

Involvement of β_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- α and Interleukin-1

Toshio Miyata,* Reiko Inagi,† Yoshiyasu Iida,* Motoyoshi Sato,* Norio Yamada,* Osamu Oda,‡ Kenji Maeda,* and Hisao Seo^{||}

*Department of Internal Medicine, The Branch Hospital, Nagoya University School of Medicine, Nagoya 461; †Department of Bacteriology, Osaka University Medical School, Osaka 565; ‡The Bio-Dynamics Research Institute, Nagoya Memorial Hospital, Nagoya 468; and ^{||}Department of Endocrinology and Metabolism, Research Institute of Environmental Medicine, Nagoya University, Nagoya 464, Japan

Abstract

β_2 -Microglobulin (β_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 β_2 M in HAA remains to be determined. Recently, we demonstrated that β_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 β_2 M (AGE- β_2 M) in the pathogenesis of HAA. AGE- and normal- β_2 M were purified from urine of long-term hemodialysis patients. AGE- β_2 M enhanced directed migration (chemotaxis) and random cell migration (chemokinesis) of human monocytes in a dose-dependent manner. However, normal- β_2 M did not enhance any migratory activity. AGE- β_2 M, but not normal- β_2 M, increased the secretion of TNF- α and IL-1 β from macrophages. Similar effects were also induced by in vitro prepared AGE- β_2 M (normal- β_2 M incubated with glucose in vitro for 30 d). When TNF- α or IL-1 β was added to cultured human synovial cells in an amount equivalent to that secreted from macrophages in the presence of AGE- β_2 M, a significant increase in the synthesis of collagenase and morphological changes in cell shape were observed. These findings suggested that AGE- β_2 M, a major component in amyloid deposits, participates in the pathogenesis of HAA as foci where monocyte/macrophage accumulate 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 • cytokines • human synovial cell • collagenase

Introduction

Hemodialysis-associated amyloidosis (HAA)¹ 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 β_2 -microglobulin (β_2 M) is a major constituent of the amyloid fibrils in HAA (2-4), the pathological role of β_2 M is not well understood. The serum β_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 β_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 β_2 M level. Thus, despite the fact that the prolonged elevation of serum β_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 β_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 isoform (referred to as acidic β_2 M) was also detectable in a small fraction of β_2 M in the serum and urine of these patients, but not in healthy individuals (8). We purified acidic β_2 M as well as normal β_2 M from the urine of long-term hemodialysis patients. Physicochemical and immunochemical analyses with anti-advanced glycation end products (AGEs) antibody showed that the acidic β_2 M, a dominant constituent of the amyloid deposits in HAA, is generated by the modification of β_2 M with AGEs of the Maillard reaction (8).

Through a series of chemical rearrangements and dehydration 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 β_2 M (8). Among the postulated structures for AGEs so far are 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI) (13), pyrrole aldehyde (14), pentosidine (15), and crosslines (16). In vitro prepared AGE-BSA induces transendothelial human mono-

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

Received for publication 9 August 1993 and in revised form 18 October 1993.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/94/02/0521/08 \$2.00

Volume 93, February 1994, 521-528

1. Abbreviations used in this paper: AGE- β_2 M, advanced glycation end product-modified β_2 -microglobulin; FFI, 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole; HAA, hemodialysis-associated amyloidosis.

cyte chemotaxis (17), and stimulates monocytes/macrophages via AGE receptors (18, 19), to secrete cytokines such as PDGF (17), TNF- α , and IL-1 β (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- β_2 M is a dominant constituent of amyloid deposits in HAA, strongly suggest that the modification of β_2 M with AGEs plays an important role in the pathogenesis of HAA. To elucidate the potential role of β_2 M modified with AGEs (referred to as AGE- β_2 M), we examined the effect of AGE- β_2 M on human monocyte migration and macrophage production of TNF- α and IL-1 β , 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- β_2 M induces the chemotaxis for monocytes and the secretion of cytokines from macrophages, which suffices to synthesize collagenase in synovial cells. This implicates that AGE- β_2 M 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- β_2 M. We purified normal- and AGE- β_2 M from 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 mEq/liter of bicarbonate and 8 mEq/liter of acetate. The purification procedure and the physicochemical and immunochemical properties of the normal- and AGE- β_2 M were described previously (8). The purified normal- or AGE- β_2 M migrated as a single spot with a different pI value on two-dimensional polyacrylamide gel electrophoresis. The AGE- β_2 M, but not normal- β_2 M, was brown in color and fluoresced. The color and fluorescence are characteristics of the AGEs of the Maillard reaction (9, 10). The AGE- β_2 M 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- β_2 M was also prepared in vitro by our previous method (8). Briefly, 2 mg/ml of the purified normal- β_2 M 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 μ g/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- β_2 M shifted its electrophoretic mobility to a position similar to that of AGE- β_2 M and it showed a similar fluorescence spectrum with that of AGE- β_2 M, though the intensity of fluorescence detected in in vitro prepared AGE- β_2 M was weaker than that in AGE- β_2 M (8).

