β2-microglobulin modified with advanced glycation end products modulates collagen synthesis by human fibroblasts

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β2-microglobulin modified with advanced glycation end products modulates collagen synthesis by human fibroblasts. β2-microglobulin amyloidosis (Aβ₂m) is a serious complication for patients undergoing long-term dialysis. β2-microglobulin modified with advanced glycation end products (β₂m-AGE) is a major component of the amyloid in Aβ₂m. It is not completely understood whether β₂m-AGE plays an active role in the pathogenesis of Aβ₂m, or if its presence is a secondary event of the disease. β2-microglobulin amyloid is mainly located in tendon and osteo-articular structures that are rich in collagen, and local fibroblasts constitute the principal cell population in the synthesis and metabolism of collagen. Recent identification of AGE binding proteins on human fibroblasts lead to the hypothesis that the fibroblast may be a target for the biological action of β₂m-AGE. The present study demonstrated that two human fibroblast cell lines exhibited a decrease in procollagen type I mRNA and type I collagen synthesis after exposure to β₂m-AGE for 72 hours. Similar results were observed using AGE-modified albumin. Antibody against the RAGE, the receptor for AGE, attenuated this decrease in synthesis, indicating that the response was partially mediated by RAGE. In addition, antibody against epidermal growth factor (EGF) attenuated the decrease in type I procollagen mRNA and type I collagen induced by β₂m-AGE, suggesting that EGF acts as an intermediate factor. These findings support the hypothesis that β₂m-AGE actively participates in connective tissue and bone remodeling via a pathway involving fibroblast RAGE, and at least one interposed mediator, the growth factor EGF.

β2-microglobulin amyloidosis (Aβ₂m) is a progressive and incapacitating comorbid condition affecting patients with chronic renal failure [1–3]. Amyloid deposits are predominantly found in tendons, synovium, and bone, resulting in shoulder periarthritis, hand flexor tenosynovitis, carpal tunnel syndrome, destructive spondyloarthropathy, and cystic bone lesions [1–3]. Recent biochemical and immunochemical studies have demonstrated that β2-microglobulin modified with advanced glycation end products (β₂m-AGE) is a major component of the amyloid fibrils in Aβ₂m

[4]. However, it is not completely understood whether β₂m-AGE actively contributes to osteo-articular destruction or are innocent bystanders accumulating during the course of the disease [5]. Supporting the hypothesis that β₂m-AGE is an indirect provocateur is the observation that it stimulates production of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 by macrophages [6, 7]. Indeed, recent studies have suggested that enhanced interaction of β₂m-AGE with macrophage AGE receptor (RAGE) results in increased macrophage migration and activation, as measured by increased production of TNF-α mRNA and protein [8–10].

Tendon and osteo-articular tissues, the preferential sites of β₂m amyloid deposition, are rich in collagen. In normal life processes and during inflammation, fibroblasts and related mesenchymal cells are the principal site for collagen synthesis and metabolism. Previous identification of AGE binding proteins on human fibroblasts [11] lead us to postulate that fibroblasts may be a direct target for the agonist actions of β₂m-AGE. Pursuant to this hypothesis, we evaluated the capacity of β₂m-AGE to affect collagen synthesis by human skin fibroblasts. Reported herein is that β₂m-AGE decreased synthesis of type I collagen in a dose- and time-dependent manner. The inhibition of this effect by antibody against RAGE suggested a receptor-mediated effect. These findings support a novel agonist role for β₂m-AGE that may adversely influence repair and remodeling of connective tissues in Aβ₂m.

METHODS

Fibroblast culture

A normal human, non-fetal skin fibroblast cell line (GM05757A) was obtained from NIGMS Human Genetic Mutant Cell Repository (Camden, NJ, USA). The cells were grown to confluence at 37°C in 95% air and 5% CO₂ in Dulbecco's modified Eagle medium; Nutrient Mixture F-12 (DMEM/F-12) contains L-glutamine (GIBCO Life Technologies, Grand Island, NY, USA) with 10% (vol/vol) fetal calf serum (FCS; GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA). Confluent cultures of fibroblasts were harvested by incubation with 0.1% trypsin/0.02% EDTA (Sigma) and subcultured at a 1:3 split ratio. Cells between passages 7 to 10 were harvested for experiments by incubation with 0.1% trypsin/0.02%

EDTA for three minutes at 37°C to 167°C, followed by extensive washing in DMEM/F-12 and resuspension in DMEM/F-12 containing 10% FCS at a concentration of 10^6 cells/ml. Since phenotypic modulation is a concern with cell passage, we compared the response to β_2m-AGE between two strains of human skin fibroblast (GM05757A and GM03377C) and between different passages (passages 4, 8, and 12). Culture conditions and harvesting procedures were as described earlier.

In vitro preparation of advanced glycation end product-modified proteins *in vitro*

Advanced glycation end product-modified proteins were prepared *in vitro* as previously described [12, 13]. Briefly, 1.75 mg/ml of purified normal human β_2m (Cortex Biochem, San Leandro, CA, USA), normal human serum albumin (HSA; Sigma), or bovine serum albumin (BSA; Sigma) were incubated at 37°C for 30 days with 100 mM D-glucose in 100 mM phosphate buffer containing 200 U/ml penicillin, 70 μg/ml gentamicin, and 1.5 mM PMSF (Sigma). Samples incubated in an identical manner in the absence of glucose were used as controls. After incubation, all samples were dialyzed against phosphate buffer (pH 7.4). AGE-modified proteins were characterized by ELISA using anti-KLH-AGE antibody at 1:2500 dilution (gift of Dr. John W. Baynes, University of South Carolina, Columbia, SC, USA) and fluorospectrometry as described [4, 13, 14]. The AGE content was quantitated by fluorospectrometry. For β_2m-AGE, it was 26.4 units/mg protein; for HSA-AGE 26.1 units/mg protein; for BSA-AGE 26.0 units/mg protein; and for β_2m, HSA and BSA controls 0.9 units/mg protein. The endotoxin content in all samples was measured by Limulus amoebocyte lysate assay (E-toxate, Sigma) and found to be < 0.2 ng/ml.

