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β_2-Microglobulin modified with advanced glycation end products delays monocyte apoptosis

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Background. A local inflammatory reaction to β_2-microglobulin (β_2m) amyloid deposits by monocytes/macrophages is a characteristic histologic feature of dialysis-related amyloidosis (DRA). Since β_2m modified with advanced glycation end products (AGE-β_2m) is a major constituent of amyloid in DRA, we tested the hypothesis that AGE-β_2m affects apoptosis and phenotype of human monocytes.

Methods. Human peripheral blood monocytes were incubated with or without in vitro-derived AGE-β_2m, and their viability, extent of apoptosis, morphology, and function examined over the subsequent four days.

Results. AGE-modified but not unmodified β_2m significantly delayed spontaneous apoptosis of human peripheral blood monocytes in adherent and nonadherent cultures. The effect of AGE-β_2m on monocytes apoptosis was time- and dose-dependent and was attenuated by a blocking antibody directed against the human AGE receptor (RAGE). There was no difference in effect between AGE-β_2m and that of AGE-modified human serum albumin. Culture of monocytes with AGE-β_2m did not alter membrane expression of Fas or Fas ligand. Monocytes cultured with AGE-β_2m underwent substantial changes in morphology similar to those observed when monocytes differentiate into macrophages. The cultured cells increased in size and vacuolization, and their content of β-glucuronidase and acid phosphatase increased by 5- to 10-fold at day 4. Expression of the monocyte–macrophage membrane antigens HLA-DR, CD11b, and CD11c also increased at day 4. Although exhibiting phenotypic characteristics of macrophages, monocytes cultured with AGE-β_2m functioned differently than macrophages cultured with serum. Superoxide production in response to phorbol myristic acetate was maintained in monocytes cultured with AGE-β_2m, but declined with time in cells cultured with serum. Constitutive synthesis of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and prostaglandin E_2 (PGE_2) increased in monocytes cultured for four to six days with AGE-β_2m.

Conclusions. These findings support a novel role for AGE-modified proteins such as AGE-β_2m that may contribute to the development of a local inflammatory response, with predominant accumulation of monocytes/macrophages, in DRA.

Key words: amyloidosis, cell death, dialysis, inflammation, chronic renal failure.

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Dialysis-related amyloidosis (DRA) is a progressive and incapacitating condition that affects patients with chronic renal failure [1, 2]. Amyloid fibrils that contain the subunit protein β_2-microglobulin (β_2m) deposit predominantly in osteoarticular tissue, causing shoulder periarthritis, carpal tunnel syndrome, hand flexor tenosynovitis, destructive spondyloarthropathy, and cystic bone lesions [1, 2]. Histologic examination of osteoarticular tissue from patients with DRA reveals a local inflammatory reaction to deposited amyloid by monocytes/macrophages [3–5]. The predominant location of these infiltrating cells around blood vessels or adjacent to the amyloid deposits suggests that the infiltrating monocytes are recruited from the peripheral blood. The observation that they produce interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) suggests that they are primed [6, 7].

A subtle balance between recruitment and constitutive death of inflammatory cells regulates inflammatory responses within the microenvironment [8]. Monocytes and macrophages play significant roles in the exacerbation and resolution of acute and chronic inflammation. Thus, regulation of monocyte/macrophage survival and maturation might be critical in controlling their presence, and hence their biologic affect, in inflammatory lesions [9]. Human peripheral blood monocytes undergo programmed cell death, or apoptosis, when cultured in the absence of appropriate stimuli. Certain inflammatory cy-

tokines, such as IL-1β and TNF-α, but not chemotactic factors, prevent monocyte apoptosis and thus maintain cell viability in vitro [10]. Because monocyte differentiation into macrophages occurs over a period of several days [11], monocyte survival is a fundamental component of their maturation.

β_2m modified with advanced glycation end products (AGE) is a major component of amyloid deposits in DRA [12]. Human monocytes/macrophages express specific receptors for AGE [13]. Interaction of AGE-modified β_2m (AGE-β_2m) with the receptor for AGE (RAGE) on monocytes/macrophages increases monocyte migration and priming [14, 15]. The display of these proinflammatory functions by infiltrating mononuclear phagocytes depends on their continued survival within the tissue microenvironment. Therefore, we examined the effect of AGE-β_2m on monocyte survival and maturation in vitro. The demonstration of an effect of AGE-β_2m on monocyte apoptosis would offer a novel regulatory step in the pathobiology of this debilitating complication of renal failure.

METHODS

In vitro preparation of AGE-modified proteins, CML, and pentosidine

AGE-modified proteins were prepared in vitro as previously described with slight modification [16]. Briefly, 2.5 mg/mL of purified normal human β_2m (BiosPacific, Emeryville, CA, USA) or human serum albumin (HAS; Sigma, St. Louis, MO, USA) was incubated at 37°C for eight weeks with 200 mmol/L D-glucose in 100 mmol/L phosphate buffer containing penicillin and gentamicin. 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 modification was characterized by ELISA using an anti-AGE antibody (gift of Dr. John W. Baynes, University of South Carolina, Columbia, SC, USA) [12, 16]. This antibody specifically reacts with AGE but not with the early products of the Maillard reaction [17]. AGE-modified proteins were also characterized and quantitated by fluorospectrometry as previously described [12, 16]. The AGE content of the products was 42.1 U/mg protein for AGE-β_2m, 43.0 U/mg protein for AGE-HSA and 0.8 U/mg protein for β_2m control. The pentosidine content, as measured by high-performance liquid chromatography (HPLC) [18], was 135 pmol/mg for AGE-β_2m and 1.4 pmol/mg for β_2m control. The carboxymethyllysine (CML) content, as determined by mass spectrometry [18], was 43.8 nmol/mg for AGE-β_2m and less than 0.1 nmol/mg for β_2m control (lower limit of detection of the assay). These results confirm the presence of chemically identified AGE in AGE-modified β_2m, but not in the control sample.