Removal of endotoxin from the purified β_2 M. To perform the experiments in endotoxin-free materials, we carefully removed endotoxin from the purified normal- and AGE- β_2 M, as well as in vitro prepared AGE- β_2 M as follows. The specimens were diluted with endotoxin-free distilled water (Otsuka Pharmaceutical, Tokyo, Japan) and ultrafiltered 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[®]; Daicel Chemical Industries, Ltd., Tokyo, Japan) for 2 h at 4°C and filtered through a 0.22- μ m-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; Nacalai Tesque Kyoto, Japan) and sterilized with a 0.22- μ m-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 (Nacalai Tesque) and centrifugation through Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden), and suspended in HBSS at 6×10^5 cells/ml. The analysis by a FACS[®] (Becton Dickinson & Co., Mountain View, CA) revealed that 22.5–27% of cells in these preparations were monocytes. Endotoxin-free normal- or AGE- β_2 M 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- μ m-pore size filter (Costar Corp., Cambridge, MA). A human mononuclear leukocyte suspension (0.1 ml) was added to the top compartment and β_2 M 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 ($\times 400$) 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-1 β assays. Fresh human peripheral blood monocytes were isolated by a Percoll gradient centrifugation from mononuclear leukocytes obtained as described above. The monocytes (2.5×10^5 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 μ g/ml (final concentration) of normal- β_2 M or AGE- β_2 M, or 50 μ g/ml of in vitro prepared AGE- β_2 M. 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 (Sumiloin; 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-1 β) 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-1 β mRNAs 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 μ l solution containing 50 mM Tris hydrochloride (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 100 μ g/ml BSA, and 3 mM MgCl₂.

PCR amplification was performed in a total volume of 50 μ l (with 2 μ l of the reverse-transcribed products) containing 67 mM Tris hydrochloride (pH 8.8), 17 mM ammonium sulfate, 6.7 mM MgCl₂, 10 mM β -mercaptoethanol, 6.7 μ M EDTA, 0.67 mg/ml BSA, 1.5 mM each of the four deoxynucleoside triphosphates (Pharmacia), 10 μ Ci [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-1 β were: sense, 5'-ATG-GCAGAAGTACCTGAGCTC-3'; and antisense, 5'-TTCCTTGAG-GCCCAAGGCCAC-3'. The expected size of the amplified fragment was 540 bp. The primers for β -actin were: sense, 5'-ACCTTCAACACC-CCAGCCATG-3'; and antisense, 5'-GGCCATCTCTTGCTCGAA-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 Fujix 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 U/ml penicillin G, 50 μ g/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 (Multiwell™ [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 26 h. Cells were then stimulated with several concentrations of either TNF- α (Asahi Chemical Industry Co. Ltd., Tokyo, Japan) or IL-1 β (Oncogene Science Inc., Uniondale, NY) for 12 h at 37°C. Total RNA (6.5 μ g) was mixed with 20% formaldehyde and 6 \times 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 \times SSPE, 2 \times Denhardt's reagent, 0.1% SDS, and 100 μ g/ml of herring sperm DNA for 20 h at 42°C. Hybridization was performed overnight at 42°C after the denatured radiolabeled probe for human collagenase (10⁶ 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 \times SSC, followed by two 30-min washes in 2 \times SSC, 1% SDS at 65°C, and two 10-min washes in 0.1% SDS 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- β_2 M is chemotactic for human monocytes. To test whether AGE- β_2 M could be a potent chemotactic factor for peripheral human monocytes, their migration toward AGE- β_2 M 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- β_2 M exhibited direct migratory activity in a dose-dependent manner (Fig. 1). However, normal- β_2 M did not en-

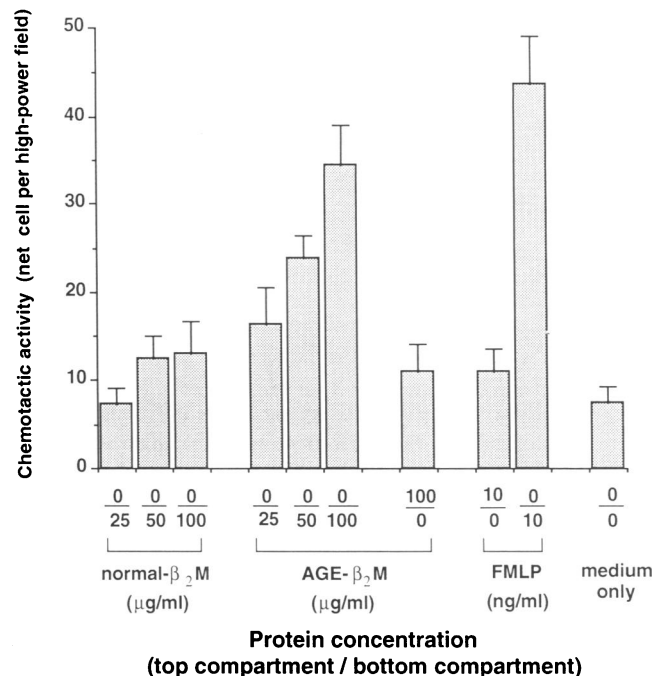


Figure 1. Monocyte chemotaxis to AGE- β_2 M. Monocyte migration was measured in response to increased concentrations (25, 50, and 100 μ g/ml) of normal- and AGE- β_2 M using a modified Boyden chamber (see Methods). FMLP at 10 ng/ml served as positive control. Chemotactic activity was defined as the average number of cells that had migrated in response to the test substance. The experiment was repeated three times using mononuclear leukocyte preparations from three healthy males. The number of cells in four high-power fields ($\times 400$) was counted for each of the duplicate chemotaxis chamber filters. In all the experiment, AGE- β_2 M exhibited direct migratory activity in a dose-dependent manner. Representative data from the three experiments are shown. The data are expressed as means \pm SD.

hance the migratory activity of human monocytes. The activity of AGE- β_2 M was significantly higher than that of normal- β_2 M at all concentrations tested ($P < 0.001$). The response induced by 100 μ g/ml of AGE- β_2 M 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- β_2 M enhanced both directed chemotaxis and random cell migration (chemokinesis).

