Receptor for Advanced Glycation End Products on Human Synovial Fibroblasts: Role in the Pathogenesis of Dialysis-Related Amyloidosis

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Abstract. An important component of amyloid fibrils in dialysis-related amyloidosis (DRA) is β-2-microglobulin (β-2-m) modified with advanced glycation end products (AGE). The amyloid deposits are located principally in joint structures, with adjacent chronic inflammatory reaction characterized by monocyte infiltration. This study examined the interaction of AGE-β-2-m with human synovial fibroblasts and investigated the proinflammatory effects of that interaction. It was demonstrated that human synovial fibroblasts constitutively expressed the receptor for AGE (RAGE). RAGE expression was detected mainly in synovial intima and was upregulated in DRA synovium. 125I-AGE-β-2-m bound to immobilized human synovial fibroblasts in a specific, dose-dependent manner (Kd of approximately 138.0 nM), and binding was inhibited by anti-RAGE IgG. Incubation of human synovial fibroblasts with AGE-β-2-m induced degradation of this AGE-modified protein, as well as increased monocyte chemoattractant protein-1 (MCP-1) mRNA and protein expression. The amount of MCP-1 produced by AGE-β-2-m-stimulated human synovial fibroblasts was sufficient to induce the chemotaxis of monocytes. MCP-1 synthesis resulted from engagement of RAGE, because the increase in MCP-1 synthesis was attenuated by preincubation of human synovial fibroblasts with anti-RAGE IgG. These data provide evidence of RAGE-mediated perturbation of human synoviocytes, which may be involved in the pathogenesis of inflammatory processes associated with DRA.

Dialysis-related amyloidosis (DRA) is a serious, often incapacitating complication for patients undergoing maintenance dialysis (1,2). Amyloid deposits, composed of β-2-microglobulin (β-2-m) (3), are located mainly in joints and periarticular bones and can result in carpal tunnel syndrome, periarticular bone cysts, and destructive spondyloarthropathy (1,2,4).

The pathogenesis of DRA is incompletely understood. Although recent histologic studies demonstrated the accumulation of monocytes/macrophages around amyloid deposits (5,6), the factors causing their infiltration and pathologic involvement have yet to be fully elucidated. Because β-2-m is a major constituent of amyloid fibrils, it has been the target molecule in some efforts to elucidate the pathogenesis of DRA. However, no correlation between serum levels of β-2-m and the occurrence of DRA has been observed (7,8).

Recent studies demonstrated that advanced glycation end products (AGE), formed by nonenzymatic Maillard reactions between proteins and carbohydrates, are present in β-2-m amyloid fibrils in DRA (9,10). In diseases such as diabetes mellitus and Alzheimer’s disease, excessive deposition of these covalent adducts has been demonstrated to contribute to tissue injury via direct chemical crosslinking, as well as through cell surface receptor-mediated pathways (11). Articular structures, such as the synovium, are the predominant sites of early β-2-m amyloid deposition. The synovial intima consists of macrophage-like (type A) and fibroblast-like (type B) cells (12). Synovial fibroblasts, which account for 70% of the cellular constituents of the synovial intima (12), are exposed directly to AGE-modified β-2-m (AGE-β-2-m) during the natural history of DRA. Our previous identification of the receptor for AGE (RAGE) on human skin fibroblasts (13) led us to postulate that synovial fibroblasts might be a direct target for the agonist actions of AGE-β-2-m. On the basis of this hypothesis, we examined the interaction of AGE-β-2-m with human synovial fibroblasts and investigated the proinflammatory effects of this interaction.

Materials and Methods

Isolation of Human Synovial Fibroblasts

Synovial tissues were obtained from healthy subjects during knee operations or lower-extremity amputations after traumatic injuries. Synovial fibroblasts were isolated and cultured using conventional
techniques, as described previously (14). Briefly, synovial tissue was minced, digested with collagenase (Sigma Chemical Co., St. Louis, MO) followed by trypsin (Life Technologies, Grand Island, NY), and then processed through a 120-μm stainless steel mesh. The crude cell suspension was pelleted and resuspended in a trypsin-ethylenediaminetetraacetaet (EDTA) solution (Life Technologies), to quench the collagenase activity. The isolated cells were grown at 37°C in 95% air/5% CO₂, in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) (Life Technologies). Confluent cultures were collected by incubation with 0.1% trypsin/0.02% EDTA (Sigma) and were subcultured at a 1:3 split ratio. For further characterization of the cells, ultrastructural examinations were performed at the fourth passage (15). Immunofluorescence or immunohistochemical staining for markers specific for fibroblasts (vascular cell adhesion molecule-1 and vimentin) or macrophages (CD68 and CD14) was performed at the same passages as noted previously (16,17).