Identification of fibroblast advanced glycation end produce receptors

Immunoblot analysis. The presence of AGE receptors (RAGE) on fibroblasts was assessed by immunoblot analysis as described [15]. Briefly, 10^8 dispersed fibroblasts were solubilized in Tris-HCl (20 mM, pH 7.4), NaCl (0.1 M), octyl-β-glucoside (1%), and PMSF (1 mM), and the lysate eluted through a 10 ml hydroxylapatite column (IBF Biotechnics, Columbia, MD, USA). The column was washed with Tris-buffered saline (Tris, 20 mM, pH 7.4; NaCl, 0.1 M) containing octyl-β-glucoside (0.1%), and the protein extract eluted in the same buffer with the NaCl concentration increased to 1.0 M. The membrane-enriched extract was subjected to SDS-PAGE (10%) followed by Western blotting using the Blotto procedure [16]. After incubation with 45 μg/ml of rabbit anti-human RAGE IgG [8] or nonimmune rabbit IgG (Sigma) for 60 minutes at room temperature, sites of IgG binding were visualized by chemiluminescence using peroxidase-conjugated secondary antibody (Amersham Corp., Arlington Heights, IL, USA).

Immunofluorescent Analysis. Fibroblasts were allowed to adhere to 12-mm coverslips for 24 hours at 37°C. The cells were washed with PBS (pH 7.4) and fixed in 2% paraformaldehyde for 15 minutes at 4°C. The coverslips were incubated wtih rabbit anti-human RAGE antibody (33.5 μg/ml) for 45 minutes at 37°C, washed with PBS (pH 7.4), and incubated with FITC-conjugated goat anti-rabbit IgG (Sigma) at 1:1000 dilution for 45 minutes at 37°C. Nonimmune rabbit IgG replaced anti-RAGE IgG at the same concentration as a negative control. The cells were observed using phase contrast and fluorescence microscopy, then photographed (Nikon, Labophot-2, Japan).

Extraction and analysis of procollagen mRNA and type I collagen

Fibroblasts were plated onto 75 cm^2 flasks in DMEM/F-12 with 10% FCS and grown to confluence. The monolayers were washed three times with Hanks balanced salt solution without divalent cations (HBSS$^-$) to remove traces of serum. For analysis of dose-dependence, the monolayers were cultured for 72 hours with incremental concentrations of β_2m-AGE or unmodified β_2m in serum-free DMEM/F-12 with 0.2% lactalbumin (DMEM-LH; Gibco Life Technologies, Grand Island, NY, USA). To evaluate the time-dependence of the response, cells were incubated with 50 μg/ml β_2m-AGE or unmodified β_2m for different durations. Negative controls were incubated with DMEM-LH alone, and plates incubated with 60 ng/ml of recombinant human IL-1β (Genzyme, Cambridge, MA, USA) served as positive controls. To compare the effect of various AGE-modified proteins, fibroblasts were incubated with 50 μg/ml of β_2m-AGE, HSA-AGE, BSA-AGE, or their unmodified forms for 72 hours. At the endpoints of incubation, the supernatants were collected for quantitation of type I collagen. The corresponding monolayers were washed and trypsinized. The cells were counted, and Trypan blue dye exclusion was performed to test cell viability. Greater than 98% of the cells were Trypan blue excluding in all experiments. Then cells were lysed in 1 ml TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA). The lysate was collected, and phase separated by the addition of 0.2 ml of chloroform. The aqueous phase was used for total RNA isolation, and the interphase and organic phases were used for protein analysis as described [17].

For steady-state mRNA analysis, 5 to 10 μg of total RNA from representative samples were electrophoresed on a 1.2% agarose/6% formaldehyde gel and transferred onto a nylon membrane [18]. For slot-blotting, 5 to 8 μg of total RNA was denatured at 68°C for 15 minutes in 20% formaldehyde and 6× SSC, serially diluted at the desired concentration, and slotted onto a nylon membrane using the BIO-DOT SF apparatus (BIO-RAD, Hercules, CA, USA). The filters were baked at 80°C and prehybridized [6]. After the denatured [^{32}P] labeled probe for human type I collagen α1 (10^6 cpm/ml; ATCC, Rockville, MD, USA) was added directly to the prehybridization fluid, hybridization was performed overnight at 42°C. The filters were washed and exposed to Kodak X-Omat. The densitometric scanning of the slot-blot autoradiograms was performed using a densitometer (Molecular Dynamics, Sunnyvale, CA, USA). The results were corrected by the amount of RNA loaded and expressed as density unit/μg RNA.