Synthesized pentosidine or CML were prepared in vitro according to our previous method [19] Bovine serum albumin (BSA) modified with pentosidine (pentosidine-BSA) or CML (CML-BSA) was prepared by conjugating synthesized pentosidine or CML with BSA [19].

All samples contained <0.05 U/mL (5 pg/mL) of endotoxin as measured by a limulus amebocyte lysate assay (Sigma) at $\times 2$ the working dilution used for the experiments.

Isolation and culture of human monocytes

Human mononuclear leukocytes, from the whole blood of healthy volunteers not ingesting steroidal or nonsteroidal anti-inflammatory drugs, were separated from other cell types by sedimentation in 6% dextran and centrifugation through Ficoll-Paque [20]. Monocytes were isolated and purified by CD14 positive immunomagnetic selection using VS^+ columns and Vario MACS system according to the manufacturer's technical guidelines (Milienvi Bioiec, Auburn, CA, USA). Final monocyte purification was reproducibly >99% as demonstrated by Wright's stain morphology and phenotypic characterization ($CD11c^+$, $CD25^-$) analyzed by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Monocytes were cultured in RPMI 1640 (GIBCO BRL, Gaithersburg, MD, USA) with 20 mmol/L L-glutamine, 25 mmol/L HEPES buffer (GIBCO BRL), 10 μmol/L 2-mercaptoethanol (GIBCO BRL), penicillin, and streptomycin, either containing various amounts of AGE-β_2m, unmodified β_2m, 10% autologous serum, or no additives. The monocytes were cultured in 17×100 mm polypropylene tubes (nonadherent cultures of 2×10^6/0.5 mL tubes) or in 24-well polystyrene plates (adherent cultures of 2×10^6/1.0 mL wells) at 37°C in 5% CO_2 and high humidity. All media and reagents contained <0.05 U/mL endotoxin.

Analysis of monocytes/macrophages viability

At different times after culture initiation, the cell in nonadherent cultures were centrifuged and resuspended in phosphate buffer (pH 7.4). The adherent cultured cells were detached from the plates by vigorous pipetting after incubation with versene 1/5000 (GIBCO BRL) for 5 to 10 minutes. The harvested cells were centrifuged with their supernatants that contained floating cells and resuspended in phosphate buffer. One volume of trypan blue (0.4%) was added to five volumes of cells suspension. After five minutes of incubation, the cells were counted in duplicate and considered viable if able to exclude trypan blue.

Assessment of apoptosis

Apoptosis was assessed by the following three methods.

Measurement of cellular DNA contents. The percentage of apoptotic cells was quantitated by DNA content

analysis after extraction of the degraded DNA from the cells [21]. Briefly, freshly isolated or cultured cells were fixed in 70% (vol/vol) ice-cold ethanol and stored at −20°C for one to two days. The fixed cells were pelleted, resuspended in 0.5 mL of phosphate buffer, and incubated at room temperature for five minutes with 1.0 mL of DNA extraction buffer. The cells were spun and suspended in 250 μL of phosphate buffer containing 20 μg/mL of propidium iodide (Sigma) and 25 μg/mL DNase-free RNase (Boehringer Mannheim, Indianapolis, IN, USA). Cells were held for 30 minutes in the dark at room temperature and then stored at 4°C for eight hours. DNA content was analyzed using a FACScan flow cytometry (FACs).

Fluorescent TUNEL and Hoechst 33258 staining. DNA strand breaks in individual cells were identified using an in situ nick/end-labeling technique (TUNEL) [22]. Morphological changes in cells undergoing apoptosis were detected simultaneously by counterstaining cells with Hoechst 33258. Briefly, cytospins of harvested cells were fixed for 10 minutes in 2% paraformaldehydrate followed by methanol. After washing with TdT buffer (GIBCO BRL), cells were incubated in TdT buffer containing 20 U/μL TdT (GIBCO BRL) and 20 nmol/L biotinylated dUTP (Boehringer Mannheim) at 37°C for 60 minutes. The enzymatic reaction was terminated by placing the slides in terminal buffer (30 mmol/L sodium citrate, 300 mmol/L sodium chloride). The slides were then blocked in phosphate buffer supplemented with 2% bovine serum albumin (BSA) and followed by incubation with streptavidin-FITC (Fisher, Pittsburgh, PA, USA) and 0.5 μg/mL of Hoechst 33258 (Sigma) for 60 minutes. Slides were examined by fluorescent microscopy (Nikon FXA).

DNA electrophoresis. DNA extraction and electrophoresis were performed as described previously [23]. In brief, cells were collected and lysed by a DNA lysing buffer [20 mmol/L Tris, pH 7.4, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), and 0.4% Triton X-100]. After centrifugation at 14,000 r.p.m. for 10 minutes, supernatants were extracted with a 25 phenol:24 chloroform:1 isoamyl alcohol mixture (GIBCO BRL). Next, 100 μL 5 mol/L NaCl and 500 μL isopropanol were added to each tube before incubating overnight at −70°C. Samples were centrifuged, washed once with 70% ethanol, and dried in a Speed-Vac. The lyophilized pellet were resuspended in 30 μL of Tris-EDTA buffer containing 0.1 mg/mL RNase A (Sigma) and incubated at 37°C for 30 minutes. Equal amounts of DNA were separated on 0.8% agarose gels and visualized by ethidium bromide staining under ultraviolet light.

Morphology and ultrastructure examination

Freshly isolated monocytes were adhered to 24-well polystyrene plates for two days (day 0) or incubated for four days with RPMI 1640 with 10% autologous serum or with serum-free medium containing 100 μg/mL of AGE-β_2m. Morphologic changes of the cells were observed though an inverted microscopy (Nikon TMS). At the end point of culture, the cells were detached from the plates as described above and fixed in 1.25% glutaraldehyde in cacodylate buffer containing 1% $CaCl_2$. Fixed cells were washed in Sabatini's solution and postfixed in 1% osmium tetroxide. After washing, cells were dehydrated in ethanol followed by treatment with propylene oxide. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by a transmission electron microscopy (JEOL 100CX; JEOL, Tokyo, Japan).