**Identification of RAGE on Human Synovial Fibroblasts**

**Immunofluorescence Analyses.** Human synovial fibroblasts were allowed to adhere to 12-mm coverglasses for 2 h at 37°C. The cells were then washed with phosphate-buffered saline (PBS) (pH 7.4) and fixed in 2% paraformaldehyde. Fixed synovial fibroblasts were incubated with rabbit anti-human RAGE (40 μg/ml) (13,18) or nonimmune rabbit IgG (40 μg/ml) for 60 min at 37°C. After washing the primary antibody, the surface distribution of the primary antibody was detected with FITC-conjugated goat anti-rabbit IgG (Sigma).

**Immunohistochemical Analyses.** Synovial samples were obtained from healthy subjects (n = 5; mean age, 51 yr) and from nondiabetic patients with DRA (n = 4; mean age, 60 yr) who had undergone hemodialysis for 7 to 12 yr. DRA synovial samples were obtained during surgical intervention (carpal tunnel release or rotator cuff repair). Tissue samples were snap-frozen in OCT compound (Life Technologies) and were stored at −80°C. Frozen tissue sections (6 μm) were air-dried at room temperature, on poly-t-lysine-coated glass slides, for 6 to 16 h. Sections were fixed in chloroform/acetone (1:1) for 10 min at room temperature, air-dried, and rehydrated in 0.05 M Tris-HCl for at least 5 min.

Serial sections of each specimen were subjected to both single and double immunohistochemical analyses with the Dako EnVision double-stain system (Dako, Glostrup, Denmark), according to the technical guidelines provided by the manufacturer. Single immunohistochemical analyses were performed for β₂m and CD68. β₂m amyloid deposits were identified on the basis of alkaline Congo Red and anti-β₂m (dilution, 1/100; Dako) staining. Monocyte/macrophage infiltration was identified on the basis of anti-CD68 (dilution, 1/50; Dako) staining. Double immunohistochemical analyses were performed for vimentin and RAGE, CD68 and RAGE, and RAGE and monocyte chemoattractant protein-1 (MCP-1). The primary antibodies used in the study were as follows: mouse anti-human vimentin (dilution, 1/50; Dako), rabbit anti-human RAGE (40 μg/ml), mouse anti-human CD68 (dilution, 1/50; Dako), and mouse anti-human MCP-1 (dilution, 1/40; Pharmingen, San Diego, CA). The following controls were always included: anti-vimentin and mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD68 and mouse IgG1 (Pharmingen), and RAGE and rabbit IgG.

**Cell Membrane Extraction and Western Blotting.** Cell membrane-enriched fractions were prepared as described previously (19). Synovial fibroblast monolayers were detached with PBS containing EDTA (5 mM), centrifuged, and resuspended in lysis buffer [PBS containing 200 mM Hepes, 1 mM EDTA, 300 mM KCl, 3 mM MgCl₂, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 10 μg/ml aprotonin)]. Cells were homogenized at 4°C, and unbroken cells were removed after centrifugation at 12,000 × g for 10 min at 4°C. The supernatants were subjected to further centrifugation at 97,000 × g for 1 h. The membrane-enriched pellets were resuspended in lysis buffer.

For Western blot analysis, the cell membrane extracts (20 μg of protein/lane) were subjected to electrophoresis on 12% sodium dodecyl sulfate (SDS)-Tris-glycine gels (BioRad, Hercules, CA), under reducing conditions, and were electroblotted onto nitrocellulose membranes (BioRad). Nonspecific binding sites were blocked with 5% albumin solution. The primary rabbit anti-RAGE antibody (45 μg/ml), in PBS containing 0.05% Tween-20, was added to the blots and incubated at room temperature for 2 h. Nonimmune rabbit IgG was used as a negative control. At the end of the incubation, a peroxidase-conjugated, secondary anti-rabbit IgG antibody (IgG) was added and the blots were developed with ECL reagents (Amersham, Arlington Heights, IL).

**Reverse Transcription-PCR.** Synovial fibroblasts were detached and washed. Total RNA was extracted by using a single-step method of RNA isolation via acid guanidinium-thiocyanate-phenol-chloroform extraction (20). RNA contents were measured, and 5 μg of RNA was used to reverse transcription (RT) reactions, which were performed by using a first-strand cDNA synthesis kit (Genset, Singapore). A sample of cDNA was then placed in a PCR instrument (with Taq polymerase and dNTP purchased from Genset). The specific primer pairs for RAGE were as follows: sense, AGGCCCTCCCATGACTACT; antisense, GGGCACGGGGACACGATTG (Institute of Biophysics of China, Peking, China). The expected size of the RT-PCR product was 1.2 kb. The PCR included 32 cycles. After PCR, the products were separated on a 1% agarose gel and stained with ethidium bromide. At least three RT-PCR were performed, using synovial fibroblasts from different isolations.

