intermediate which breaks down by two pathways to yield α-aspartate (Asp) and its β-isomer (isoAsp) in a ratio of about 1:3. A previous study using a synthetic peptide demonstrated a rapid deamidation reaction at Asn-Gly sequences with a half-life of only 1.4 days at 37°C, pH 7.4 [26], suggesting that Asn residues may be hot spots for nonenzymatic degradation of most proteins and can limit their biological lifetime. Thus, deamidation at Asn residues is not a modification specifically occurring in β_2m, but a generally occurring reaction with a variety of proteins. Furthermore, as deamidation is a spontaneous degradation reaction occurring easily under physiological conditions [28], some fraction of deamidated β_2m in the acidic β_2m preparation may be generated during the purification procedure. As far as we have investigated, deamidated β_2m exerts no biological activity towards monocytes/macrophages.

In regard to the amyloid formation (amyloidogenesis) of β_2m, Linke et al demonstrated that proteases can cleave some lysine residues of β_2m, thus increasing its hydrophobic tendency and contributing to amyloidogenesis [30]. Therefore, although fragmented β_2m did not exhibit the biological activity, proteolytically cleaved β_2m might favor amyloid formation. Although some researchers recently suggested the possible involvement of nonenzymatic glycation in the amyloidogenesis of β-amyloid peptide in Alzheimer's disease [31, 32], the role of AGEs or Amadori products in the amyloidogenesis of β_2m is unclear.

The pathological role of pentosidine in DRA is an issue of particular interest. Although our experiments concerning the biological activity of pentosidine-modified β_2m in the native form *in vivo* are as yet inconclusive, the results thus far obtained suggest that pentosidine is neutral with respect to cytokine induction in macrophages. Previous studies have demonstrated that pentosidine formation may be involved in oxidative stress [11, 23, 33], and that increased glycation promotes the formation of free radicals and enhances oxidative damage in proteins and lipids [34]. It was demonstrated that there is a close association between pentosidine level and occurrence of diabetic complications [35–37]. Therefore, the oxidative stress associated with pentosidine formation in amyloid fibrils might be involved in the development of bone and joint destruction of DRA.

Thus, precise structure of AGEs in acidic β_2m responsible for monocyte chemotaxis and cytokine induction from macrophages remains unknown. It was demonstrated that neither 2-(2-furoyl)-4 (5)-(2-furanyl)-1*H*-imidazole (FFI) [38], pyrraline [39], nor their BSA conjugates showed ligand activity for the AGE-receptor of rat peritoneal macrophages [22, 40], suggesting these AGE-structures may not be responsible for the biological activity toward monocytes/macrophages. In accord with the current findings, the chemical structure responsible for the biological activity may be different from the AGE-structures postulated so far. Our previous study [6] showed that acidic β_2m reacted with anti-AGE antibody [22]. However, the epitope is neither pentosidine, FFI, nor pyrraline (unpublished data), indicating the presence of another AGE-structure distinct from those postulated so far. A preliminary study by Makita et al [41] has also shown that neither pentosidine, FFI, nor pyrraline cross react with their anti-AGE antibody. At present, the structure of epitope of these anti-AGE antibodies remains unknown, and we do even not know whether or not this epitope really contributes to the biological activity toward monocytes/macrophages. Taking into account the findings of several reports concerning the biological effects of AGEs toward monocytes/macrophages [12–16], endothelial cells [42], and mesangial cells [43, 44], a further characterization of the remaining AGE-structure(s) and its biological significance is imperative to better understand the molecular mechanisms of the pathophysiological role of AGEs in diabetic complications, aging, and DRA.

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Appendix

Abbreviations used in this paper are: AGEs, advanced glycation end products; β_2m, β_2-microglobulin; BSA, bovine serum albumin; Endo Lys-C, Endoproteinase Lys-C; ESI, electrospray ionization; FAB, fast atom bombardment; FFI, 2-(2-furoyl)-4-(5)-(2-furanyl)-1*H*-imidazole; FMLP, *N*-formylmethionylleucylphenyl-alanine; DRA, dialysis-related amyloidosis; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; IL-1β, interleukin-1β; IL-6, interleukin-6; INF-γ, interferon-γ; MS, mass spectrometry; $NaBH_4$, sodium borohydride; $NaBH_3CN$, sodium cyanoborohydride; TNF-α, tumor necrosis factor-α.

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