AGE- β_2 M induces TNF- α synthesis and secretion in macrophages. Next, we examined the effect of AGE- β_2 M on the production of cytokines (TNF- α and IL-1 β) from macrophages. Freshly isolated human monocytes were incubated for 24 h in endotoxin-free medium with endotoxin-free normal- β_2 M, AGE- β_2 M, or in vitro prepared AGE- β_2 M, followed by determination of TNF- α in the culture supernatant with an ELISA system. As shown in Fig. 2, addition of AGE- β_2 M resulted in a dose-dependent increase of TNF- α in the culture medium. By contrast, the medium from the cells incubated with normal- β_2 M contained little TNF- α . The small amount of TNF- α detected in the culture medium from normal- β_2 M could be due to contamination with a trace amount of AGE- β_2 M during purification. Neither INF- γ nor medium alone induced the secretion of a detectable amount of TNF- α from the cells. Note

Table I. Checkerboard Analysis of Monocyte Chemotactic Response to AGE- β_2 M

AGE- β_2 M in bottom compartment	AGE- β_2 M in top compartment		
	0	50	100
	$\mu\text{g/ml}$		
0	2.0 \pm 1.4	3.0 \pm 2.5	5.5 \pm 1.8
50	16.3 \pm 4.3	9.5 \pm 1.8	8.5 \pm 3.4
100	22.0 \pm 4.2	15.8 \pm 4.0	11.0 \pm 3.1

Chemotactic activity was defined as the average number of cells that had migrated in response to AGE- β_2 M. The number of cells in four high-power fields ($\times 400$) 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 \pm SD. Representative data from three experiments are shown.

that AGE- β_2 M obtained by in vitro glycation of normal- β_2 M 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- β_2 M induces IL-1 β synthesis and secretion in macrophages. We examined the effect of AGE- β_2 M on macrophage secretion of IL-1 β , another important cytokine in the inflammatory process. Monocytes were incubated for 24 h with nor-

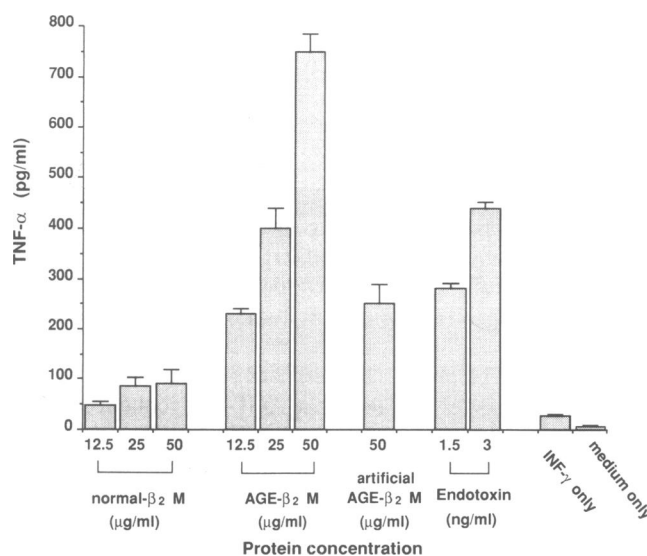


Figure 2. TNF- α secretion by human macrophages in response to AGE- β_2 M. Monocytes were incubated in medium containing human INF- γ (50 U) in the presence of normal- and AGE- β_2 M (12.5, 25, and 50 $\mu\text{g/ml}$), and in vitro prepared AGE- β_2 M (50 $\mu\text{g/ml}$) for 24 h at 37°C. TNF- α level in each culture medium was measured by an ELISA (see Methods). In some wells, cells were incubated in medium containing B₄ 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- β_2 M 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 mean \pm range.

mal- β_2 M, AGE- β_2 M, or in vitro prepared AGE- β_2 M, and IL-1 β levels in the culture supernatant were measured with an ELISA. As Fig. 3 shows, a dose-dependent increase of IL-1 β level was observed in the medium from the cells incubated with AGE- β_2 M. On the other hand, IL-1 β levels were much lower in the medium from the cells incubated with normal- β_2 M. IL-1 β was not detected in the culture medium from the cells incubated with INF- γ or medium alone. A slight induction of IL-1 β by normal- β_2 M at the concentration of 50 $\mu\text{g/ml}$ could be due to a trace amount of AGE- β_2 M contamination during preparation. In vitro prepared AGE- β_2 M could also induce the secretion of IL-1 β . This finding is consistent with a previous report that AGE-BSA can stimulate macrophages to secrete IL-1 β (20).