**Preparation of AGE-Modified Proteins**

AGE-modified proteins were prepared in vitro as described previously (13). Briefly, 1.75 mg/ml purified normal human β₂m (Cortex Biochem, San Leandro, CA) or normal human serum albumin (HSA) (Sigma) was incubated at 37°C for 56 d with 200 mM D-glucose in 100 mM phosphate buffer containing 200 U/ml penicillin, 70 μg/ml gentamicin, and 1.5 mM phenylmethylsulfonyl fluoride. Samples incubated in an identical manner in the absence of glucose were used as controls. After incubation, all samples were dialyzed against PBS (pH 7.4). Endotoxin was removed from the samples as described previously (21). AGE-modified proteins were characterized by using an enzyme-linked immunosorbent assay (ELISA) and fluorospectrometry, as described (22). The AGE content was quantitated fluorospectrometrically. The AGE content for AGE-β₂m was 66.3 U/mg protein, that for AGE-HSA was 70.1 U/mg protein, and that for both β₂m and HSA controls was 0.9 U/mg protein. Endotoxin levels in all samples were measured with an E-toxat kit (Sigma) and were found to be below the limit of detection (<0.5 U/ml).

**125I-AGE-β₂m Binding Assays**

AGE-β₂m was radiolabeled by using the Iodo-Gen method (23), with carrier-free 125I (Institute of Atomic Energy of China, Peking, China). Samples were dialyzed against 0.01 M phosphate (pH 7.0)/0.15 M NaCl until >95% of the radioactivity was TCA-precipitable and the samples were free of iodide. Protein concentrations were determined by using the BioRad assay (BioRad).
For binding studies, synovial fibroblasts (2 × 10^4/well) were seeded onto 24-well plates and incubated overnight. Cells were then washed with binding buffer (Hanks’ buffered salt solution containing 0.02% FCS and 25 mM Hepes) and incubated with the indicated concentrations of radiolabeled AGE-β_m in the presence or absence of a 25-fold molar excess of unlabeled AGE-β_m or nonglycated β_m for 3 h at 4°C. Where indicated, synovial fibroblasts were preincubated with the indicated concentrations of rabbit anti-human RAGE antibody or nonimmune rabbit IgG for 2 h at 4°C, before addition of radiolabeled AGE-β_m. At the end of the incubation, the radioligand-containing medium was aspirated. The cells were washed three times with fresh medium, lysed by addition of 0.1 M NaOH, and counted in a γ-counter (LKB, Gaithersburg, MD). Specific binding was calculated as the difference between total and nonspecific (the radioligand bound in the presence of a 25-fold molar excess of unlabeled ligand) binding. Transformation of saturation binding data was performed according to the method described by Vlassara (23), for estimation of the receptor number and binding affinity constant.

For determination of 125I-AGE-β_m uptake and degradation by synovial fibroblasts, monolayers were washed with DMEM containing 0.02% FCS and 25 mM Hepes and were incubated with 125I-AGE-β_m (50 μg) at 37°C for 22 h, in the presence or absence of unlabeled AGE-β_m. Culture supernatants were collected and precipitated with an equal volume of 20% TCA. The amount of cell-degraded 125I-AGE-β_m was calculated from the non-TCA-precipitable radioactivity value, as described previously (24).

**MCP-1 Protein and mRNA Analyses**

**Cell Preparation.** Human synovial fibroblasts isolated from each patient were plated at 5 × 10^5 cells/well in 24-well plates and were grown in DMEM with 10% FCS for 3 d, until confluent. The medium was changed to DMEM supplemented with 0.2% lactalbumin (Life Technologies) and 0.05% (vol/vol) lipids (Sigma) for 18 h; the medium was changed to DMEM supplemented with 0.2% lactalbumin according to the method described by Vlassara (23), for estimation of binding. Transformation of saturation binding data was performed as described by Vlassara (23), for estimation of the receptor number and binding affinity constant.

For determination of 125I-AGE-β_m uptake and degradation by synovial fibroblasts, monolayers were washed with DMEM containing 0.02% FCS and 25 mM Hepes and were incubated with 125I-AGE-β_m (50 μg) at 37°C for 22 h, in the presence or absence of unlabeled AGE-β_m. Culture supernatants were collected and precipitated with an equal volume of 20% TCA. The amount of cell-degraded 125I-AGE-β_m was calculated from the non-TCA-precipitable radioactivity value, as described previously (24).

**ELISA.** The levels of MCP-1 in the supernatants were quantitated with a sandwich ELISA, as described previously (25). Briefly, plates were coated with a mouse anti-human MCP-1 monoclonal antibody (5-D3-F7; PharMingen), at a concentration of 5 μg/ml, for 12 h at 4°C. Nonspecific binding sites were blocked with 0.3% (wt/vol) gelatin in PBS containing 0.05% (vol/vol) lipids (Sigma) for 18 h; the cells were then incubated in the same medium with increasing concentrations of AGE-β_m, AGE-HSA, native HSA, or unmodified β_m. In separate experiments, synovial fibroblasts were preincubated with 5.0 μg/ml rabbit anti-human RAGE or nonimmune rabbit IgG for 2 h at 4°C. The cells were washed with DMEM with 0.2% lactalbumin and incubated for 48 h with AGE-β_m (50 μg/ml). At the end of the incubations, the supernatants were collected and quantitated for MCP-1. The corresponding monolayers were washed and trypsinized, and the cells were counted.