Type I collagen in the cell supernatants and in the extracts of the cell layer were quantitated by ELISA using the ELISA Starter Kit (Pierce, Rockford, IL, USA). Ninety-six-well polystyrene plates were coated with monoclonal anti-human type I collagen antibody (ICN Biomedicals, Inc., Aurora, OH, USA) at 10 μg/ml by incubation for 12 hours at 4°C. Each well was washed three times with washing buffer, and nonspecific binding sites were blocked with blocking buffer. Human type I collagen (Gibco Life Technologies) at incremental concentrations, properly diluted samples were added, and the plates were incubated for 60 minutes
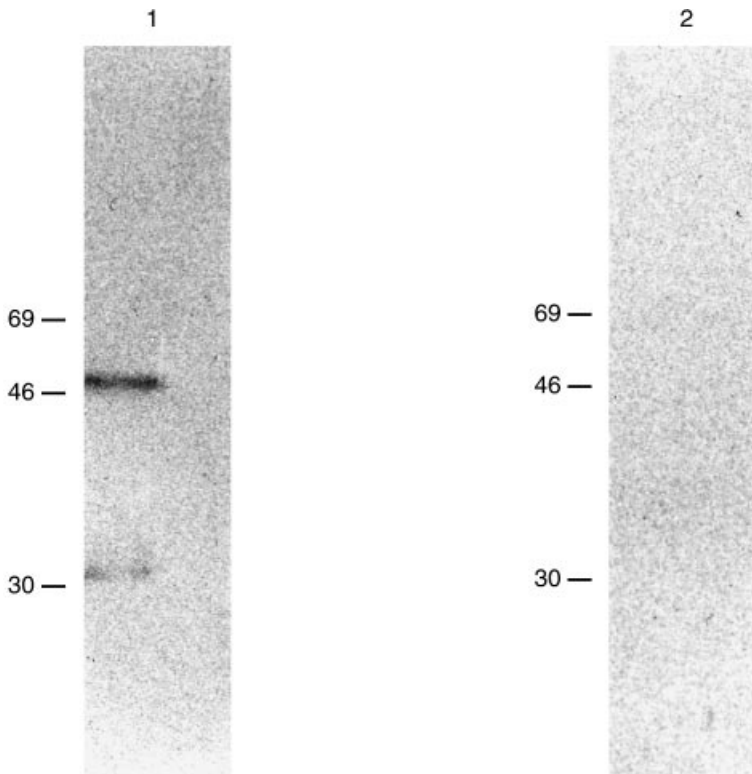
Fig. 1. Immunoblotting of detergent extracts of human fibroblasts with anti-human RAGE IgG. Lane 1. Detergent extract of fibroblasts was subjected to 10% SDS-PAGE and electroblotting. The blots were reacted with anti-human RAGE IgG (45 μg/ml). Sites of primary antibody binding were visualized with an peroxidase-conjugated secondary antibody. Lane 2. The same experiment was performed using detergent extract of fibroblasts, but nonimmune rabbit IgG (45 μg/ml) was used as the primary antibody. The experiment was repeated two times with identical findings.

at room temperature. After washing the plates, a polyclonal rabbit anti-human type I collagen antibody (ICN Biomedicals, Inc.) at 1:1000 dilution was incubated with the plates for one hour at room temperature. The wells were then washed, incubated with a goat anti-rabbit IgG-peroxidase antibody (Pierce) at 1:5000 dilution, followed by addition of the substrate 2,2′-azino-di-3-ethylbenz-thiazoline-6-sulfonic acid. The absorbance at 405 nm was measured on a micro-ELISA plate reader (Titertek Multiskan, Mcc/340). For control studies, blocking buffer alone was used instead of samples, and nonimmune rabbit IgG was substituted for rabbit anti-human type I collagen. The amount of type I collagen measured was corrected for the number of cells harvested.

Effect of antibodies to RAGE, EGF or IL-1β on type I collagen synthesis

To determine the effect of anti-RAGE IgG on type I collagen synthesis, fibroblasts were plated in six-well plates, grown to confluence, and preincubated with serum-free DMEM-LH containing 50 μg/ml of rabbit anti-human RAGE or nonimmune rabbit IgG for two hours at 37°C. This concentration was based on preliminary experiments demonstrating a plateau in activity at concentrations $\geq$ 50 μg/ml (data not shown). The monolayers were washed three times with DMEM-LH and then incubated with β_2m-AGE (50 μg/ml). After 72 hours incubation, the supernatants were collected, and the total RNA and protein were extracted for analysis.

To determine the effect of anti-EGF IgG, confluent fibroblasts in six-well plates were incubated with serum-free DMEM-LH containing 50 μg/ml of β_2m-AGE and 50 μg/ml of goat neutralizing antibody for human epidermal growth factor (EGF; R&D System, Minneapolis, MN, USA) or 50 μg/ml of β_2m-AGE plus

50 μg/ml of nonimmune goat IgG (Sigma). After 72 hours of incubation, the supernatants were collected and the total RNA and protein were extracted. In separate experiments, fibroblasts were preincubated with goat anti-human EGF neutralizing antibody (50 μg/ml) or nonimmune goat IgG (50 μg/ml) for two hours. The cells were washed with DMEM-LH and incubated for 72 hours with β_2m-AGE (50 μg/ml). The supernatants were collected, and the total RNA and protein were extracted.

To quantitate IL-1β synthesis from fibroblasts, washed monolayers were incubated with β_2m-AGE or HSA-AGE (50 μg/ml) for 12 to 72 hours at 37°C. At the endpoint of incubation, the supernatant was collected, and IL-1β was quantitated by using the Predicta Human IL-1β ELISA Kit (Genzyme) with detection limits of 3.0 pg/ml. To determine the effect of anti-IL-1β, fibroblasts were simultaneously incubated with serum-free DMEM-LH containing 50 μg/ml of β_2m-AGE and 25 to 250 μg/ml of rabbit neutralizing antibody for human IL-1β (Genzyme) for 72 hours. Type I procollagen mRNA and type I collagen were analyzed as described above.