Not only secreted IL-1 β 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-1 β . Thus, we analyzed IL-1 β 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-1 β 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- β_2 M (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-1 β mRNA was detected in the cells incubated with AGE- β_2 M (lane 2), in vitro prepared AGE- β_2 M (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-1 β cDNA, this amplified 540-bp

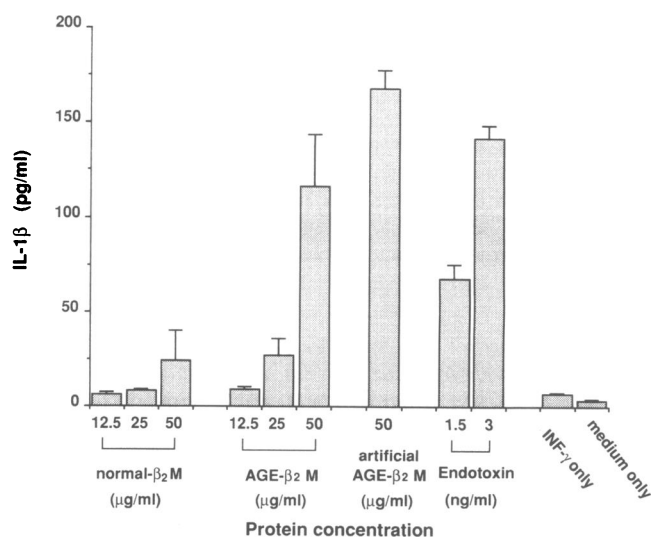


Figure 3. IL-1 β secretion by human macrophages in response to AGE- β_2 M. Monocytes were incubated in medium containing INF- γ with normal- and AGE- β_2 M, and in vitro prepared AGE- β_2 M for 24 h at 37°C (see Fig. 2 legend). The culture supernatants were used for measuring IL-1 β levels by an ELISA. The experiment was repeated three times using monocyte preparations from three healthy males. In the entire experiment, addition of AGE- β_2 M resulted in a dose-dependent increase of IL-1 β in the culture medium. Representative data from the three experiments are shown. The data are expressed as means \pm range.

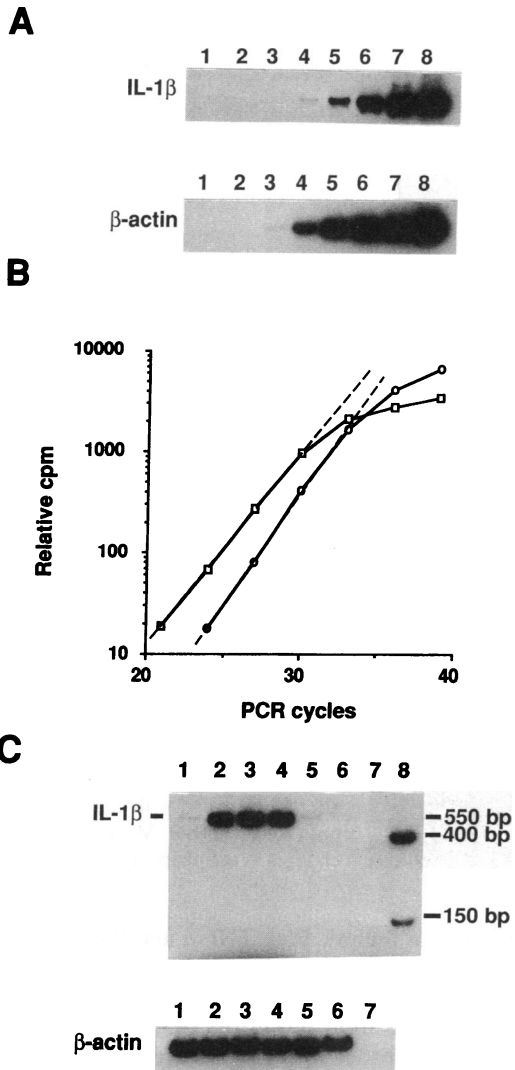


Figure 4. IL-1 β mRNA synthesized by macrophages in response to AGE- β_2 M. (A) Reverse transcription was performed on total RNA from cells incubated with 50 μ g/ml AGE- β_2 M as described in Methods, and IL-1 β (top) and β -actin (bottom) cDNAs were amplified by PCR for 18–39 cycles and analyzed on a 6% polyacrylamide gel. Lanes 1–8 correspond to 18, 21, 24, 27, 30, 33, 36, and 39 cycles, respectively. (B) The log of relative cpm incorporated into the IL-1 β (circles) and β -actin (squares) products was plotted against the number of cycles to give relative amplification rates. (C) Total RNAs from the cells incubated with 50 μ g/ml normal- β_2 M (lane 1), AGE- β_2 M (lane 2), and in vitro prepared AGE- β_2 M (lane 3), 3 ng/ml endotoxin (lane 4), INF- γ alone (lane 5), and medium alone (lane 6) were reverse transcribed, and IL-1 β and β -actin cDNAs were amplified by PCR for 30 and 27 cycles, respectively, and analyzed on a 6% polyacrylamide gel. The IL-1 β cDNAs (top) were normalized to yield equivalent amounts of β -actin (bottom) PCR product to standardize the amount of total cellular mRNA in each PCR reaction. PCR was performed on the total RNA from the cells incubated with 50 μ g/ml AGE- β_2 M without reverse transcription (lane 7). (Lane 8) Fragments of the IL-1 β PCR products (lane 2) digested with HindIII. Representative data from the two experiments are shown.

fragment should have a HindIII site, generating two fragments of 400 and 140 bp. Indeed, the PCR product from the cells incubated with AGE- β_2 M was digested with HindIII (Fig. 4 C,

lane 8). These findings, together with the results of ELISA, indicate that AGE- β_2 M induces the synthesis and secretion of IL-1 β in monocyte-derived macrophages.