**Northern Blot Analyses.** Human synovial fibroblasts were incubated with 50 μg/ml AGE-β_m, AGE-HSA, unmodified β_m, or unmodified HSA for 48 h. RNA was extracted with TRIZOL (Life Technologies) (20). Twenty micrograms of total RNA were subjected to electrophoresis in each lane of an agarose/formaldehyde gel, transferred to nitrocellulose membranes, and hybridized with a 32P-labeled cDNA probe for human MCP-1 (American Type Culture Collection, Manassas, VA) at 42°C for 16 h. After hybridization, the membranes were washed twice with 2× SSC/0.1% SDS at room temperature for 30 min each time and twice with 0.1× SSC/0.1% SDS at 50°C for 15 min each time. The membranes were exposed to x-ray film (Kodak, Xiamen, China). The autoradiographs were scanned, and densitometric analysis was performed by using metamorphic software (Universal Imaging Corp., England). The membranes were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase, for comparison of RNA loading.

**Monocyte Chemotaxis Assay**

Chemotaxis assays were performed in 48-well microchemotaxis chambers (Neuro-Probe, Bethesda, MD) containing polycarbonate membranes (5 μm; Nucleopore, Pleasanton, CA), using human peripheral blood-derived monocytes (18). Monocytes were suspended in RPMI 1640 medium containing 1% FCS. Ten thousands cells/well were added to the upper chamber, and the lower chamber contained MCP-1 (4.4 ng/ml), medium alone (negative controls), or 10⁻⁶ M formylmethionylleucylphenylalanine (positive controls). Assays were performed in triplicate or quadruplicate with a 4-h incubation period at 37°C, after which nonmigrating cells were removed, membranes were fixed in methanol, and migrating cells were observed with Wright's stain. Cells in nine high-power fields were counted. The experiment was repeated three times, using monocytes from three healthy volunteers.

**Statistical Analyses**

All experiments were performed in triplicate. Continuous variables, expressed as mean ± SD, were compared by using ANOVA. Multiplicative terms were included for evaluation of interactions among explanatory variables. The Student-Newman-Keuls procedure was used to evaluate pairwise comparisons. Two-tailed P values of <0.05 were considered statistically significant.

**Results**

**Characterization of Human Synovial Fibroblasts**

Human synovial fibroblasts were isolated from normal synovium. As assessed with electron microscopy, the ultrastructure of the cells from the fourth passage exhibited broad processes containing rough endoplasmic reticulum, which is phenotypically characteristic of fibroblast-like cells (12) (Figure 1A). Supporting these ultrastructural findings were the results of the immunofluorescence and histochemical staining assays. By the fourth passage, all cells were positive for vascular cell adhesion molecule-1 (Figure 1B) and vimentin (Figure 1C). No CD68 or CD14 expression was observed (data not shown), confirming pure fibroblast lineage. There was no change in phenotypic or immunohistochemical characteristics of cells between the fourth and seventh passages. Because of these preliminary findings, all subsequent experiments were performed with fourth- to seventh-passage cells, to ensure pure fibroblast cell populations.

**Demonstration of RAGE in Human Synovium and on Cultured Synovial Fibroblasts**

β_m amyloid deposits were observed in synovial specimens from hemodialysis patients but not in specimens from healthy
RAGE immunoreactivity was markedly enhanced on fibroblasts (Figure 2C) and was observed on monocytes/macrophages surrounding the amyloid deposits (Figure 2F). No immunostaining of synovium from either healthy subjects or patients with DRA was evident with nonimmune IgG (Figure 2, B, E, F, H, and I).

For confirmation of the presence of RAGE on human synovial fibroblasts, membrane-enriched fractions were generated from isolated synovial fibroblasts and were analyzed for immunoreactive material with polyclonal anti-human RAGE IgG. Two bands were visible in Western blots, corresponding to relative molecular masses of 32 and 50 kD (Figure 3A, left). Irrelevant antibody was without immunoreactivity (Figure 3A, right). The presence of two different immunoreactive RAGE polypeptides in the membrane fraction probably reflects post-translational processing/cleavage; multiple forms of RAGE, with different molecular masses, are observed in cells transfected with full-length RAGE cDNA (26), as well as in the membrane fraction of human skin fibroblasts (13).

For assessment of the distribution of RAGE on synovial fibroblasts, isolated cells were studied with immunofluorescence staining. Synovial fibroblasts incubated with anti-human RAGE IgG demonstrated a diffuse pattern of membrane staining (Figure 3B), compared with the nonimmune rabbit IgG control (data not shown).