Immunophenotype analysis

Fresh isolated monocytes or cells cultured for four days in RPMI 1640 with 10% autologous serum or serum-free medium containing 100 μg/mL of AGE-β_2m were detached from the plates. The cells were washed, resuspended in PBS with 0.5% human serum albumin, and incubated for 60 minutes in ice with 1:50 diluted monoclonal antibody (mAb) against HLA-DR (PharMingen, San Diego, CA, USA), CD11b (Mac-1; PharMingen), CD11c (P150; PharMingen), or isotype-matched irrelevant controls [24]. Primary mAb were detected using 1:50 diluted FITC-conjugated rat antimouse Ig, κ light chain (PharMingen). Mean fluorescence intensity (MFI) and forward light scatter (FSC; correlated with cell size) were analyzed with FAScan [25, 26]. The results are presented as overlaid histograms and the magnitude increase of MFI (MFI units of tested mAb staining/MFI units of matched isotype control mAb staining).

For determination of Fas and Fas ligand (FasL) expression, 1×10^6 monocytes were seeded in 24-well plates and cultured with RPMI 1640–10% autologous serum or with serum-free medium in the presence or absence of 50 to 200 μg/mL of AGE-β_2m for 10 to 48 hours. The cells were then incubated with 1:50 diluted mouse anti-human Fas (PharMingen), mouse anti-human FasL (PharMingen), or an equal amount of mouse IgG_1 (isotype control) for 60 minutes in ice. Immunoreactivity was detected by 1:50 diluted FITC-conjugated rat anti-mouse Ig, κ chain.

Quantitation of intracellular lysosomal enzymes

Acid phosphatase and β-glucuronidase were assayed as previously described [22, 24]. In briefly, fresh isolated monocytes were adhered to plates for two hours or incubated for four days in RPMI 1640 with 10% autologous serum or serum-free medium containing AGE-β_2m (100 μg/mL). The cells were then harvested as described previously in this article, counted, and lyzed with 0.1% Triton X-100. The microassay was performed in 96-well microtiter plates using p-nitrophenolphosphate (for acid phosphatase assay; Sigma) or p-nitrophenyl-β-D-glu-

curonide (for β-glucuronidase assay; Sigma) as enzyme substrates. The plates were incubated at 37°C for one hour, and the absorbency was determined at 405 nm in an ELISA reader (Titertek Multiskan Mcc/340). One unit of enzyme activity represented cleavage of 1 μmol of substrate per hour.

Assay for superoxide production

Superoxide release in response to stimulation with 10 μg/mL of phorbol myristate acetate (PMA; Sigma) was determined as has been described by Pick and Mizel [27]. Monocytes were cultured in 96-well plates with RPMI 1640 with 10% autologous serum or serum-free medium containing 100 μg/mL AGE-β_2m for two hours to six days. The culture medium was removed and replaced with Hanks' buffer containing 160 μmol/L of ferricytochrome C (Sigma), with and without PMA, and with and without 300 U/mL superoxide dismutase (Sigma). The plates were incubated at 37°C for one hour and the absorbency was determined at 540 nmol/L. The results are expressed as nanomoles O_2^- per 10^6 cells per hour.

Quantitation of TNF-α, IL-1β, and PGE_2 production

Monocytes (1×10^6) were cultured in duplicate in RPMI 1640 with 10% autologous serum or with serum-free medium containing 100 μg/mL of AGE-β_2m from two hours to six days. The monolayers were washed two times with PBS and then fresh medium with or without 1 μg/mL of lipopolysaccharide (LPS; from *Escherichia coli* 026:B6; Sigma) or 100 μg/mL of AGE-β_2m was added. The culture was performed in 24-well plates. After 18 hours (for LPS stimulation) or 24 hours (for AGE-β_2m stimulation), supernatants were harvested and centrifuged at $400 \times g$ for five minutes. The cells were detached and counted as described above. TNF-α and IL-1β levels were measured in duplicate in each supernatant with ELISA kits according to the manufacturer's technical guidelines (TNF-α, Genzyme, Cambridge, MA, USA; IL-1β, Endogen, Woburn, MA, USA). PGE_2 was quantitated by radioimmunoassay using rabbit anti-PGE_2 (Sigma) according to the manufacturer's protocol.

Statistical analysis

All experiments were performed in triplicate. Continuous variables, expressed as mean ± SD, were compared using analysis of variance (ANOVA). Multiplicative interaction terms were included to evaluate for interaction terms 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 analyzes were conducted with SAS (The SAS Institute, Cary, NC, USA).

RESULTS

Effect of AGE-β_2m on monocyte apoptosis

Immediately after isolation, only 2 to 3% of the monocytes were nonviable, as determined by permeability to trypan blue (Fig. 1A, C, and apoptotic cells were less than 1% as analyzed by flow cytometry (Fig. 1B, D). The percentage of trypan blue-positive monocytes and apoptotic cells increased over time when the cells were cultured in suspension in RPMI 1640 alone. Incubation of nonadherent monocytes with 10% autologous serum had no effect on viability and apoptosis. In contrast, incubation of nonadherent monocytes with AGE-β_2m maintained monocyte viability (Fig. 1A) and significantly decreased apoptosis (Fig. 1B). A similar number of monocytes were recovered from cultures with or without AGE-β_2m (difference <16%), indicating that the cells were not undergoing autolysis.

Since adherence impacts selected monocyte functions [28], such as cytokine elaboration, we examined the effect of AGE-β_2m on adherent monocytes. When incubated in serum-free medium, cells adhering to tissue culture plates underwent cell death and apoptosis at approximately the same rate as nonadherent monocytes (Fig. 1C, D). However, when autologous serum (10%) was added to the cultures, more than 90% of cells survived for 72 hours without undergoing apoptosis ($P < 0.0001$). AGE-β_2m also attenuated cell death and apoptosis in adherent monocytes ($P < 0.0001$). After 72 hours of culture in the presence of AGE-β_2m (100 μg/mL), about 60% of monocytes were viable and had not undergone apoptosis.