TNF- α and IL-1 β 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-1 β , in such amounts that are produced from macrophages by AGE- β_2 M, 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-1 β (Fig. 5 A). The equivalent amounts of TNF- α and IL-1 β produced from macrophages by 50 μ g/ml of AGE- β_2 M sufficed to cause a dose-dependent induction of collagenase. Interestingly, this collagenase synthesis by TNF- α and IL-1 β 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- β_2 M is a dominant constituent of the amyloid deposits in HAA. In this study we tested whether AGE- β_2 M could participate in the pathogenesis of HAA, and provided evidence that AGE- β_2 M may function as a pathogenic factor that induces the inflammatory response leading to joint/bone destruction in HAA (Fig. 6). First, AGE- β_2 M enhanced the chemotaxis and chemokinesis of human monocytes, but normal- β_2 M did not. This finding gives an explanation for the preferential localization of monocytes/macrophages to amyloid deposits. Amyloid deposits, in which AGE- β_2 M 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- β_2 M, but not normal- β_2 M, stimulated macrophages to secrete inflammatory cytokines such as TNF- α and IL-1 β . In vitro prepared AGE- β_2 M obtained by incubating normal- β_2 M 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 FFI induces the secretion of these cytokines from macrophages. To our knowledge, this study demonstrates for the first time that purified AGE proteins (AGE- β_2 M) formed in vivo do have the same biological effect. The induction of these cytokines would be due to the interaction of AGE- β_2 M 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-1 β , and IL-1 β is a potent inducer of TNF- α vice versa (42), the induction of TNF- α and IL-1 β observed by AGE- β_2 M is not due to a single effect of AGE- β_2 M,

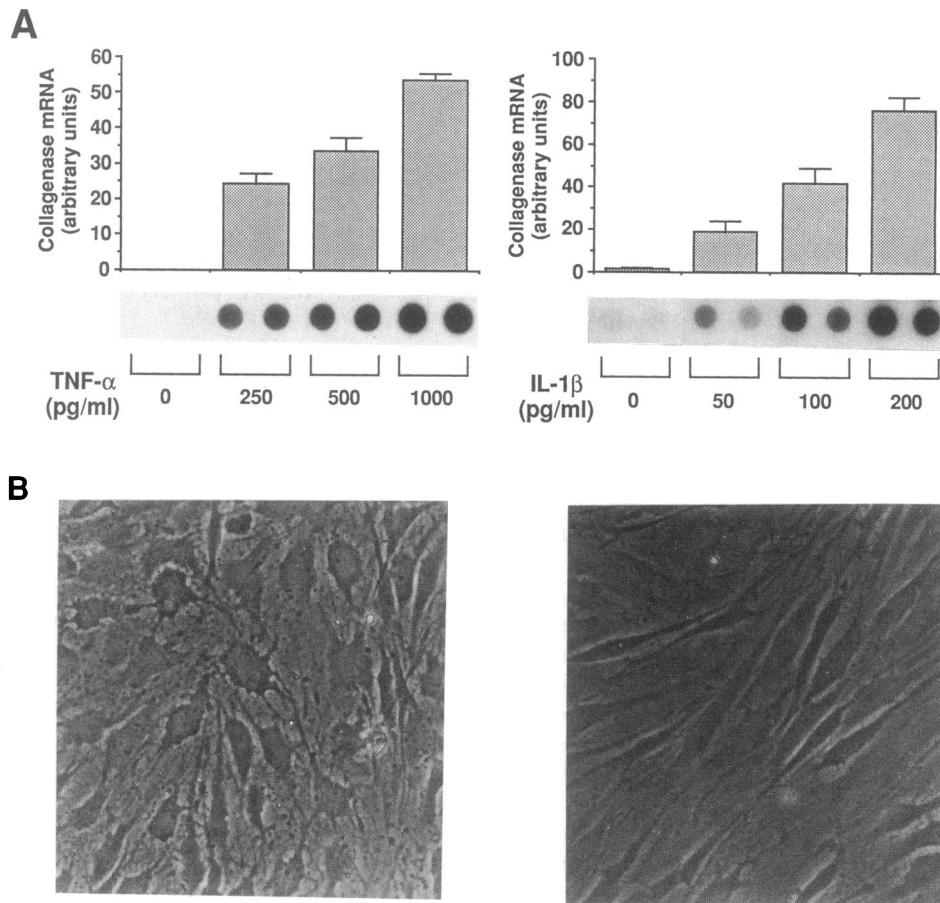


Figure 5. Effect of TNF- α and IL-1 β on the mRNA levels for collagenase in cultured human synovial cells. (A) Total cellular RNA was prepared from synovial cells treated with TNF- α or IL-1 β at the concentrations indicated. 6.5- μ g samples of RNA were analyzed by dot blotting using a specific cDNA probe. The autoradiograph was exposed for 22 h (TNF- α) and 60 h (IL-1 β). Collagenase mRNAs were analyzed by densitometry, and changes in mRNA are expressed in arbitrary units. The values are expressed as means \pm range of the duplicate determinations. Representative data from the two experiments are shown. (B) Cells were photographed through a light microscope after 12 h treated with (left) or without (right) TNF- α . Morphological changes in cell shape are shown.