To demonstrate the transcription of RAGE in synovial fibroblasts, RAGE mRNA expression was analyzed by RT-PCR. A signal was observed for isolated human synovial fibroblasts (Figure 3C, lane 2). The RNA sample that had not been reverse-transcribed did not yield PCR products (Figure 3C, lane 1), confirming the absence of extraneous cDNA or PCR products contaminating the samples.

**RAGE Mediation of the Binding of AGE-\( \beta_2 \text{m} \) to Synovial Fibroblasts**

In view of the presence of RAGE on the surface of human synovial fibroblasts, we considered whether they could mediate interactions with extracellular AGE. Incubation of \( ^{125}\text{I}-\text{AGE-}\beta_2 \text{m} \) with synovial fibroblasts resulted in dose-dependent, specific binding with a \( K_d \) of 138.0 ± 0.16 nM (Figure 4A), which is lower than the binding affinity observed previously for skin fibroblasts (27). The radioligand binding was inhibited in the presence of excess amounts of unlabeled AGE-\( \beta_2 \text{m} \) but not unmodified \( \beta_2 \text{m} \). The hypothesis that RAGE are involved in the interaction of AGE-\( \beta_2 \text{m} \) with synovial fibroblasts was supported by preincubation studies using anti-RAGE IgG. Anti-RAGE IgG, but not nonimmune IgG, largely inhibited the binding of \( ^{125}\text{I}-\text{AGE-}\beta_2 \text{m} \) to synovial fibroblasts, in a dose-dependent manner (\( P < 0.001 \)) (Figure 4B).

For analysis of the uptake and degradation of AGE-\( \beta_2 \text{m} \), synovial fibroblasts were incubated with 50 \( \mu \text{g/ml} \) \( ^{125}\text{I}-\text{AGE-}\beta_2 \text{m} \) for 22 h. The mean amount of degraded radiolabeled ligand demonstrated as TCA-soluble material was 4.8 ± 1.1 ng/\( \mu \text{g} \) cell protein (\( n = 3 \)). \( ^{125}\text{I}-\text{AGE-}\beta_2 \text{m} \) degradation by synovial fibroblasts was reduced to 1.4 ± 0.3 ng/\( \mu \text{g} \) cell protein (\( n = 3 \)) when excess unlabeled ligand was added (\( P < 0.001 \)).
0.01, compared with the absence of excess unlabeled ligand) and to 2.1 ± 0.5 ng/μg cell protein (n = 3) when cells were preincubated with 50 μg/ml anti-RAGE before the addition of radiolabeled ligand (P < 0.02, compared with the nonpreincubation group). These results suggest that the degradation of AGE-β2m by synovial fibroblasts was partially receptor-dependent and was mediated through RAGE.

**Figure 2.** Immunohistochemical examination of synovial tissue. Peroxidase-labeled polymer detection of vimentin and CD68 is indicated by brown staining, and alkaline phosphatase-labeled polymer detection of the receptor for advanced glycation end products (RAGE) is indicated by red staining. (A) Double-staining with anti-vimentin and anti-RAGE in a synovial specimen from a healthy control subject (age, 58 yr). Some vimentin-positive cells stained for RAGE. (B) Double-staining with anti-vimentin and control rabbit IgG in the same region as shown in A. Vimentin staining remained unchanged, and RAGE staining was absent. (C) Double staining with control mouse IgG and control rabbit IgG in the same region as shown in A. (D) Double-staining with anti-vimentin and anti-RAGE in synovial tissue from a 60-yr-old patient with dialysis-related amyloidosis (DRA). Most vimentin-positive cells stained for RAGE. (E) Double-staining with anti-vimentin and control rabbit IgG in the same region as shown in C. Staining for vimentin was present, and staining for RAGE was absent. (F) Double-staining with control mouse IgG1 and control rabbit IgG in the same region as shown in C. (G) Double-staining with anti-CD68 and anti-RAGE in synovium from a patient with DRA. CD68-positive cells around amyloid deposits expressed RAGE. (H) Double-staining with anti-CD68 and control rabbit IgG in the same region as shown in F. CD68 staining was present, and RAGE staining was absent. (I) Double-staining with control mouse IgG1 and rabbit IgG in the same region as shown in F. Magnification, ×200.