Statistical analysis

All experiments were performed in triplicate. Continuous variables, expressed as mean $\pm$ SD, were compared using analysis of variance (ANOVA). Multiplicative terms were included to evaluate for interaction among explanatory variables. The Student-Newman-Keuls procedure was used to evaluate pairwise comparisons. Two-tailed P values < 0.05 were considered statistically significant. Statistical analyses were conducted with SAS (The SAS Institute, Cary, NC, USA).
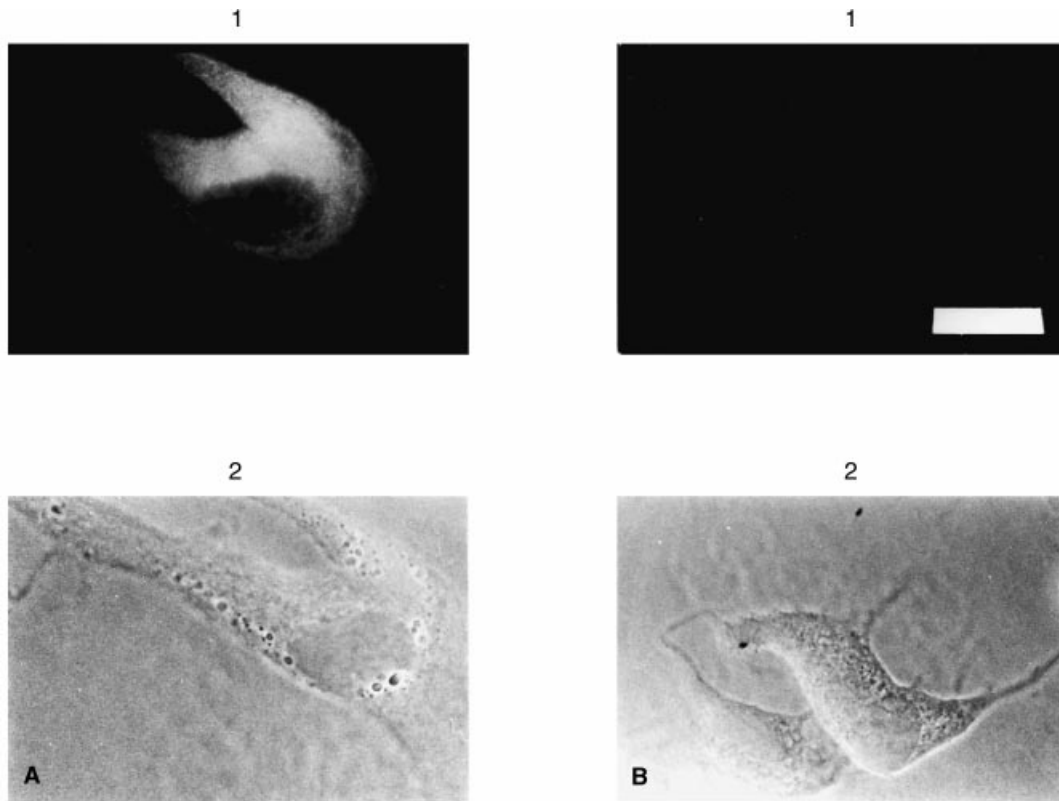
Fig. 2. Detection of RAGE on human fibroblasts by indirect immunofluorescence. Fibroblasts were stained with rabbit anti-RAGE IgG (lane A) or nonimmune rabbit IgG (lane B) as described in the text. Panels 1: Immunofluorescence. Panels 2: Phase contrast. The experiment was repeated two times with identical findings (bar = 10 μm).

RESULTS

Demonstration of RAGE on fibroblasts

To ascertain the presence of RAGE on human fibroblasts, membrane-enriched fractions were generated from human fibroblasts and were analyzed for immunoreactive material using polyclonal anti-human RAGE IgG. Two bands were visible by immunoblot analysis, corresponding to relative molecular masses of 32 and 50 kDa (Fig. 1, lane 1). Irrelevant antibody was without immunoreactivity (Fig. 1, lane 2). The presence of two different size immunoreactive RAGE polypeptides in the membrane fraction probably reflects post-translational processing/cleavage, in that multiple molecular mass forms of RAGE are observed in cells transfected with full-length RAGE cDNA [10]. Immunofluorescence studies demonstrated a cell surface pattern of staining for RAGE. Fibroblasts incubated with anti-human RAGE IgG showed a diffuse pattern of membrane staining (Fig. 2A) as compared with nonimmune rabbit IgG control (Fig. 2B).

Effect of β₂m-AGE on type I procollagen mRNA expression and synthesis of type I collagen

Type I procollagen mRNA was detectable in cells incubated with medium alone (921 $\pm$ 126 density units/μg RNA), and was taken as 100% (the negative controls). After a 72 hour incubation with β_2m-AGE (50 μg/ml), type I procollagen mRNA levels decreased to 66 $\pm$ 9% (Fig. 3, lane 4) of that observed with cells incubated in medium alone (the negative controls; Fig. 3, lane 1). In contrast, cells incubated with β_2m showed no significant change

in level of mRNA coding for type I procollagen (104 $\pm$ 8% of the negative controls; Fig. 3, lane 3). β-actin mRNA levels were comparable for fibroblasts incubated with medium alone, β_2m, and β_2m-AGE (Fig. 3). Fibroblasts were incubated with IL-1β as a positive control for down-regulation of collagen biosynthesis (Fig. 3, lane 2).

The decrease in type I procollagen mRNA was dependent upon the concentration of β_2m-AGE contained in the medium (Fig. 4A). Steady-state mRNA levels were reduced by 25% upon incubation with 0.5 μg/ml of β_2m-AGE and 40% upon incubation with 50 μg/ml. A decrement in steady-state mRNA for type I procollagen was not detectable before 48 hours incubation with β_2m-AGE (Fig. 4B).

To determine whether the observed decrease in type I procollagen mRNA was associated with a decrease in the synthesis of collagen, type I collagen levels were quantitated both in the supernatants and in the extracts of the cell layer. In fibroblasts incubated with medium alone, the quantities of type I collagen in the supernatants and in the extracts of the cell layer were 195 $\pm$ 22 ng/10⁴ cell and 181 $\pm$ 16 ng/10⁴ cell, respectively, and were taken as 100% (Fig. 5). The decrease in type I collagen levels corresponded to the decrement in steady-state mRNA. The quantities of type I collagen in the supernatants and the extracts of cell layer were reduced by 51% and 67% upon incubation with 50 μg/ml of β_2m-AGE.