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-1 β stimulates bone resorption (23–26), which requires the presence of osteoblasts to activate osteoclasts to resorb bone (43, 44). The mechanism

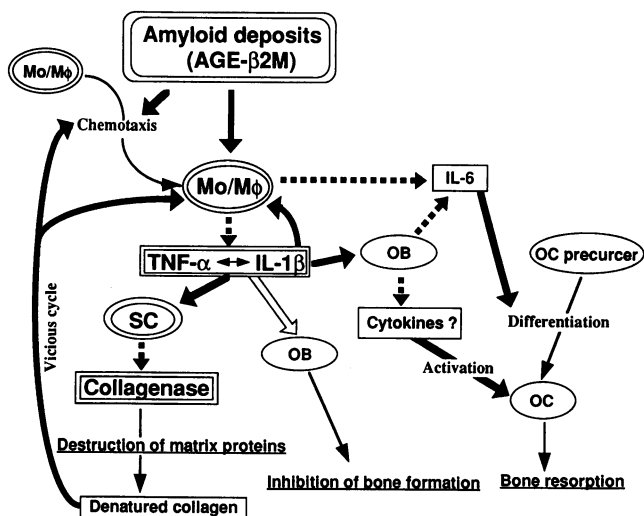


Figure 6. Roles of AGE- β_2 M in the pathogenesis of HAA. Mo, monocyte; M ϕ , macrophage; SC, synovial cell; OB, osteoblast; OC, osteoclast. Thick arrows, stimulation; open arrow, inhibition; broken arrows, secretion.

postulated so far is as follows. Both TNF- α and IL-1 β 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-1 β (43, 44). Furthermore, bone formation is inhibited by either TNF- α or IL-1 β (23, 24, 42). These findings together with our present observation that AGE- β_2 M stimulates the secretion of these cytokines from monocytes/macrophages, suggest that AGE- β_2 M 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-1 β produced by AGE- β_2 M 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- β_2 M 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- β_2 M enhances the migration of monocytes toward amyloid deposits and stimulates them to secrete TNF- α and IL-1 β . 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- β_2 M 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 β_2 M leads to the polymerization of β_2 M 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 β_2 M correlates with the formation of amyloid deposits to occur. Connors et al. (51) demonstrated that a high concentration of intact β_2 M could change into amyloid fibrils in vitro by salt-free dialysis. Thus, one possibility could be that AGE modification accelerates the amyloid formation of β_2 M. Or, alternatively, monocytes/macrophages may actively participate in the amyloid formation of AGE- β_2 M, either by degrading or by modifying AGE- β_2 M. 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.

Acknowledgments

We thank Drs. Seikoh Horiuchi, Naoyuki Taniguchi, and Takashi Miyazaki for helpful discussions and valuable comments on the manuscript, and Yasuhiko Ueda and Yuko Izuhara for excellent technical assistance. We also thank Dr. Arthur Z. Eizen for providing us collagenase cDNA.

