Involvement of $\beta_2$-Microglobulin Modified with Advanced Glycation End Products in the Pathogenesis of Hemodialysis-associated Amyloidosis

Induction of Human Monocyte Chemotaxis and Macrophage Secretion of Tumor Necrosis Factor-$\alpha$ and Interleukin-1

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Abstract

$\beta_2$-Microglobulin ($\beta_2$M) is a major constituent of amyloid fibrils in hemodialysis-associated amyloidosis (HAA), a complication of long-term hemodialysis. However, the pathological role of $\beta_2$M in HAA remains to be determined. Recently, we demonstrated that $\beta_2$M in the amyloid deposits of HAA is modified with advanced glycation end products (AGEs) of the Maillard reaction. Since AGEs have been implicated in tissue damage associated with diabetic complications and aging, we investigated the possible involvement of AGE-modified $\beta_2$M (AGE-$\beta_2$M) in the pathogenesis of HAA. AGE- and normal-$\beta_2$M were purified from urine of long-term hemodialysis patients. AGE-$\beta_2$M enhanced directed migration (chemotaxis) and random cell migration (chemokinesis) of human monocytes in a dose-dependent manner. However, normal-$\beta_2$M did not enhance any migratory activity. AGE-$\beta_2$M, but not normal-$\beta_2$M, increased the secretion of TNF-$\alpha$ and IL-1$\beta$ from macrophages. Similar effects were also induced by in vitro prepared AGE-$\beta_2$M (normal-$\beta_2$M incubated with glucose in vitro for 30 d). When TNF-$\alpha$ or IL-1$\beta$ was added to cultured human synovial cells in an amount equivalent to that secreted from macrophages in the presence of AGE-$\beta_2$M, a significant increase in the synthesis of collagenase and morphological changes in cell shape were observed. These findings suggested that AGE-$\beta_2$M, a major component in amyloid deposits, participates in the pathogenesis of HAA as foci where monocyte/macrophage accumulation and initiate an inflammatory response that leads to bone/joint destruction. (J. Clin. Invest. 1994. 93:521–528.)

Key words: long-term hemodialysis patient • monocyte/macrophage • cytokine • human synovial cell • collagenase

Introduction

Hemodialysis-associated amyloidosis (HAA) is a serious complication among patients on long-term hemodialysis (1). Its clinical and radiological manifestations include chronic arthralgia, carpal tunnel syndrome, periarticular soft-tissue swelling, diffuse destructive arthropathy, spondylarthropathy, and subchondral bone erosions and cysts (1). The most distinctive histological feature of HAA is the deposition of amyloid fibrils in the affected joint structures, especially in periarticular bones.

Although recent biochemical and immunohistological studies have demonstrated that $\beta_2$-microglobulin ($\beta_2$M) is a major constituent of the amyloid fibrils in HAA (2–4), the pathological role of $\beta_2$M is not well understood. The serum $\beta_2$M level is markedly higher (usually > 30-fold) in these patients as compared with healthy individuals (5). However, there is no statistical correlation between the serum concentration of $\beta_2$M 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 an important factor in HAA, some unknown factor(s) has been suggested to be involved in the pathogenesis (5, 6). Histologically, amyloid deposits are surrounded by macrophages and other inflammatory cells, implicating a potential involvement of these cells in the pathogenesis of HAA (6, 7).

Recently, we observed that $\beta_2$M isolated from amyloid deposits of patients with HAA was subjected to electrophoresis at a much more acidic position than its normal counterpart and that this isof orm (referred to as acidic $\beta_2$M) was also detectable in a small fraction of $\beta_2$M in the serum and urine of these patients, but not in healthy individuals (8). We purified acidic $\beta_2$M as well as normal $\beta_2$M from the urine of long-term hemodialysis patients. Physicochemical and immunohistological analyses with anti–advanced glycation end products (AGEs) antibody showed that the acidic $\beta_2$M, a dominant constituent of the amyloid deposits in HAA, is generated by the modification of $\beta_2$M with AGEs of the Maillard reaction (8).