Although the magnitude of the absolute response to β_2m-AGE was somewhat variable in different lines and passages (cell line
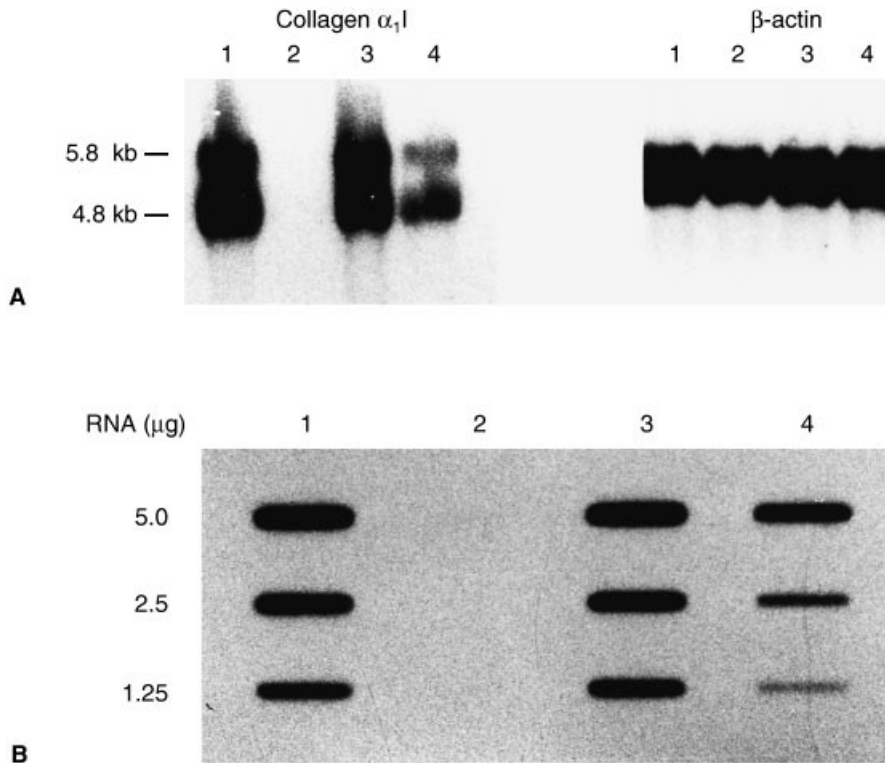
Fig. 3. Effect of β_2m-AGE on type I procollagen mRNA level expressed by human fibroblasts. Fibroblasts were incubated with DMEM-LH alone (lane 1), 60 ng/ml IL-1β (lane 2), 50 μg/ml β_2m (lane 3), and 50 μg/ml β_2m-AGE (lane 4) for 72 hours. Type I procollagen mRNA levels were analyzed by Northern blot (*A*) and slot-blot (*B*) using a cDNA probe specific for human α1 chain of type I collagen. The experiments were repeated three times with the same findings.

GM05757A had decreased type I procollagen mRNA of 61.0 $\pm$ 8.2%, 65.0 $\pm$ 7.8%, and 67.1 $\pm$ 6.2% at passages 4, 8 and 12; cell line GM03377C had decreased type I procollagen mRNA of 52.8 $\pm$ 5.8%, 60.5 $\pm$ 6.6%, and 61.2 $\pm$ 7.2% at passages 4, 8 and 12, $N = 3$), the decrease in type I procollagen mRNA expression was a uniform phenomenon.

To test the specificity of the effect of β_2m-AGE on type I collagen synthesis, fibroblasts were incubated with other AGE-modified proteins. No significant difference was seen in type I procollagen mRNA or type I collagen levels between cells treated with β_2m-AGE, HSA-AGE and BSA-AGE (Fig. 6). Unmodified HSA or BSA had no effect on type I collagen synthesis.

Effect of anti-RAGE, anti-EGF, and anti-IL-1β antibodies on β_2m-AGE induced decrease of type I collagen synthesis

Preincubation of the human fibroblast cultures with anti-human RAGE IgG prior to addition of β_2m-AGE attenuated the β_2m-AGE-induced decrease of type I procollagen mRNA levels (Fig. 7, lanes 2 and 4, respectively, and Table 1). There was no change in procollagen mRNA levels when fibroblasts were pretreated with nonimmune rabbit IgG (Fig. 7, lane 3, and Table 1). Similar effects were observed for type I collagen synthesis by the fibroblast cultures treated with anti-RAGE IgG or nonimmune rabbit IgG, respectively (Table 1).

The simultaneous addition of anti-EGF IgG to the incubation solution blunted the β_2m-AGE-induced decrease in both type I procollagen mRNA and type I collagen levels (Table 1). In contrast, addition of nonimmune goat IgG had no effect on β_2m-AGE-induced decrease in type I procollagen mRNA. Instead, if fibroblasts were pretreated with anti-EGF IgG, washed, and followed by incubation with β_2m-AGE, no significant change

could be seen in type I procollagen mRNA expression, or type I collagen synthesis in comparison to cells exposed to β_2m-AGE without pretreatment (data not shown). Neither anti-human RAGE IgG nor anti-human EGF IgG affected type I procollagen mRNA levels or type I collagen synthesis in fibroblasts incubated with medium alone or with β_2m (data not shown).

Using an ELISA with a limit of detection of 3.0 pg/ml, no IL-1β was detected in the supernatants of fibroblasts cultured for 12 to 72 hours with β_2m-AGE (50 μg/ml) or HSA-AGE (50 μg/ml). Furthermore, the addition of anti-IL-1β antibody did not alter the β_2m-AGE induced decrease in type I collagen synthesis (Table 1).