To determine the dose-related effect of AGE-β_2m on monocyte viability, monocytes were cultured in suspension in serum-free medium containing various concentrations of AGE-β_2m (Fig. 2). As assessed by FACs, AGE-β_2m treatment delayed apoptosis in a dose-dependent manner ($P < 0.0001$). Experiments performed using adherent monocytes yielded similar results. AGE-β_2m at concentrations of 50 μg/mL, 100 μg/mL, and 200 μg/mL decreased the proportion of apoptotic cells to 79, 47, and 42%, respectively, of that observed with medium alone (data not shown).

Monocytes cultured in serum-free medium developed morphologic characteristics of apoptosis, including pyknotic nuclei, chromatin condensation, and nuclear fragmentation, as identified by Hoechst 33258 staining (Fig. 3C). DNA strand breaks in these cells were demonstrated by TUNEL (Fig. 3D). Culture of monocytes with 100 μg/mL AGE-β_2m attenuated the development of morphologic features of apoptosis (Fig. 3G) and of DNA breaks (Fig. 3H), whereas unmodified β_2m had no effect on either morphologic signs of monocyte apoptosis (Fig. 3E) or DNA breaks (Fig. 3F). AGE-β_2m, but not unmodified β_2m, also diminished DNA internucleosomal fragmenta-

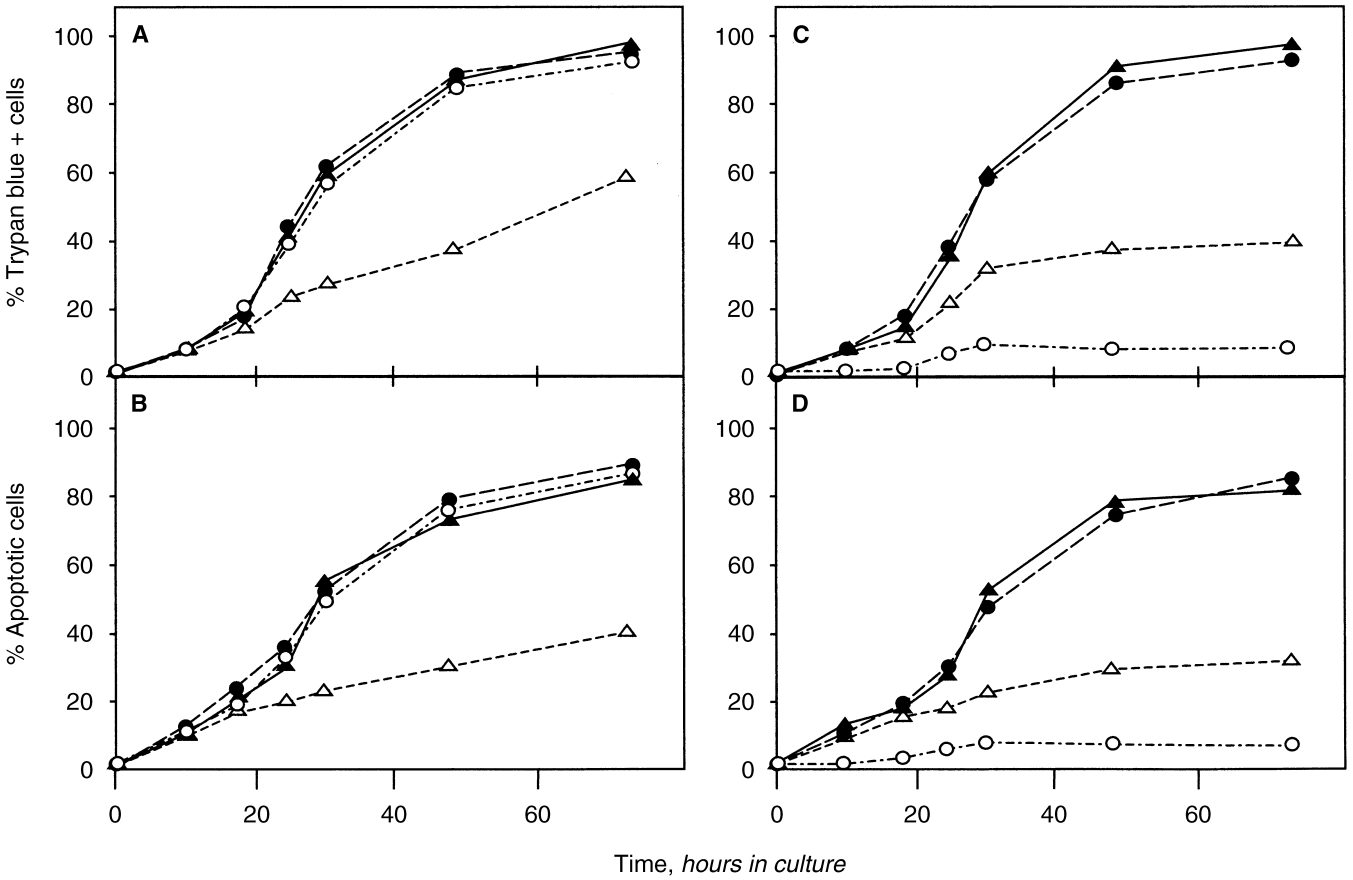


Fig. 1. Kinetics of cell death and apoptosis in monocytes. Monocytes were cultured in polypropylene tubes (*A* and *B*) or in 24-well polystyrene plates (*C* and *D*) in RPMI 1640 with no added stimulant (●), 10% autologous serum (○), β_2m (▲), or 100 μg/mL of AGE-β_2m (△). The percentages of nonviable (trypan blue positive cells; A and C) and apoptotic cells (B and D) were determined. Apoptosis was quantitated by flow cytometry. Monocytes from the same donor preparation were used in each experiment to assure compatible conditions Data are the mean of triplicate experiments that did not vary by more than 15% (ANOVA, $P < 0.0001$; duration of incubation, $P < 0.0001$; culture condition, $P < 0.0001$; duration of incubation × culture condition, $P < 0.0001$).

tion, as identified by electrophoresis on DNA gels (data not shown).