References

- Drüeke, T. B. 1991. Beta-2-microglobulin amyloidosis and renal bone disease. *Miner. Electrolyte Metab.* 17:261-272.
- Gejyo, F., T. Yamada, S. Odani, Y. Nakagawa, M. Arakawa, T. Kunitomo, H. Kataoka, M. Suzuki, Y. Hirasawa, T. Shirahama, A. S. Cohen, and K. Schmid. 1985. A new form of amyloid protein associated with chronic hemodialysis was identified as β_2 -microglobulin. *Biochem. Biophys. Res. Commun.* 129:701-706.
- Gorevic, P. D., P. C. Munoz, T. T. Casey, C. R. DiRaimondo, W. J. Stone, F. C. Prelli, M. M. Rodrigues, M. D. Poulik, and B. Frangione. 1986. Polymerization of intact β_2 -microglobulin in tissue causes amyloidosis in patients on chronic hemodialysis. *Proc. Natl. Acad. Sci. USA.* 83:7908-7912.
- Shirahama, T., M. Skinner, A. S. Cohen, F. Gejyo, M. Arakawa, M. Suzuki, and Y. Hirasawa. 1985. Histochemical and immunohistochemical characterization of amyloid associated with chronic hemodialysis as β_2 -microglobulin. *Lab. Invest.* 53:705-709.
- Gejyo, F., S. Odani, T. Yamada, N. Honma, H. Saito, Y. Suzuki, Y. Nakagawa, H. Kobayashi, Y. Maruyama, Y. Hirasawa, M. Suzuki, and M. Arakawa. 1986. β_2 -microglobulin: a new form of amyloid protein associated with chronic hemodialysis. *Kidney Int.* 30:385-390.
- Depierreux, M., M. Goldman, I. Fayt, C. Richard, J. Quintin, M. Dhaene, and J.-L. Van Herweghem. 1988. Osteoarticular amyloidosis associated with haemodialysis: an immunohistochemical study. *J. Clin. Pathol.* 41:158-162.
- Theaker, J. M., A. E. G. Raine, A. J. Rainey, A. Heryet, A. Clark, and D. O. Oliver. 1987. Systemic amyloidosis of β_2 -microglobulin type: a complication of long term haemodialysis. *J. Clin. Pathol.* 40:1247-1251.
- Miyata, T., O. Oda, R. Inagi, Y. Iida, N. Araki, N. Yamada, S. Horiuchi, N. Taniguchi, K. Maeda, and T. Kinoshita. 1993. β_2 -Microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis. *J. Clin. Invest.* 92:1243-1252.
- Brownlee, M., A. Cerami, and H. Vlassara. 1988. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* 318:1315-1321.
- Baynes, J. W., and V. M. Monnier. 1989. The Maillard reaction in aging, diabetes and nutrition. *Prog. Clin. Biol. Res.* 304:1-410.
- Araki, N., N. Ueno, B. Chakrabarti, Y. Morino, and S. Horiuchi. 1992. Immunochemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. *J. Biol. Chem.* 267:10211-10214.
- Makita, Z., H. Vlassara, E., Rayfield, K. Cartwright, E. Friedman, R. Rodby, A. Cerami, and R. Bucala. 1992. Hemoglobin-AGE: a circulating marker of advanced glycosylation. *Science (Wash. DC).* 258:651-653.
- Pongor, S., P. C. Ulrich, F. A. Bencsath, and A. Cerami. 1984. Aging of proteins: isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. *Proc. Natl. Acad. Sci. USA.* 81:2684-2688.
- Njoroge, F. G., L. M. Sayre, and V. M. Monnier. 1987. Detection of glucose-derived pyrrole compounds during Maillard reaction under physiological conditions. *Carbohydr. Res.* 167:211-220.
- Sell, D. R., and V. M. Monnier. 1989. Structure elucidation of a senescence cross-link from human extracellular matrix. *J. Biol. Chem.* 264:21597-21602.
- Nakamura, K., T. Hasegawa, Y. Fukunaga, and K. Ienaga. 1992. Cross-lines A and B as candidates for the fluorophores in age- and diabetes-related cross-linked proteins, and their diacetates produced by Maillard reaction of α -N-acetyl-L-lysine with D-glucose. *J. Chem. Soc. Chem. Commun.* 14:992-994.
- Kirstein, M., J. Brett, S. Radoff, S. Ogawa, D. Stern, and H. Vlassara. 1990. Advanced protein glycosylation induces transendothelial human monocyte chemotaxis and secretion of platelet-derived growth factor: role in vascular disease of diabetes and aging. *Proc. Natl. Acad. Sci. USA.* 87:9010-9014.
- Vlassara, H., M. Brownlee, and A. Cerami. 1985. High-affinity-receptor-mediated uptake and degradation of glucose-modified proteins: a potential mechanism for the removal of senescent macromolecules. *Proc. Natl. Acad. Sci. USA.* 82:5588-5592.
- Vlassara, H., L. Moldawer, and B. Chan. 1989. Macrophage/monocyte receptor for nonenzymatically glycosylated proteins is upregulated by cachectin/tumor necrosis factor. *J. Clin. Invest.* 84:1813-1820.
- Vlassara, H., M. Brownlee, K. R. Manogue, C. A. Dinarello, and A. Pasagian. 1988. Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science (Wash. DC).* 240:1546-1548.
- Skolnik, E. Y., Z. Yang, Z. Makita, S. Radoff, M. Kirstein, and H. Vlassara. 1991. Human and rat mesangial cell receptors for glucose-modified proteins: potential role in kidney tissue remodeling and diabetic nephropathy. *J. Exp. Med.* 174:931-939.
- Miyata, S., and V. M. Monnier. 1992. Immunohistochemical detection of advanced glycosylation end products in diabetic tissues using monoclonal antibody to pyrraline. *J. Clin. Invest.* 89:1102-1112.
- Saklatvala, J. 1986. Tumour necrosis factor α stimulates resorption and inhibits synthesis of proteoglycan in cartilage. *Nature (Lond.).* 322:547-549.
- Bertolini, D. R., G. E. Nedwin, T. S. Bringman, D. D. Smith, and G. R. Mundy. 1986. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumor necrosis factors. *Nature (Lond.).* 319:516-518.
- Gowen, M., D. D. Wood, E. J. Ihrie, M. K. B. McGuire, and R. G. G. Russell. 1983. An interleukin 1 like factor stimulates bone resorption in vitro. *Nature (Lond.).* 306:378-380.
- Pacifici, R., L. Rifas, S. Teitelbaum, E. Slatopolsky, R. McCracken, M. Bergfeld, W. Lee, L. V. Avioli, and W. A. Peck. 1987. Spontaneous release of interleukin 1 from human blood monocytes reflects bone formation in idiopathic osteoporosis. *Proc. Natl. Acad. Sci. USA.* 84:4616-4620.
- Dayer, J.-M., B. Beutler, and A. Cerami. 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts. *J. Exp. Med.* 162:2163-2168.
- Horiuchi, S., N. Araki, and Y. Morino. 1991. Immunochemical approach to characterize advanced glycation end products of the Maillard reaction. *J. Biol. Chem.* 266:7329-7332.
- Falk, W., R. H. Goodwin, Jr., and E. J. Leonard. 1980. A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods.* 33:239-247.
- Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin. 1983. Identification of interferon- γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* 158:670-689.
- Pace, J. L., S. W. Russell, R. D. Schreiber, A. Altman, and D. H. Katz. 1983. Macrophage activation: Priming activity from a T-cell hybridoma is attributable to interferon- γ . *Proc. Natl. Acad. Sci. USA.* 80:3782-3786.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Dayer, J.-M., S. M. Krane, R. G. G. Russell, and D. R. Robinson. 1976. Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells. *Proc. Natl. Acad. Sci. USA.* 73:945-949.
- Goldberg, G. I., S. M. Wilhelm, A. Kronberger, E. A. Bauer, G. A. Grant, and A. Z. Eisen. 1986. Human fibroblast collagenase: complete primary structure and homology to an oncogene transformation-induced rat protein. *J. Biol. Chem.* 261:6600-6605.
- Altman, L. C., C. T. Furukawa, and S. J. Klebanoff. 1977. Depressed