**AGE-β2m Induction of MCP-1 Expression by Synovial Fibroblasts**

MCP-1 is a well characterized chemokine that selectively attracts and activates monocytes. We examined the effect of AGE-β2m on synovial fibroblast secretion of MCP-1 (25). Synovial fibroblasts were incubated for 48 h with AGE-β2m, and the levels of MCP-1 in the supernatants were measured by
Figure 3. Identification of RAGE on cultured human synovial fibroblasts. (A) Western blot analysis of cell membrane extracts of human synovial fibroblasts. (Left) The membrane extract was subjected to 12% sodium dodecyl sulfate-Tris-glycine gel electrophoresis and electroblotting. The blots were reacted with anti-human RAGE IgG (45 μg/ml). Sites of primary antibody binding were detected with a peroxidase-conjugated secondary antibody. (Right) The same experiment was performed by using nonimmune rabbit IgG (45 μg/ml) as the primary antibody. (B) Detection of RAGE on human synovial fibroblasts by indirect immunofluorescence analysis. The cells were stained with rabbit anti-RAGE IgG (40 μg/ml) as described in the text. (C) Demonstration of the transcription of RAGE in synovial fibroblasts. Lane 1, PCR product of sample RNA that had not been reverse transcribed. Lane 2, reverse transcription-PCR product of RAGE mRNA from human synovial fibroblasts. Lane 3, markers.

Figure 4. Binding of advanced glycation end products (AGE)-β2-microglobulin (β2-m) to immobilized synovial fibroblasts. (A) 125I-AGE-β2-m, alone (total binding) or in the presence of a 25-fold molar excess of unlabeled AGE-β2-m (nonspecific binding), was incubated with immobilized human synovial fibroblasts, as described in the text. Specific binding (total minus nonspecific binding) was plotted against the concentration of free added 125I-AGE-β2-m. Nonspecific binding was ≤25% of the total binding in all cases. Data were analyzed with a nonlinear least-squares method. Parameters of binding for immobilized synovial fibroblasts included a $K_d$ of 138.0 ± 0.16 nM. (Inset) Scatchard plot analysis. (B) Synovial fibroblasts were preincubated with the indicated concentration of anti-RAGE IgG or nonimmune rabbit IgG for 2 h at 4°C. 125I-AGE-β2-m (alone or in the presence of a 25-fold molar excess of unlabeled AGE-β2-m) was then added, and a binding assay was performed as described above. Data (percentages of maximal specific binding; 100% is defined as specific binding in the absence of added anti-RAGE IgG) are expressed as means ± SD of duplicate determinations, and the experiments were repeated three times. ANOVA, $P < 0.0001$; concentration of α-RAGE IgG, $P < 0.001$. The α-RAGE-treated group was significantly different from the rabbit IgG-treated ($P < 0.001$) and AGE-β2-m alone-treated groups ($P < 0.001$).
ELISA. As presented in Figure 5A, a dose-dependent increase in MCP-1 levels ($P < 0.001$) was observed in the medium from cells incubated with AGE-β$_2$m. In contrast, trace concentrations of MCP-1 were detected in the culture medium from cells incubated with unmodified β$_2$m or medium alone. AGE-modified HSA, but not unmodified HSA, also induced MCP-1 production. There was no significant difference in MCP-1 levels in supernatants of cells treated with AGE-β$_2$m versus AGE-HSA ($P > 0.05$). AGE-β$_2$m-induced MCP-1 secretion was time-dependent and peaked at 48 h (Figure 5B).

To determine whether the AGE-β$_2$m-induced increase in MCP-1 protein release was associated with an increase in mRNA, Northern blot analysis was performed with isolated human synovial fibroblasts. The results indicated that AGE-β$_2$m and AGE-HSA increased the expression of MCP-1 mRNA, compared with their unmodified forms (Figure 6).

Attenuation of the Effect of AGE-β$_2$m with Blocking of RAGE

To further confirm the role of RAGE in the interaction of AGE-β$_2$m with synovial fibroblasts, adherent synovial fibroblasts were preincubated with 50 μg/ml rabbit anti-human RAGE or nonimmune rabbit IgG for 2 h. This concentration of anti-human RAGE was previously demonstrated to block other effects of AGE-β$_2$m in vitro (13). The synovial fibroblasts were then cultured for 48 h with 50 μg/ml AGE-β$_2$m. The antibody that blocks the interaction of AGE-β$_2$m and RAGE in other systems (13,18,28) significantly diminished the capacity of AGE-β$_2$m to induce MCP-1 production (Figure 7). The blocking effect of anti-RAGE was specific; nonimmune IgG had no effect on MCP-1 synthesis.

MCP-1-Induced Monocyte Chemotaxis

Monocyte recruitment and activation may play important roles in the pathogenesis of inflammatory processes in DRA (21). Therefore, we investigated whether the amount of MCP-1 produced by synovial fibroblasts stimulated with AGE-β$_2$m was adequate to induce monocyte chemotaxis in vitro. An amount of MCP-1 equivalent to that produced by 10$^5$ synovial fibroblasts after stimulation with 50 μg/ml AGE-β$_2$m was added to the chamber. This amount of MCP-1 (4.4 ng/ml) induced monocyte chemotaxis (46 ± 6 cells/high-power field, $n = 3$), in comparison with negative controls with medium alone (10 ± 2 cells/high-power field, $n = 3$; $P < 0.001$). The formylmethionylleucylphenylalanine positive controls induced an equivalent amount of chemotaxis (38 ± 5 cells/high-power field, $n = 3$; $P > 0.05$, compared with MCP-1).