Blocking of RAGE attenuates the effect of AGE-β_2m

To ascertain the specificity of the effect of AGE-β_2m on monocyte apoptosis, adherent monocytes were preincubated with 50 μg/mL of rabbit anti-human RAGE or nonimmune rabbit IgG for two hours. This concentration of anti-human RAGE has previously been demonstrated to block other effects of AGE-β_2m in vitro [29]. The monocytes were then cultured with 100 μg/mL of AGE-β_2m for 30 hours. The antibody that blocks the interaction of AGE-β_2m and RAGE in other systems [14, 16, 29] significantly diminished the capacity of AGE-β_2m to prevent monocyte apoptosis ($P < 0.0001$; Fig. 4). These findings are not a consequence of LPS contamination, since polymyxin B (10 μg/mL) did not change the effect of AGE-β_2m on monocyte survival (data not shown). In addition, unmodified β_2m, which was prepared using the same conditions as in the preparation of AGE-β_2m, had no effect on monocyte apoptosis. The lack of a difference between the effect of AGE-β_2m and that of AGE-HSA (Fig. 4), indicates that the observed effect on the inhibition of apoptosis was due to AGE-modification of the protein and was not a property specific to β_2m.

Chemical nature of the AGE

To clarify the chemical nature of the AGE responsible for the effect on monocyte apoptosis, adherent monocytes were cultured with serum-free medium, AGE-β_2m (100 μg/mL) pentosidine-BSA (100 μg/mL), CML-BSA (100 μg/mL), synthesized pentosidine (10 or 1000 pmol) and synthesized CML (2 or 200 nmol) for 30 hours. Percent trypan blue positive cells and apoptotic cells were quantitated as described previously in this article (Table 1). AGE-β_2m containing cultures had less apoptotic (24.3 ± 3.5%) and trypan blue positive cells (31.5 ± 3.1%) compared with cultures containing medium alone (controls, 52.0 ± 4.0% and 56.3 ± 4.5%,

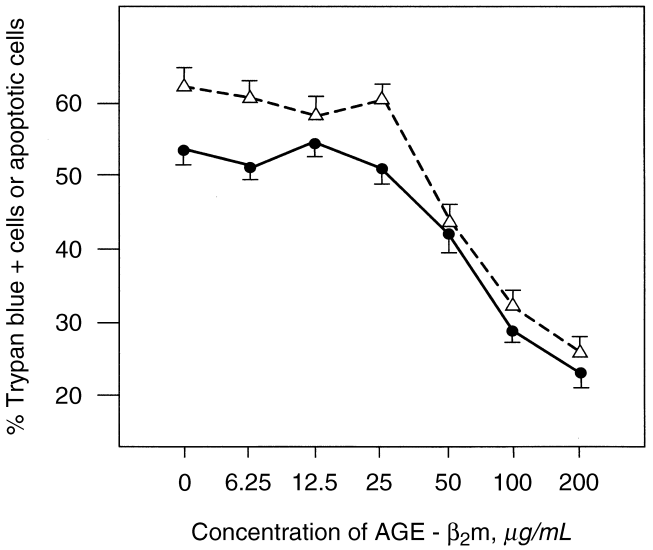


Fig. 2. Dose-dependent effect of AGE-β_2m on apoptosis in monocytes. Monocytes were cultured for 30 hours in serum-free RPMI 1640 in polypropylene tubes containing the indicated concentration of AGE-β_2m. Percent trypan blue positive cells (△) and apoptotic cells (●) were then determined as described in the text. Data are expressed as mean ± SD of triplicate cultures from one donor and are representative of data from three donors (ANOVA, $P < 0.0001$). Data values from cultures stimulated with ≥50 μg/mL AGE-β_2m are significantly different from controls without AGE-β_2m.

$N = 3$, $P < 0.01$). In contrast, monocytes cultured with pentosidine-BSA or CML-BSA underwent apoptosis (49.1 ± 7.5% and 50.6 ± 9.1%, respectively) and cell death (54.3 ± 8.1% and 53.0 ± 12.1%, respectively) at rates no different from controls ($P > 0.05$). Similarly, neither free pentosidine nor free CML could protect monocytes from apoptosis or cell death. Apoptotic cells were 50.0 ± 8.7% and 50.6 ± 9.5% when cultured in the presence of 10 pmol and 1000 pmol of pentosidine, respectively; 49.6 ± 8.0% and 51.3 ± 8.3% in 2 nmol and 200 nmol of CML, respectively ($N = 3$, $P > 0.05$ compared with controls). Trypan blue positive cells were 55.0 ± 7.8 and 53.3 ± 10.5% in 10 pmol and 1000 pmol of pentosidine, respectively; 52.3 ± 9.0% and 55.6 ± 7.9% in 2 nmol and 200 nmol of CML, respectively ($N = 3$, $P > 0.05$ compared with controls).

Duration of AGE-β_2m effect on apoptosis

To determine whether inhibition of monocyte apoptosis persists after removal of AGE-β_2m, monocytes were cultured in 24-well polystyrene plates with AGE-β_2m (100 μg/mL) for two hours to six days. The cells were washed, detached from the wells, resuspended in fresh serum-free medium without AGE-β_2m, and further cultured in the polystyrene plates at 1×10^5 cells/1.0 mL/well for an additional 48 hours. Removing AGE-β_2m from the culture at or before 48 hours resulted in a rapid decrease in monocyte viability (Fig. 5). However, when monocytes were cultured with AGE-β_2m for longer than four days, their survival was extended even after AGE-β_2m was removed from the culture. By day 4 of culture with AGE-β_2m, the cells no longer required the presence of AGE-β_2m to survive. Parallel experiments with cultures using 10% autologous serum yielded similar results. Monocytes cultured with serum for four days no longer required serum for survival after that time (results not shown).