DISCUSSION

The amyloidosis of chronic renal failure is unique, consisting of β_2m and localizing mainly in bones and synovium [1–3]. Subchondral bone resorption and growing bone cysts are hallmarks of the clinical presentation of Aβ_2m. Among maintenance dialysis patients, the signs of hyperparathyroidism decrease with time, but the size and number of subchondral bone cysts increase. Biopsy of these cysts yields β_2m amyloid [19]. Controversy exists about whether β_2m deposition plays an active role in inducing bone destruction or if it is only a secondary event of the disease [5]. In favor of active participation is the observation that β_2m increases collagenase synthesis by cultured rabbit synovial cells, suggesting an autocrine function for β_2m [20]. Similarly, other studies demonstrate that human β_2m stimulates DNA production, and synthesis of collagen and non-collagen proteins in isolated osteoblast cultures [21, 22]. However, these observations are not uniform [23, 24]. In the current analysis, unmodified β_2m did not effect the synthesis of type I collagen by human skin fibroblasts.
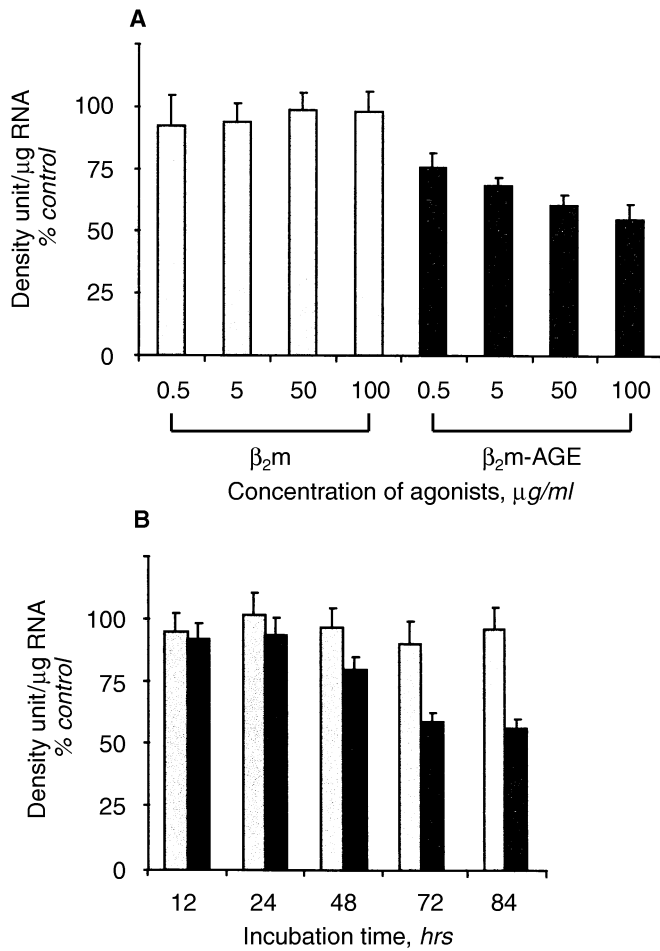
A

B

Fig. 4. Dose-related effect of β_2m and β_2m-AGE on type I procollagen mRNA level expressed by human fibroblasts (*A*). Confluent cultures of fibroblasts were incubated with incremental concentrations of β_2m or β_2m-AGE for 72 hours. Total RNA was slotted and hybridized with a cDNA probe specific for human α1 chain of type I collagen. The scanned results are expressed as the percentage of the control values without agonists, taken as 100%. Data from three independent experiments are shown as mean $\pm$ SD. The error bars indicate the SD from the mean. ANOVA, $P < 0.0001$; AGE modification, $P < 0.0001$; concentration of β_2m, $P =$ NS; AGE $\times$ dose interaction, $P = 0.02$. (*B*) Time course of the effect of β_2m-AGE on type I procollagen mRNA level expressed by human fibroblasts. Cells were incubated with 50 μg/ml β_2m-AGE (■) or unmodified β2M (▨) for different durations. Type I procollagen mRNA levels were analyzed by slot-blot. Data from three independent experiments are represented as mean $\pm$ SD. The error bars indicate the SD from the mean. ANOVA, $P < 0.0001$; AGE modification, $P < 0.0001$; duration of incubation, $P = 0.0006$; AGE $\times$ duration interaction, $P = 0.01$.

AGE-modified β_2m is a major component of Aβ_2m [4]. Its pathobiologic role is suggested by the finding that β_2m-AGE induces chemotaxis of human monocytes and stimulates production of TNF-α and IL-1β by macrophages [6, 8]. In turn, both cytokines have multiple effects on bone, including stimulation of cell-mediated resorption and inhibition of bone formation [25, 26]. In addition, AGE-modified β_2m stimulates the synthesis of TNF-α and IL-1β, cytokines that enhance collagenase mRNA expression in cultured human synovial cells [6]. This may subsequently lead to collagen degradation and connective tissue breakdown. Thus, it is hypothesized that β_2m-AGE may be involved in bone and joint destruction by a secondary effector mechanism in
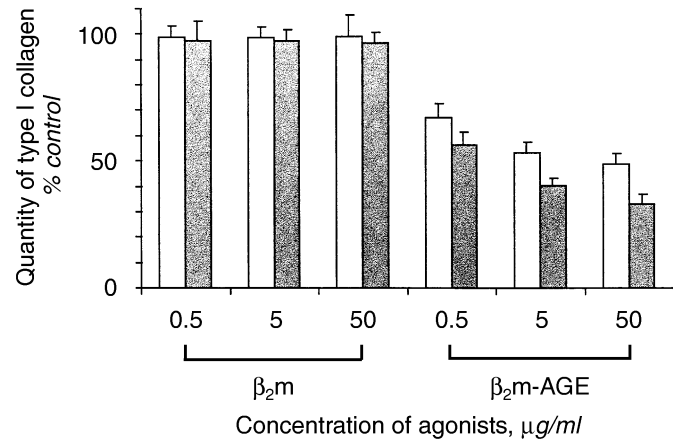

Fig. 5. Quantity of type I collagen in medium (▨) and cell layer (■) of human fibroblasts incubated with unmodified β_2m or β_2m-AGE for 72 hours. Data were expressed as percent of the quantity of protein in cells exposed to DMEM-LH alone and represent the mean $\pm$ SD of three independent experiments. The error bars indicate the SD from the mean. ANOVA, $P < 0.0001$; AGE modification, $P < 0.0001$; concentration of β_2m-AGE, $P < 0.0001$.
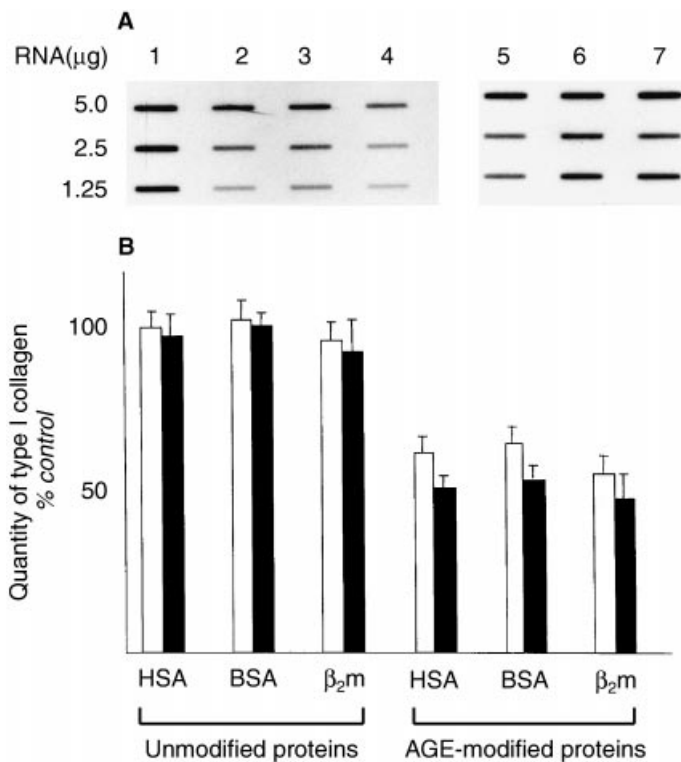
A