Through a series of chemical rearrangements and dehydro reactions, some Amadori products are converted to AGEs that accumulate on long-lived proteins (9, 10). Recent studies have demonstrated the in vivo presence of AGEs in human lens crystallin (11), human hemoglobin (12), and human $\beta_2$M (8). Among the postulated structures for AGEs so far are 2-(2-furanylidene)-4(5)-(2-furanylidene)-1H-imidazole (FFI) (13), pyrone aldehyde (14), pentosidin (15), and crosslines (16). In vitro prepared AGE-BSA induces transendothelial human mono-
cyte chemotaxis (17), and stimulates monocytes/macrophages via AGE receptors (18, 19), to secrete cytokines such as PDGF (17), TNF-α, and IL-1β (20). AGE-BSA is also known to stimulate mesangial cells to produce fibronectin (21). These findings suggested that AGE proteins may normally play a role in tissue remodeling, i.e., the removal and replacement of senescent extracellular matrix components (17, 20). However, under pathological conditions, the accumulation of AGE proteins may induce the subsequent pathogenic events seen in the vascular disease associated with diabetes and aging (17, 22).

These findings together with our previous observation that AGE-β2M is a dominant constituent of amyloid deposits in HAA, strongly suggest that the modification of β2M with AGEs plays an important role in the pathogenesis of HAA. To elucidate the potential role of β2M modified with AGEs referred to as AGE-β2M, we examined the effect of AGE-β2M on human monocyte migration and macrophage production of TNF-α and IL-1β, all of which play important roles in the pathogenesis of bone/joint diseases such as rheumatoid arthritis and osteoporosis (23–27). The results indicated that AGE-β2M induces the chemotaxis for monocytes and the secretion of cytokines from macrophages, which suffices to synthesize collagenase in synovial cells. This implicates that AGE-β2M in amyloid deposits functions as a pathogenic factor that recruits monocyte/macrophage in situ and initiates the inflammatory response, i.e., the production of cytokines leading to bone resorption and the synthesis of collagenase leading to matrix protein destruction.

Methods

Purification of normal- and AGE-β2M. We purified normal- and AGE-β2M from the urine of two long-term hemodialysis patients (56-yr-old male undergoing hemodialysis for 8 yr and 44-yr-old female undergoing hemodialysis for 5 yr). These patients had been on regular hemodialysis using a cuprophane dialyzer and a dialysate containing 30 mM/liter of bicarbonate and 8 mM/liter of acetate. The purification procedure and the physicochemical and immunological properties of the normal- and AGE-β2M were described previously (8). The purified normal- or AGE-β2M migrated as a single spot with a different pl value on two-dimensional polyacrylamide gel electrophoresis. The AGE-β2M, but not normal-β2M, was brown in color and fluoresced. The color and fluorescence are characteristics of the AGEs of the Maillard reaction (9, 10). The AGE-β2M also reacted with an anti-AGE antibody that recognizes AGE-BSA, AGE-HSA, and AGE-hemoglobin, but not nonglycated proteins and the early products of the Maillard reaction (28).

AGE-β2M was also purified in vitro by our previous method (8). Briefly, 2 mg of the purified normal-β2M was incubated at 37°C for 30 d with 0.1 M d-glucose (Wako Pure Chemicals, Tokyo, Japan) in 100 mM phosphate buffer containing 200 U/ml of penicillin, 80 μg/ml of gentamicin, and 1.5 mM of PMSF. When the extent of modification was estimated by two-dimensional polyacrylamide gel electrophoresis and fluorospectrometry, a major portion of in vitro prepared AGE-β2M shifted its electrophoretic mobility to a position similar to that of AGE-β2M and it showed a similar fluorescence spectrum with that of AGE-β2M, though the intensity of fluorescence detected in in vitro prepared AGE-β2M was weaker than that in AGE-β2M (8).