Effect of AGE-β_2m on Fas and FasL expression on monocytes

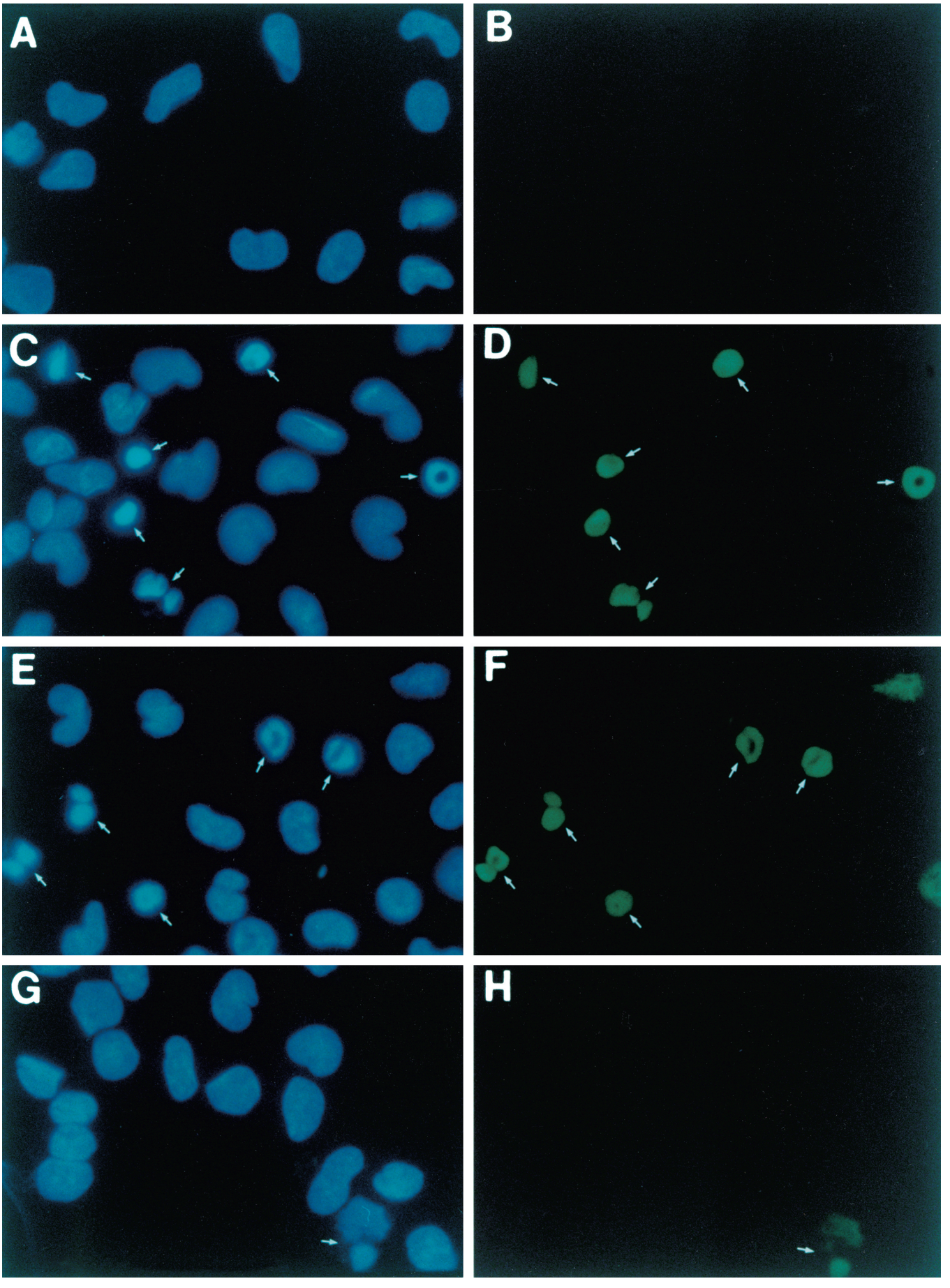
Because it has been suggested that Fas–FasL interaction is involved in the induction of spontaneous apoptosis of monocytes [30], we tested the effect of AGE-β_2m on monocyte expression of Fas and FasL. Monocytes were incubated in polypropylene tubes in serum-free medium or in medium with 10% autologous serum, with or without AGE-β_2m for 10 to 48 hours. FACs analysis showed that AGE-β_2m did not affect the expression of Fas or FasL on monocytes, cultured either in serum-free medium or in medium with 10% autologous serum, at any time tested (data not shown).

Morphology and ultrastructure of monocytes cultured with AGE-β_2m

Monocytes cultured with serum for longer than three days differentiate into macrophage-like cells [24, 26]. Eighty percent of monocytes cultured with serum-free medium or medium containing unmodified β_2m were apoptotic within 48 hours (Fig. 1) and incapable of differentiation in vitro. Since 60% of adherent monocytes cultured with AGE-β_2m were viable at day 3 of the cultures, we examined the morphologic and functional characteristics of these cells. Using inverted microscopy, monocytes cultured with AGE-β_2m (Fig. 6C) or with 10% autologous serum (Fig. 6B) for four days appeared to be larger than freshly isolated monocytes (Fig. 6A) and were predominantly circular with increased numbers of lipid inclusions. However, monocytes cultured with AGE-β_2m (Fig. 6C) were smaller than monocytes cultured with 10% autologous serum (Fig. 6B) and had more pseudopodia. Electron microscopic examination demonstrated that after incubation for four days, the ultrastructural changes of monocytes cultured with AGE-β_2m were similar to those of cells cultured with 10% autologous serum. Monocytes cultured with either AGE-β_2m or 10% autologous serum displayed morphology typical of mature macrophages with increased and thickened cytoplasmic processes, increased phagocytic vacuoles, crystal inclusions, and secondary lysosomes in the cytoplasm (Fig.6 E, F).