mononuclear leukocyte chemotaxis in thermally injured patients. *J. Immunol.* 119:199-205.

36. Territo, M. C., and M. J. Cline. 1976. Monocyte function in man. *J. Immunol.* 118:187-192.

37. Kurt-Jones, E. A., J.-M. Kiely, and E. R. Unanue. 1985. Conditions required for expression of membrane IL 1 on B cells. *J. Immunol.* 135:1548-1350.

38. Dinarello, C. A. 1988. Biology of interleukin 1. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:108-115.

39. Harris, E. D., Jr. 1985. Pathogenesis of rheumatoid arthritis. In *Textbook of Rheumatology*. W. N. Kelley, E. D. Harris, Jr., S. Ruddy, and C. B. Sledge, editors. W. B. Saunders Company, Philadelphia. 886-914.

40. Aggeler, J., S. M. Frisch, and Z. Werb. 1984. Changes in cell shape correlate with collagenase gene expression in rabbit synovial fibroblasts. *J. Cell Biol.* 98:1662-1671.

41. Woolley, D. E., E. D. Harris Jr., C. L. Mainardi, and C. E. Brinckerhoff. 1978. Collagenase immunolocalization in cultures of rheumatoid synovial cells. *Science (Wash. DC)*. 200:773-775.

42. Akira, S., T. Hirano, T. Taga, and T. Kishimoto. 1990. Biology of multi-functional cytokines: IL 6 and related molecules (IL 1 and TNF). *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:2860-2867.

43. Horowitz, M. C. 1993. Cytokines and estrogen in bone: anti-osteoporotic effects. *Science (Wash. DC)*. 260:626-627.

44. Horowitz, M. C., D. L. Coleman, P. M. Flood, T. S. Kupper, and R. L. Jilka. 1989. Parathyroid hormone and lipopolysaccharide induce murine osteoblast-like cells to secrete a cytokine indistinguishable from granulocyte-macrophage colony-stimulating factor. *J. Clin. Invest.* 83:149-157.

45. Girasole, G., R. L. Jilka, G. Passeri, S. Boswell, G. Boder, D. C. Williams, and S. C. Manolagas. 1992. β -Estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: potential mechanism for the antiosteoporotic effect of estrogens. *J. Clin. Invest.* 89:883-891.

46. Löwik, C. W. G. M., G. van der Pluijm, H. Bloys, K. Hoekman, O. L. M. Bijvoet, L. A. Aarden, and S. E. Papapoulos. 1989. Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogenesis. *Biochem. Biophys. Res. Commun.* 162:1546-1552.

47. Postlethwaite, A. E., and A. H. Kang. 1976. Collagen and collagen peptide-induced chemotaxis of human blood monocytes. *J. Exp. Med.* 143:1299-1307.

48. Pacifici, R., A. Carano, S. A. Santoro, L. Rifas, J. J. Jeffrey, J. D. Malone, R. McCracken, and L. V. Avioli. 1991. Bone matrix constituents stimulate interleukin-1 release from human blood mononuclear cells. *J. Clin. Invest.* 87:221-228.

49. Shin, D. B., F. Hayase, and H. Kato. 1988. Polymerization of proteins caused by the reaction with sugars and the formation of 3-deoxyglucosone under physiological conditions. *Agric. Biol. Chem.* 52:1451-1458.

50. Sell, D. R., and V. M. Monnier. 1990. End-stage renal disease and diabetes catalyze the formation of a pentose-derived crosslink from aging human collagen. *J. Clin. Invest.* 85:380-384.

51. Connors, L. H., T. Shirahama, M. Skinner, A. Fenves, and A. S. Cohen. 1985. In vitro formation of amyloid fibrils from intact β_2 -microglobulin. *Biochem. Biophys. Res. Commun.* 131:1063-1068.