Increased Expression of MCP-1 on DRA Synovium

Double-labeling immunostaining for RAGE and MCP-1 and for vimentin and MCP-1 was performed for all DRA and control specimens. A majority of the cells that expressed RAGE in DRA also stained positively for MCP-1 (Figure 8, A and B). MCP-1 staining was present on vimentin-positive cells in DRA specimens (Figure 8, C and D) but not in samples from healthy control subjects (Figure 8E).
Nonenzymatic glycation status, specifically immunologic and molecular probes corresponding to RAGE on human synovial fibroblasts was confirmed by using specific immunologic and molecular probes corresponding to RAGE on human synovial fibroblasts. Synovial fibroblasts were incubated for 48 h with medium alone (lane 1), 50 μg/ml unmodified HSA (lane 2), 50 μg/ml AGE-HSA (lane 3), 50 μg/ml native β2m (lane 4), or 50 μg/ml AGE-β2m (lane 5). MCP-1 mRNA levels were analyzed by Northern blotting, as described in the text. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data from three independent experiments are presented as means ± SD. ANOVA, P < 0.01; protein nonenzymatic glycation status, P < 0.001.

Discussion

AGE-modified β2m is observed in the amyloid fibrils in DRA and has been suggested to be a provocative agent in this condition (9,21). AGE-modified macromolecules are subject to endocytosis by AGE-specific receptors present in a variety of cell systems, especially monocytes/macrophages, which are thought to facilitate AGE removal and elimination (18,23,28). In addition to serving in the scavenging of senescent macromolecules, cellular interactions with AGE have been demonstrated to promote tissue damage via pathways involving chemical crosslinking and proinflammatory reactions (11,13,18). Such interactions may occur in normal aging and in diseases such as diabetes mellitus and Alzheimer’s disease. Although links between the AGE-laden amyloid deposits and the synovial infiltration of monocytes in DRA have been reported (28), the biologic effects of AGE in human articular structures and their role in DRA-associated tissue damage have not been fully elucidated. As suggested by previous evidence of time-dependent AGE formation in synovial constituents such as collagen (29) and of AGE deposition in amyloid synovium, an AGE-specific cellular pathway may be important in both normal homeostasis and the pathogenesis of joint damage in DRA.

As indicated by immunohistochemical analysis of synovial sections from healthy adult subjects, RAGE were expressed constitutively and distributed on synovial fibroblasts. For further identification of the cell type that expresses RAGE, human synovial fibroblasts (the most abundant cells in the synovial intima) were isolated and cultured in vitro. The presence of RAGE on human synovial fibroblasts was confirmed by using specific immunologic and molecular probes corresponding to previously characterized RAGE (30). Binding to other resident cell types in the synovium is currently unknown.

Several AGE-binding molecules have been identified, such as F’60, P’90 (31), galectin-3 (19), the macrophage scavenger receptor (32), and RAGE. The most well characterized of these is RAGE, a member of the Ig superfamily that is expressed by mononuclear phagocytes, endothelial cells, and smooth muscle cells (33), as well as certain neurons and other cells. RAGE has been demonstrated to mediate the interactions of AGE-modified proteins with endothelium, being responsible for the initial rapid phase of clearance observed after the infusion of AGE-albumin and the induction of interleukin-6 transcripts (34). This receptor may also have a central role in AGE-induced perturbations of mononuclear phagocytes, including enhanced chemotaxis and release of proinflammatory cytokines (28). In the context of the biologic processes of skin fibroblasts, RAGE binds AGE, which is accompanied by changes in collagen synthesis (13). Reported herein is the first study examining the functional pathway of RAGE on human synovial fibroblasts. Our data suggested that AGE-modified β2m, which accumulates in the joints of patients with DRA, interacted with RAGE on the surface of synovial fibroblasts. AGE-β2m bound to synovial fibroblasts in a specific, dose-dependent manner, and that process was largely inhibited in the presence of RAGE blockade. The incomplete blockade by anti-RAGE may be a consequence of receptor recycling or the involvement of AGE-binding proteins other than RAGE. The RAGE-mediated en-

Figure 6. Effect of AGE-β2m on MCP-1 mRNA levels expressed by human synovial fibroblasts. Synovial fibroblasts were incubated for 48 h with medium alone (lane 1), 50 μg/ml unmodified HSA (lane 2), 50 μg/ml AGE-HSA (lane 3), 50 μg/ml native β2m (lane 4), or 50 μg/ml AGE-β2m (lane 5). MCP-1 mRNA levels were analyzed by Northern blotting, as described in the text. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data from three independent experiments are presented as means ± SD. ANOVA, P < 0.01; protein nonenzymatic glycation status, P < 0.001.