Effect of culture with AGE-β_2m on macrophage membrane antigen development

Monocytes cultured for four days with AGE-β_2m were analyzed for expression of the macrophage surface anti-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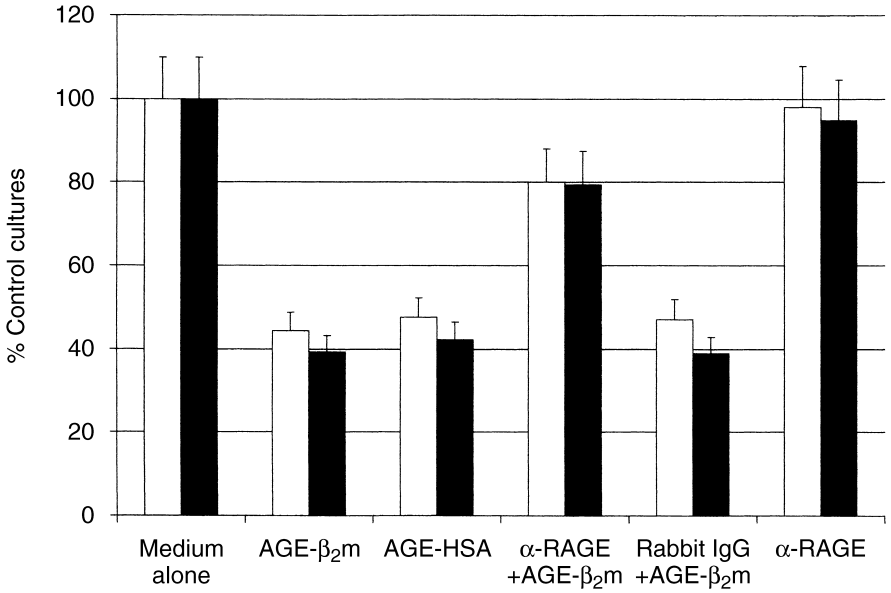

Fig. 4. Effect of AGE-β_2m, AGE-HAS, and anti-RAGE on monocyte apoptosis. Monocytes were cultured in RPMI 1640 alone (control), 100 μg/mL AGE-β_2m, or 100 μg/mL AGE-HSA for 30 hours. In indicated wells, cells were preincubated with 50 μg/mL rabbit anti-human RAGE IgG or nonimmune rabbit IgG for 2 hours and then cultured with 100 μg/ml AGE-β_2m for 30 hours. Percent trypan blue positive cells (□) and apoptotic cells (■) were determined. Data are mean ± SD of triplicate experiments and are presented as percentage trypan blue positive cells and apoptotic cells in control cultures (ANOVA, $P < 0.0001$). The RAGE-treated group is significantly different from rabbit IgG treated and AGE-β_2m alone group.

gens, HLA-DR, CD11b, and CD11c. Monocytes cultured with AGE-β_2m or with 10% autologous serum exhibited a shift in the mean fluorescence intensity when stained for these membrane epitopes. However, the relative increase in mean fluorescence intensity was less for monocytes cultured with AGE-β_2m than for those cultured with 10% autologous serum (Table 2).

Enhancement of intracellular lysosomal enzyme content of monocytes cultured with AGE-β_2m

Increased levels of lysosomal enzymes accompany phenotypic transformation of monocytes into macrophages [24, 31]. To evaluate the effect of AGE-β_2m on this component of transformation, acid phosphatase and β-glucuronidase activity were assessed in monocytes cultured with AGE-β_2m or with 10% autologous serum for four days. Freshly isolated monocytes contained 180.0 ± 3.0 units of acid phosphatase per 10^6 viable cells. After four days of culture with 100 μg/mL of AGE-β_2m or 10% autologous serum, acid phosphatase activity increased to 2130.0 ± 130.0 and 3120.0 ± 90.0 U/10^6 viable cells, respectively ($N = 3, P < 0.0001$). Freshly isolated monocytes contained 7.0 ± 0.3 units of β-glucuronidase per 10^6 viable cells, which increased to 35.0 ± 10.0 and 50.4 ± 0.7 U/10^6 viable cells by four days of culture with AGE-β_2m and 10% autologous serum, respectively ($P < 0.0001$).

Superoxide production in monocytes cultured with AGE-β_2m

Superoxide production in serum-containing cultures declines as monocytes change phenotype into macrophages [30]. After stimulation with PMA, monocytes freshly isolated or cultured for four or six days with 10% autologous serum elaborated 52.4 ± 4.1, 25.8 ± 2.7, and 11.7 ± 1.9 nmol/L of superoxide per 60 minutes per 10^6 cells, respectively ($N = 3$). In contrast, monocytes cultured with 100 μg/mL of AGE-β_2m generated significantly more superoxide after PMA stimulation at four and six days. The cells produced 52.9 ± 3.3, 49.8 ± 3.8, and 44.3 ± 4.2 nmol/L of superoxide per 60 minutes per 10^6 cells at the same time points, respectively ($N = 3$, ANOVA, $P < 0.0001$; duration of incubation, $P < 0.0001$; culture condition, $P < 0.0001$; duration of incubation × culture condition interaction, $P < 0.0001$).

Production of TNF-α, IL-1β, and PGE_2 in monocytes developed with AGE-β_2m

A critical function of macrophages is the synthesis and secretion of proinflammatory cytokines and lipid inflammatory mediators in response to biologic stimuli. Therefore, we quantitated the production of TNF-α, IL-1β, and PGE_2 in response to LPS or AGE-β_2m. Preliminary experiments demonstrated that LPS-induced

Fig. 3. Detection of morphological signs of apoptosis and DNA strand breaks in monocytes cultured in polypropylene tubes. Fresh isolated monocytes (*A* and *B*), monocytes cultured for 30 hours in serum-free medium (*C* and *D*), in 100 μg/mL of unmodified β_2m (*E* and *F*), and in 100 μg/mL of AGE-β_2m (*G* and *H*) were counterstained with Hoechst 33258 and fluorescent TUNEL. The photographs were taken using an fluorescence microscopy (×100). The arrows indicate apoptotic monocytes. Reproduction of these illustrations in color was made possible by the support of Amgen, Inc., Thousand Oaks, CA, USA.