Removal of endotoxin from the purified β2M. To perform the experiments in endotoxin-free materials, we carefully removed endotoxin from the purified normal- and AGE-β2M, as well as in vitro prepared AGE-β2M as follows. The specimens were diluted with endotoxin-free distilled water (Otsuka Pharmaceutical, Tokyo, Japan) and ultrafiltrated three times through a filter with a cut-off value of a molecular weight of > 100,000 (Ultrafree C3-THK; Nihon Millipore Ltd., Tokyo, Japan). The ultrafiltrates were then mixed with endotoxin-adsorbent (Pyro Sep®; Daiel Chemical Industries, Ltd., Tokyo, Japan) for 2 h at 4°C and filtered through a 0.22-μm pore filter (Ultrafree C3-GV; Nihon Millipore Ltd.) to remove the adsorbent. The filtrates were concentrated by a filter with a cut-off value of a molecular weight of > 10,000 (Ultrafree C3-LGC; Nihon Millipore Ltd.). Finally, the condensed proteins were diluted to 1 mg/ml with DME; Nakai Tesque Kyoto, Japan) and sterilized with a 0.22-μm pore filter. The endotoxin levels were measured by a kit (Toxicolor* system; Seikagaku Corp., Tokyo, Japan), and all the materials and media used in this study were found to be endotoxin-free (< 0.5 U/ml of endotoxin).

Chemotaxis assays. Human mononuclear leukocytes from the blood of a healthy volunteer were separated from other cell types by sedimentation in 6% dextran (Nakai Tesque) and centrifugation through Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden), and suspended in HBSS at 6 × 10⁵ cells/ml. The analysis by a FACStar (Becton Dickinson & Co., Mountain View, CA) revealed that 22.5–27% of cells in these preparations were monocytes. Endotoxin-free normal- or AGE-β2M in DME was diluted with HBSS and their chemotactic activities were determined by a modification of the Boyden chamber technique (29) in a 24-well of double chambers separated by a 5-μm pore size filter (Coster Corp., Cambridge, MA). A human mononuclear leukocyte suspension (0.1 ml) was added to the top compartment and β2M in HBSS was added to the bottom (0.6 ml) or top compartment. The chambers were incubated at 37°C for 4 h, the filters were then removed, fixed in methanol, and stained with Giemsa. FMLP (Peninsula Labs, Inc., Belmont, CA), a synthetic peptide highly chemotactic for monocytes, served as a positive control. The experiment was repeated three times using mononuclear leukocyte preparations from three healthy males (Age range, 27–32 yr). Chemotactic activity was defined as the average number of cells that had migrated in response to the test substance. The number of cells in four high-power fields (×400) was counted for each of the duplicate chemotaxis chamber filters. The Student’s t test was used for the statistical analysis.

TNF-α and IL-1β assays. Fresh human peripheral blood monocytes were isolated by a Percoll gradient centrifugation from mononuclear leukocytes obtained as described above. The monocytes (2.5 × 10⁷ cells) were incubated in duplicate at 37°C for 24 h in 0.5 ml of DME with 50 U IFN-γ (Shionogi Pharmaceutical Co., Osaka, Japan), and 12.5, 25, or 50 μg/ml (final concentration) of normal-β2M or AGE-β2M, or 50 μg/ml of in vitro prepared AGE-β2M. INF-γ was added to transform peripheral monocytes into the equivalent of primed tissue macrophages (30, 31). Escherichia coli 0113:B4 endotoxin (Difco Laboratories, Detroit, MI) was used as a positive control. The culture was performed in 48-well culture plates (Sulmon; Sumitomo Bakelite Co., Ltd., Tokyo, Japan). After incubation, culture media and cells were transferred into sterile microfuge tubes and centrifuged at 400 g for 5 min. Cytokine levels (TNF-α and IL-1β) were measured in duplicate in each supernatant with ELISA kits according to the manufacturer’s technical guidelines (Amersham, Buckinghamshire, UK). The experiment was repeated three times using monocyte preparations from three healthy males. The precipitated cells were also used for reverse transcriptase (RT)-PCR analysis as described below.

RT-PCR analysis. The levels of IL-1β mRNA were semi-quantitated by RT-PCR. Total RNA was isolated from the precipitated cells as described above by a single-step guanidinium thiocyanate-phenol-chloroform method (32). 10% of the RNA was reverse transcribed using random primers (Takara, Kyoto, Japan) with 200 U of RNase H-free RT (Superscript; GIBCO BRL, Gaithersburg, MD) in a final volume of 20 μl solution containing 50 mM Tris hydrochloride (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 100 μg/ml BSA, and 3 mM MgCl₂.