Figure 7. Effect of anti-RAGE IgG on AGE-β2m-induced increases in MCP-1 secretion. Human synovial fibroblasts were maintained in medium alone or incubated with 50 μg/ml AGE-β2m for 48 h, pretreated with 50 μg/ml rabbit anti-human RAGE IgG or nonimmune rabbit IgG for 2 h and then incubated with 50 μg/ml AGE-β2m for 48 h, or preincubated with 50 μg/ml rabbit anti-human RAGE IgG or rabbit IgG for 2 h and then incubated with medium alone for 48 h. MCP-1 levels in the supernatants were determined by ELISA. Data from three independent experiments are represented as means ± SD. ANOVA, P < 0.0001; Student-Newman-Keuls test, rabbit anti-RAGE plus AGE-β2m was significantly different from AGE-β2m alone (P < 0.001) and medium alone (P < 0.001).
Docytosis and degradation of AGE-β₂m by synovial fibroblasts suggest that fibroblasts might be capable of modulating AGE-modified molecules in vivo.

MCP-1 is a well characterized member of the family of CC chemokines (35) that has been demonstrated to be one of the most potent monocyte chemoattractants and activators. MCP-1 is produced by a wide variety of cell types after stimulation with proinflammatory cytokines or even direct stimulation with whole bacteria (36). Therefore, it may be an important mediator in the regulation of monocyte influx into areas of inflammation. The interaction of synovial fibroblasts with AGE-β₂m induced marked increases in MCP-1 mRNA expression and protein release. This response was elicited by AGE modification of the protein and was not a property specific to β₂m. Other AGE-modified proteins, such as AGE-HSA, also induced MCP-1 synthesis, but unmodified β₂m or HSA did not. The capacity of AGE-β₂m to induce MCP-1 synthesis was significantly decreased by an antibody that blocks the interaction of AGE-proteins and RAGE both in vitro and in vivo (28,37), indicating that this increase in MCP-1 expression was partially mediated by RAGE. DRA is a chronic inflammatory process involving articular structures. A major factor contributing to the development and progression of joint damage is the infiltration of monocytes (5,21). AGE-modified β₂m has been speculated to be a potential causative agent in this cellular inflammatory response (21,38). Previous studies demonstrated that AGE-β₂m induces monocyte chemotaxis (28), delays monocyte apoptosis (38), and transforms monocytes to a macrophage-like morphologic phenotype (38). Therefore, it has been speculated that AGE-β₂m may contribute to the accumulation of monocytes/macrophages in DRA synovium. This study illustrates an alternative mechanism of AGE-β₂m-induced monocyte recruitment, i.e., through the release of a synoviocyte-derived chemokine. Because the amount of MCP-1 production stimulated by AGE-β₂m was sufficient to induce monocyte chemotaxis in vitro, we posit that synovial fibroblasts can actively contribute to an inflammatory response via cellular interactions with AGE-β₂m. This hypothesis was supported by the immunohistochemical analyses, in which increased expression of MCP-1 was observed on synovial fibroblasts and cells expressing RAGE in DRA synovium. MCP-1 expression on synoviocytes has also been demonstrated in other studies (39,40).

We confirmed the upregulation of RAGE in synovial intimal and subintimal cells of long-term hemodialysis patients with DRA. Consistent with this observation, previous studies demonstrated enhanced RAGE expression by mononuclear phagocytes around cutaneous β₂m amyloid deposits (28). RAGE is also expressed at high levels in diabetic vasculature (41), in which AGE are abundant. Although the factors that regulate

Figure 8. Double immunohistochemical analyses of synovial tissue from patients with DRA. Peroxidase-labeled polymer detection of vimentin and RAGE is indicated by brown staining, and alkaline phosphatase-labeled polymer detection of MCP-1 is indicated by red staining. (A) Double-staining with anti-RAGE and anti-MCP-1 in synovial tissue from a patient with DRA. Most RAGE-positive cells stained for MCP-1. (B) Double-staining with anti-RAGE and control mouse IgG1 in the same region as shown in A. (C) Double-staining with anti-vimentin and anti-MCP-1 in synovial tissue from the same patient. Many vimentin-positive cells also stained for MCP-1. (D) Double-staining with anti-vimentin and control mouse IgG1 in the same region as shown in C. (E) Double-staining with anti-vimentin and anti-MCP-1 in synovial tissue from a normal control subject. Magnification, ×100 in A and B; ×200 in C, D, and E.
RAGE expression in these situations remain to be elucidated, these findings indicate that RAGE may be a central binding site for AGE formed in vivo, and they suggest that AGE-β2-microglobulin interactions might be involved in the chronic inflammatory processes observed in DRA.

In summary, our study demonstrates the functional RAGE pathway on human synovial fibroblasts, which mediates the binding and degradation of AGE-proteins as well as the induction of MCP-1 by these cells. Through this pathway, synovial fibroblasts may be actively involved in the cellular inflammatory reactions associated with eventual joint damage and destruction in DRA.

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