B

Fig. 6. Comparison of effect of β_2m-AGE and other AGE-modified proteins on type I procollagen mRNA (*A*) and type I collagen synthesis (*B*). Fibroblasts were incubated for 72 hours with DMEM-LH alone (lane 1), 50 μg/ml of HSA-AGE (lane 2), 50 μg/ml BSA-AGE (lane 3), 50 μg/ml of β_2m-AGE (lane 4), or unmodified HSA (lane 5), BSA (lane 6) and β_2m (lane 7). Type I procollagen mRNA levels were analyzed by slot-blot and type I collagen levels in medium (□) and cell layer (■) were measured by ELISA. Data from three independent experiments are shown as the mean $\pm$ SD. The error bars indicate the SD from the mean.

which monocytes/macrophages are attracted to the microenviroment and activated to produce cytokines leading to local bone destruction [5]. However, it is noteworthy that in the location of
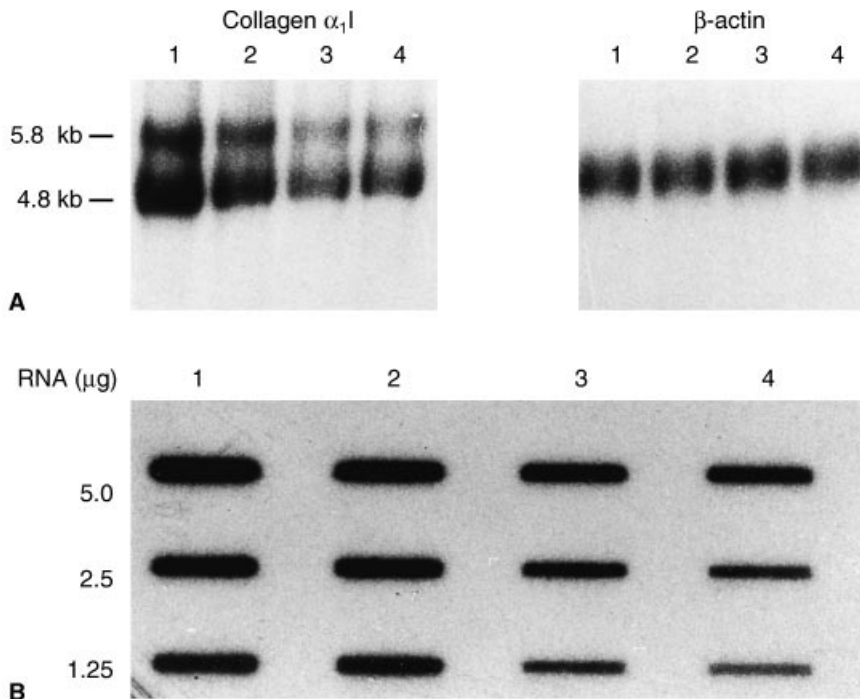
Fig. 7. Effect of anti-human RAGE IgG on β_2m-AGE induced decrease of type I procollagen mRNA. Human fibroblasts were maintained in DMEM-LH alone for 72 hours (lane 1), pretreated with 50 μg/ml of rabbit anti-human RAGE IgG for two hours followed by incubation with 50 μg/ml of β_2m-AGE for 72 hours (lane 2), pretreated with 50 μg/ml of nonimmune rabbit IgG for two hours followed by incubation with 50 μg/ml of β_2m-AGE for 72 hours (lane 3), or incubated with 50 μg/ml of β_2m-AGE for 72 hours (lane 4). Type I procollagen mRNA level were analyzed by Northern blot (*A*) and slot-blot (*B*). Data in A and B are representative of three separate experiments.

Table 1. Effect of anti-RAGE, anti-EGF, and anti-IL-1β on β_2m-AGE induced decrease of type I procollagen mRNA and type I collagen synthesis[a]

	Type I procollagen mRNA %	Type I collagen synthesis %	
		Medium	Cell layer
Medium alone	100	100	100
β_2m-AGE alone	65.0 ± 7.8	48.6 ± 4.7	38.0 ± 3.4
Rabbit α-RAGE + β_2m-AGE[b]	80.2 ± 6.4	72.7 ± 6.2	65.6 ± 4.4
Nonimmune rabbit IgG + β_2m-AGE[b]	63.0 ± 7.3	44.2 ± 3.8	36.0 ± 4.5
Goat α-EGF + β_2m-AGE[c]	75.7 ± 6.2	66.5 ± 3.2	60.8 ± 3.4
Nonimmune goat IgG + β_2m-AGE[c]	64.4 ± 6.4	46.0 ± 4.8	40.0 ± 5.8
Rabbit α-IL-1β + β_2m-AGE[d]	67.2 ± 7.2	49.4 ± 4.1	42.2 ± 4.2