PCR amplification was performed in a total volume of 50 μl (with 2 μl of the reverse-transcribed products) containing 67 mM Tris hydrochloride (pH 8.8), 17 mM ammonium sulfate, 6.7 mM MgCl₂, 10 μM β-mercaptoethanol, 6.7 μM EDTA, 0.67 mg/ml BSA, 1.5 mM each of the four deoxyribonucleoside triphosphates (Pharmacia), 10 μCi [32P]dCTP (Amersham Corp.), 50 pmol sense primer (5’ end), 50
pmol antisense primer (3' end), and 2.5 U AmpliTaq™DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). The oligonucleotides primer sequences are as follows. The primers for IL-1β were: sense, 5'-ATG-GCAGAAGTACCTGAGCTC-3'; and antisense, 5'-TTCTTGTAGGCCCAGGCGCAC-3'. The expected size of the amplified fragment was 540 bp. The primers for β-actin were: sense, 5'-ACCTTACAACCCAGCGCATG-3'; and antisense, 5'-GGCCATCTTTGCTGAA-GTC-3'. The β-actin served as an internal RNA control to allow comparison of RNA levels among different specimens. Specimens were amplified in a DNA Thermal Cycler (Perkin Elmer Cetus) for 18–39 cycles. Each cycle consisted of incubation for denaturation at 93°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. An aliquot of PCR product was then resolved by electrophoresis in a 6% polyacrylamide gel and visualized by autoradiography. PCR products were quantified using a Fuji Bio-Imaging Analysis System 2000 (Fuji Photo Film Co., Ltd., Tokyo, Japan). The relative intensity of individual bands was expressed as relative counts per minute.

Human synovial cell culture. Synovial tissues were obtained from a healthy individual during an operation on the knee joint for a traumatic injury. Synovial cells were cultured according to the method of Dayer et al. (33). Cells were grown in DME containing 10% FCS (Cell Culture Lab., Cleveland, OH), 50 μg/ml penicillin G, 50 μg/ml streptomycin sulfate (GIBCO BRL), and 1 mg/ml amphotericin B (Flow Labs., Irvine, Scotland). They were used between the seventh and eighth passage.

Determination of collagenase mRNA levels by dot blot hybridization. The cultured human synovial cells were plated into six-well (35-mm diameter) plastic culture plates (Multiflitt™ [Falcon 3046]; Becton Dickinson Labware, Lincoln Park, NJ), grown to confluence, rinsed with serum-free medium, and incubated in DME containing 1 mg/ml of BSA for 24 h. Cells were then stimulated with several concentrations of either TNF-α (Asahi Chemical Industry Co. Ltd., Tokyo, Japan) or IL-1β (Oncogene Science Inc., Uniondale, NY) for 12 h at 37°C. Total RNA (6.5 μg) was mixed with 20% formaldehyde and 6× SSC and applied to the activated nylon filter (GeneScreen Plus®, New England Nuclear, Boston, MA), and then the filter was baked for 2 h at 80°C in a vacuum oven. The blots were hybridized in 50% formamide, 5× SSPE, 2× Denhardt’s reagent, 0.1% SDS, and 100 μg/ml of herring sperm DNA for 20 h at 42°C. Hybridization was performed overnight at 42°C after the denatured radiola-beled probe for human collagenase (10 cpm/ml) was added directly to the prehybridization fluid. Collagenase cDNA was pCol 185.2 (34). Then, the filter was washed twice for 5 min in 2× SSC, followed by two 30-min washes in 2× SSC, 1% SDS at 65°C, and two 10-min washes in 0.1% SSC at room temperature. The damp filters were exposed to Kodak X-omat film with an intensifying screen at ~80°C for 22–60 h. The amount of the RNA was analyzed using an Image Analyzer (Immunomedica Co. Ltd., Shizuoka, Japan).

Results

AGE-β2M is chemotactic for human monocytes. To test whether AGE-β2M could be a potent chemotactic factor for peripheral human monocytes, their migration toward AGE-β2M was measured in modified Boyden chambers. Previous studies demonstrated that >95% of the mononuclear leukocytes that migrated in this chemotactic assay were myeloperoxidase positive, i.e., monocytes (35), and that the presence of ∼80% lymphocytes in the cell preparation had no effect on monocyte chemotaxis (36). In this study, FACS analysis showed that mononuclear leukocyte preparations contained 73–77.5% lymphocytes. Moreover, our preliminary experiments revealed that almost all migrated cells were myeloperoxidase positive (data not shown), indicating that these migrated cells were monocytes. AGE-β2M exhibited direct migratory activity in a dose-dependent manner (Fig. 1). However, normal-β2M did not enhance the migratory activity of human monocytes. The activity of AGE-β2M was significantly higher than that of normal-β2M at all concentrations tested (*P < 0.001). The response induced by 100 μg/ml of AGE-β2M corresponded to ∼80% of that elicited by 10 ng/ml of FMLP, a well-known monocyte chemotactic factor.

As shown in Table I, checkerboard analysis of the movement of monocytes revealed that AGE-β2M enhanced both directed chemotaxis and random cell migration (chemokinesis).

AGE-β2M induces TNF-α synthesis and secretion in macrophages. Next, we examined the effect of AGE-β2M on the production of cytokines (TNF-α and IL-1β) from macrophages. Freshly isolated human monocytes were incubated for 24 h in endotoxin-free medium with endotoxin-free normal-β2M, AGE-β2M, or in vitro prepared AGE-β2M, followed by determination of TNF-α in the culture supernatant with an ELISA system. As shown in Fig. 2, addition of AGE-β2M resulted in a dose-dependent increase of TNF-α in the culture medium. By contrast, the medium from the cells incubated with normal-β2M contained little TNF-α. The small amount of TNF-α detected in the culture medium from normal-β2M could be due to contamination with a trace amount of AGE-β2M during purification. Neither INF-γ nor normal alone induced the secretion of a detectable amount of TNF-α from the cells. Note...
Table 1. Checkerboard Analysis of Monocyte Chemotactic Response to AGE-β2M

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<tr>
<th>AGE-β2M in bottom compartment</th>
<th>AGE-β2M in top compartment</th>
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<tbody>
<tr>
<td>µg/ml</td>
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<tr>
<td>0</td>
<td>2.0±1.4</td>
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<tr>
<td>50</td>
<td>16.3±4.3</td>
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<td>100</td>
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Chemotactic activity was defined as the average number of cells that had migrated in response to AGE-β2M. The number of cells in four high-power fields (×400) was counted for each of the duplicate chemotaxis chamber filters. The experiment was repeated three times. Each experiment used mononuclear leukocytes obtained from a healthy subject. The data are expressed as means±SD. Representative data from three experiments are shown.

that AGE-β2M obtained by in vitro glycation of normal-β2M could also induce macrophages to produce TNF-α. These findings do agree with the previous finding by Vlassara et al. (20) that BSA modified with AGEs by in vitro incubation with glucose, glucose-6-phosphate, or FFI, could stimulate the secretion of TNF-α from macrophages.

**AGE-β2M induces IL-1β synthesis and secretion in macrophages.** We examined the effect of AGE-β2M on macrophage secretion of IL-1β, another important cytokine in the inflammatory process. Monocytes were incubated for 24 h with normal-β2M, AGE-β2M, or in vitro prepared AGE-β2M, and IL-1β levels in the culture supernatant were measured with an ELISA. As Fig. 3 shows, a dose-dependent increase of IL-1β level was observed in the medium from the cells incubated with AGE-β2M. On the other hand, IL-1β levels were much lower in the medium from the cells incubated with normal-β2M. IL-1β was not detected in the culture medium from the cells incubated with INF-γ or medium alone. A slight induction of IL-1β by normal-β2M at the concentration of 50 µg/ml could be due to a trace amount of AGE-β2M contamination during preparation. In vitro prepared AGE-β2M could also induce the secretion of IL-1β. This finding is consistent with a previous report that AGE-BSA can stimulate macrophages to secrete IL-1β (20).