Abbreviations are in the Appendix.
[a] Type I procollagen mRNA levels were analyzed by Northern blot and slot-blot. Type I collagen in the supernatants and in the extracts of the cell layer were quantified by ELISA. The results were expressed as percentages of the control values (medium alone) that were taken as 100%. Data from three separate experiments are shown as mean $\pm$ SD. For cells in medium alone, type I procollagen mRNA levels were 951 ± 121 density units/μg RNA; the quantity of type I collagen in the supernatants and in the extracts of the cell layer were 198 ± 19.1 ng/10^4 cell and 182 ± 11.7 ng/10^4 cell, respectively. ANOVA, $P < 0.0001$; SNK: rabbit α-RAGE + β_2m-AGE and goat α-EGF + β_2m-AGE are significantly different from β_2m-AGE alone and medium alone.
[b] Human fibroblasts were pretreated with 50 μg/ml of rabbit α-RAGE or nonimmune rabbit IgG for 2 hours followed by incubation with 50 μg/ml of β_2m-AGE for 72 hours.
[c] Human fibroblasts were exposed to 50 μg/ml of β_2m-AGE plus 50 μg/ml of goat α-EGF or nonimmune goat IgG for 72 hours.
[d] Human fibroblasts were incubated with 50 μg/ml of β_2m-AGE plus 100 μg/ml of rabbit α-IL-1β for 72 hours.

Aβ_2m deposits, the typical cellular inflammatory responses are not as dense as in other inflammatory processes [2, 27, 28].

The current studies demonstrate a direct pathobiologic effect of β_2m-AGE on connective tissue. Human fibroblasts exposed to β_2m-AGE exhibited a decrease in released and cell-associated type I collagen level. The decrease in type I collagen was associated with a parallel decrease in type I procollagen mRNA, suggesting that the decrease in type I collagen was a consequence of diminished synthesis, not enhanced degradation by collagenase. The effect of β_2m-AGE on type I collagen synthesis by fibroblasts was not specific, since other AGE-modified proteins also decreased type I collagen synthesis. However, the major protein component in amyloid fibrils in Aβ_2m is β_2m-AGE [4], suggesting that the osteoarticular damage in Aβ_2m is mediated by β_2m-AGE. Because of phenotypic changes that may occur with cell passage, we examined different cell lines and passages. A uniform decrease in type I collagen was observed, suggesting the more general applicability of these observations.

An effect of AGE-modified β_2m on human fibroblasts is supported by the immunohistochemical demonstration of RAGE. Several cell-surface AGE binding proteins, such as p60, p90 and galectin-3, have been identified on a variety of cells including rat and human fibroblasts [11, 29–31]. Recently, a 35 kDa member of the immunoglobulin superfamily of receptors, named RAGE, was isolated and characterized from extracts of bovine lung on the basis of its ability to bind AGEs [32]. RAGE has been found to be present on the surface of bovine endothelial cells, human peripheral mononuclear phagocytes and skeletal muscle cells [8, 10, 33]. Human β_2m-AGE binds to the extracellular domain of RAGE with a kDa ≈ 53.5 nM, and β_2m-AGE induced chemotaxis by human mononuclear phagocytes can be abrogated by anti-RAGE IgG [8]. In the present study, β_2m-AGE induced a decrease in type I collagen synthesis that was attenuated by antibody against RAGE, indicating that this decrease in type I collagen synthesis was partially mediated by RAGE. The incomplete blockade by anti-RAGE may be a consequence of receptor recycling, or the involvement of other AGE binding proteins beside RAGE. Less likely is that the β_2m-AGE effect is mediated by a conformational change in β_2m, not related to its glycation.

The requirement for $\geq$ 48 hours incubation with β_2m-AGE to down-regulate type I procollagen mRNA levels suggests an indirect effect of β_2m-AGE. Previous studies indicate that AGE-modified bovine serum albumin induces the expression of EGF and EGF-receptor mRNA in human FS4 fibroblast [11], and EGF down-regulates type I collagen synthesis in cultured skin fibroblasts [34]. The observation that the β_2m-AGE-induced decrease in type I collagen synthesis is attenuated by an antibody to EGF, suggests that this growth factor may participate as an intermediate effector of collagen synthesis. Because EGF increases B-myb mRNA in cultured bovine vascular smooth muscle cells, and B-myb down-regulates the promoter activity of type I collagen genes, it may be a critical mediator for β_2m-AGE's actions [35]. Although fibroblasts may also participate in the synthesis of IL-1, there was no detectable IL-1β in the supernatants of fibroblasts cultured with β_2m-AGE or HSA-AGE. Furthermore, addition of neutralizing IL-1β antibody did not alter the β_2m-AGE-induced decrease in type I collagen synthesis.

The ability of β_2m-AGE to down-regulate the synthesis of type I collagen may play a role in connective tissue and osteoarticular damage in Aβ_2m. Type I collagen is most abundant in skin, tendon and bone matrix where it comprises between 80 to 99% of the total collagen. Tissue fibroblasts and related mesenchymal cells play a critical role in the synthesis and metabolism of collagen [36]. Cutaneous β_2m-AGE deposits have been found on biopsy of patients who had undergone hemodialysis for > 10 years [37]. Patients with cutaneous β_2m amyloid deposits have dry, thin skin [38], a finding that may be a consequence of local β_2m-AGE deposition. β_2m-AGE located in osteoarticular structures has the same effect, so may contribute to the growth of bone cysts by suppression of the local production of type I collagen for formation of bone matrix.

In conclusion, our study demonstrates the inhibitory effect of β_2m-AGE on collagen synthesis by human fibroblasts. This finding suggests that β_2m-AGE may play an active role in the processes of connective tissue and bone remodeling in Aβ_2m. Furthermore, these findings suggest that the interaction of β_2m-AGE with RAGE may present a novel target for intervention in this disorder, as a means of restoring normal fibroblast reparative responses.

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APPENDIX

Abbreviations used in this article are: AB₂m, B₂-microglobulin amyloidosis; B₂m, B₂-microglobulin; B₂m-AGE, B₂-microglobulin-advanced glycation end products; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FCS, fetal calf serum; HSA, human serum albumin; IL, interleukin; PBS, phosphate buffered saline; RAGE, receptor for advanced glycation end products; TNF-α, tumor necrosis factor-α.

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