Not only secreted IL-1β but also its membrane-bound form are assumed to be biologically active (37, 38). However, the ELISA in this study detected only the secreted-form of IL-1β. Thus, we analyzed IL-1β mRNA expression by RT-PCR. As shown in Fig. 4, A and B, there was a linear correlation between the number of cycles (~33 cycles) and logarithm of the yield of PCR products of both IL-1β and β-actin cDNAs, indicating that within these cycles our PCR conditions can be used for semi-quantification of the respective mRNAs. Only a faint signal was observed in the cells incubated with normal-β2M (Fig. 4 C, lane 1) as well as the cells incubated with INF-γ (lane 5) or medium alone (lane 6). However, a marked increase in IL-1β mRNA was detected in the cells incubated with AGE-β2M (lane 2), in vitro prepared AGE-β2M (lane 3), or endotoxins (lane 4, positive control). The sample RNAs that had not been reverse transcribed did not yield the PCR product, as exemplified in Fig. 4 C, lane 7, confirming the absence of the extraneous cDNA or PCR product contaminating the samples. From the known sequence of IL-1β cDNA, this amplified 540-bp

![Figure 2. TNF-α secretion by human macrophages in response to AGE-β2M.](image)

Monocytes were incubated in medium containing human INF-γ (50 U) in the presence of normal- and AGE-β2M (12.5, 25, and 50 µg/ml), and in vitro prepared AGE-β2M (50 µg/ml) for 24 h at 37°C. TNF-α level in each culture medium was measured by an ELISA (see Methods). In some wells, cells were incubated in medium containing Bt endotoxin from E. coli (1.5 and 3 ng/ml; positive control) or INF-γ alone, or in medium alone. The experiment was repeated three times using monocyte preparations from three healthy males. In the entire experiment, addition of AGE-β2M resulted in a dose-dependent increase of TNF-α in the culture medium. Representative data from the three experiments are shown. The data are expressed as means±range.

![Figure 3. IL-1β secretion by human macrophages in response to AGE-β2M.](image)

Monocytes were incubated in medium containing INF-γ with normal- and AGE-β2M, and in vitro prepared AGE-β2M for 24 h at 37°C (see Fig. 2 legend). The culture supernatants were used for measuring IL-1β levels by an ELISA. The experiment was repeated three times using monocyte preparations from three healthy males. In the entire experiment, addition of AGE-β2M resulted in a dose-dependent increase of IL-1β in the culture medium. Representative data from the three experiments are shown. The data are expressed as means±range.
fragment should have a HindIII site, generating two fragments of 400 and 140 bp. Indeed, the PCR product from the cells incubated with AGE-β2M was digested with HindIII (Fig. 4 C, lane 8). These findings, together with the results of ELISA, indicate that AGE-β2M induces the synthesis and secretion of IL-1β in monocyte-derived macrophages.

TNF-α and IL-1β induce collagenase synthesis in human synovial fibroblasts. It is already established that collagenase plays a role in the pathophysiology (tissue destruction and remodeling) of bone/joint diseases, such as rheumatoid arthritis (39). Thus, we tested whether TNF-α and IL-1β, in such amounts that are produced from macrophages by AGE-β2M, do in fact induce collagenase synthesis in primary cultured human synovial cells.

Cells were serum starved for 26 h to avoid interference of serum components with collagenase mRNA induction, then stimulated with either TNF-α or IL-1β (Fig. 5 A). The equivalent amounts of TNF-α and IL-1β produced from macrophages by 50 μg/ml of AGE-β2M sufficed to cause a dose-dependent induction of collagenase. Interestingly, this collagenase synthesis by TNF-α and IL-1β was accompanied by morphological changes in cell shape (exemplified with TNF-α in Fig. 5 B). The cells treated for 12 h with these cytokines became dendritic in shape with round cytoplasm and long radial extensions, whereas the shape of the untreated cells was not altered. This is consistent with previous reports that changes in cell shape correlate well with collagenase gene expression in rabbit synovial cells (40), and that the cells dendritic in shape among human synovial cells cultured in vitro actively secrete collagenase (41).

**Discussion**

Recently, we demonstrated that AGE-β2M is a dominant constituent of the amyloid deposits in HAA. In this study we tested whether AGE-β2M could participate in the pathogenesis of HAA, and provided evidence that AGE-β2M may function as a pathogenic factor that induces the inflammatory response leading to joint/bone destruction in HAA (Fig. 6). First, AGE-β2M enhanced the chemotaxis and chemokinesis of human monocytes, but normal-β2M did not. This finding gives an explanation for the preferential localization of monocytes/macro- phages to amyloid deposits. Amyloid deposits, in which AGE-β2M is a major component, could be foci where monocytes/macrophages accumulate. This is in agreement with the reports that amyloid deposits are surrounded by macrophages and other inflammatory cells (6, 7).

Second, AGE-β2M, but not normal-β2M, stimulated macrophages to secrete inflammatory cytokines such as TNF-α and IL-1β. In vitro prepared AGE-β2M obtained by incubating normal-β2M with glucose also induced these cytokines. A previous report by Vlassara et al. (20) demonstrated that AGE-BSA prepared in vitro by incubation with glucose, glucose-6-phosphate, or FF1 induces the secretion of these cytokines from macrophages. To our knowledge, this study demonstrates for the first time that purified AGE proteins (AGE-β2M) formed in vivo do have the same biological effect. The induction of these cytokines would be due to the interaction of AGE-β2M with the AGE-receptor of monocytes/macrophages responsible for endocytic uptake of AGE-modified proteins (18, 20). Vlassara et al. (19) reported that this AGE receptor of monocytes/macrophages is upregulated several-fold by TNF-α. Since TNF-α induces IL-1β, and IL-1β is a potent inducer of TNF-α vice versa (42), the induction of TNF-α and IL-1β observed by AGE-β2M is not due to a single effect of AGE-β2M,
but is likely to reflect a secondary response augmented by these cytokines.

These cytokines are known to have diverse biological effects on several cell types (42). Among them are the effects on bone remodeling. Either TNF-α or IL-1β stimulates bone resorption (23-26), which requires the presence of osteoblasts to activate osteoclasts to resorb bone (43, 44). The mechanism postulated so far is as follows. Both TNF-α and IL-1β are potent stimulants for monocytes/macrophages (42) and osteoblasts (43, 45) to secrete IL-6, which in turn induce the differentiation of osteoclasts from precursors (43, 45, 46). These osteoclasts are then activated by the action of a particular set of cytokines that are released from osteoblasts by TNF-α and IL-1β (43, 44). Furthermore, bone formation is inhibited by either TNF-α or IL-1β (23, 24, 42). These findings together with our present observation that AGE-β2M stimulates the secretion of these cytokines from monocytes/macrophages, suggest that AGE-β2M functions as a stimulator for bone resorption and as an inhibitor of bone formation at the site of the amyloid deposit.

In addition, the amount of TNF-α and IL-1β produced by AGE-β2M was sufficient to stimulate collagenase synthesis in cultured human synovial cells. Since collagenase is an important proteolytic enzyme that degrades matrix proteins, our finding suggested the involvement of AGE-β2M in the destruction of matrix proteins. Furthermore, based on the previous reports that the degraded products of collagen are chemotactic for monocytes/macrophages (47) and stimulate them to secrete IL-1 (48), the following mechanism is possible. AGE-β2M enhances the migration of monocytes toward amyloid deposits and stimulates them to secrete TNF-α and IL-1β. This subsequently stimulates synovial cells to secrete collagenase that degrades collagen, resulting in further migration of these inflammatory cells into amyloid deposits and the production of the inflammatory cytokine. Thus, the induction of collagenase leads to the vicious cycle formed among monocytes/macrophages and synovial cells via cytokines.
Little is known about the mechanism for recruiting AGE-β2M preferentially to joint structures and periarticular bones. Of particular interest from a pathological perspective is the tendency of AGE-modified proteins to cross-link and polymerize (9, 10, 49). Thus, one possible explanation is that AGE modification of β2M leads to the polymerization of β2M and the association with some matrix protein(s) in these connective tissues. The close association of AGE-modified proteins with matrix proteins such as collagen has been emphasized (21, 22, 50). Also unknown is to what extent AGE modification of β2M correlates with the formation of amyloid deposits to occur. Connors et al. (51) demonstrated that a high concentration of intact β2M could change into amyloid fibrils in vitro by salt-free dialysis. Thus, one possibility could be that AGE modification accelerates the amyloid formation of β2M. Or, alternatively, monocytes/macrophages may actively participate in the amyloid formation of AGE-β2M, either by degrading or by modifying AGE-β2M. At present, no definite answer can be given to the intriguing questions of preferential localization of amyloid deposits and amyloidogenesis.

Several lines of evidence have emphasized a potential role of AGES in the pathogenesis of atherosclerosis in diabetes and aging. To our knowledge, this study demonstrates for the first time the pathological role of AGE modification in arthropathic complications.

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References