Table 1. Effect of various advanced glycation end products (AGE) on monocyte apoptosis

AGE	Trypan blue positive	Apoptotic cells
Serum-free medium	56.3 ± 4.5	52.0 ± 4.0
AGE-β_2m *100 μg/m*	31.5 ± 3.1	24.3 ± 3.5
Pentosidine-BSA *100 μg/mL*	54.3 ± 8.1	49.1 ± 7.5
CML-BSA *100 μg/mL*	53.0 ± 12.1	50.6 ± 9.1
Pentosidine		
10 pmol	55.0 ± 7.8	50.0 ± 8.7
1000 pmol	53.3 ± 10.5	50.6 ± 9.5
CML		
2 nmol	52.3 ± 9.0	49.6 ± 8.0
200 nmol	55.6 ± 7.9	51.3 ± 8.3

Adherent monocytes were cultured for 30 hours with serum-free medium without or containing 100 μg/mL of AGE-β_2m, pentosidine-BSA (100 μg/mL), CML-BSA (100 μg/mL), synthesized pentosidine (10 or 1,000 pmol), and synthesized CML (2 or 200 nmol). The percentages of nonviable (trypan blue positive cells) and apoptotic cells were determined. Apoptosis was quantitated by flow cytometry. The data are expressed as mean ± SD of 3 cultures.

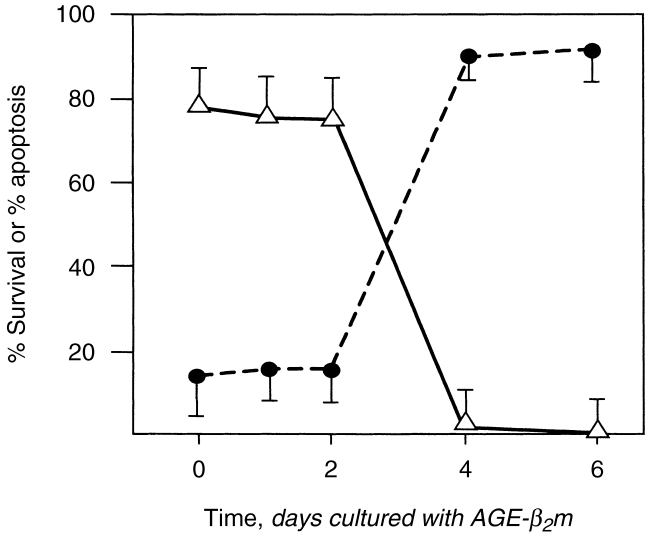

Fig. 5. Effect of duration of AGE-β_2m exposure on the survival of monocytes. Monocytes were cultured in polystyrene plates with AGE-β_2m (100 μg/mL) for the number of days indicated. The cells were washed, detached from the wells, respuspended in fresh serum-free medium, and further cultured in polystyrene plates at 1×10^5 cells/1.0 mL/well. Survival (●) and apoptotic cells (△) were quantitated after culture for an additional 48 hours. The results are expressed as mean of triplicate experiments that did not vary by more than 10%.

secretion of TNF-α, IL-1β and PGE_2 was maximal at 18 hours, whereas AGE-β_2m–induced secretion was maximal at 24 hours (data not shown). Thus, monocytes were cultured with AGE-β_2m or 10% autologous serum for two hours or for four or six days, washed, and stimulated with LPS for 18 hours or with AGE-β_2m for 24 hours. After two hours (day 0), there was no difference in cytokine levels between AGE-β_2m- and serum-containing cultures (Table 3). After four days of culture, the constitutive production of TNF-α and IL-1β decreased in cells cultured with serum, but increased in cells cultured with AGE-β_2m. Monocytes cultured with AGE-β_2m for four and six days spontaneously secreted more TNF-α and IL-1β than did cells after two hours of culture (day 0) or cells cultured with serum for the same duration of time ($P < 0.0001$). The effect of culturing monocytes with AGE-β_2m or 10% autologous serum on PGE_2 production was similar to those observed on cytokine secretion. Higher levels of PGE_2, both in the absence and in the presence of LPS, were detected in cells cultured for four to six days with AGE-β_2m as compared with those cultured with 10% autologous serum ($P < 0.0001$).

To determine how long increased cytokine secretion persists after withdrawal of AGE-β_2m, cells cultured with AGE-β_2m for four days were washed and subsequently incubated with fresh medium alone. Every 24 hours, the supernatants and the cells from two wells were harvested, and fresh medium was replaced in the remaining wells. Increased TNF-α and IL-1β production persisted for three days after removal of AGE-β_2m and subsequently returned to baseline levels after an additional four days (data not shown).

DISCUSSION

A local inflammatory reaction to β_2m amyloid deposits that involve monocytes and macrophages is a characteristic histologic feature of DRA [3, 5]. Because AGE-modified β_2m is chemotactic for human monocytes [15], and monocytes/macrophages have receptors for the endocytic uptake of AGE-modified proteins [33], AGE-β_2m–forming amyloid has been proposed to account for the preferential localization of monocytes/macrophages to these deposits [15]. However, the accumulation of short-lived cells at an inflammatory focus suggests a change in their rate of programmed cell death [8–10].

The results reported herein characterize a novel biological property of AGE-modified proteins, like AGE-β_2m. AGE-β_2m delays spontaneous apoptosis, or programmed cell death, of human monocytes in vitro. In that human monocytes undergo apoptosis when placed in culture, AGE-β_2m inhibited monocyte apoptosis in a dose- and time-dependent manner. AGE-modified proteins such as AGE-β_2m are unique in this report in that other chemotactic factors for monocytes, such as TGF-β, MCP-1, and C5a, do not maintain monocyte survival [10].

It was previously assumed that the recruitment of monocytes to an inflammatory site was insufficient to prevent monocyte apoptosis. Arguably, an additional regulatory signal(s) was needed within the microenvironment [10]. Unlike most conventional monocyte chemotactic factors, AGE-β_2m is able to provide signals for both recruitment and survival. Monocyte apoptosis is critical because it is a provocateur for cell removal and it results in the loss of functionally responsive cells [34]. Regulation of apoptosis may be a highly efficient mecha-

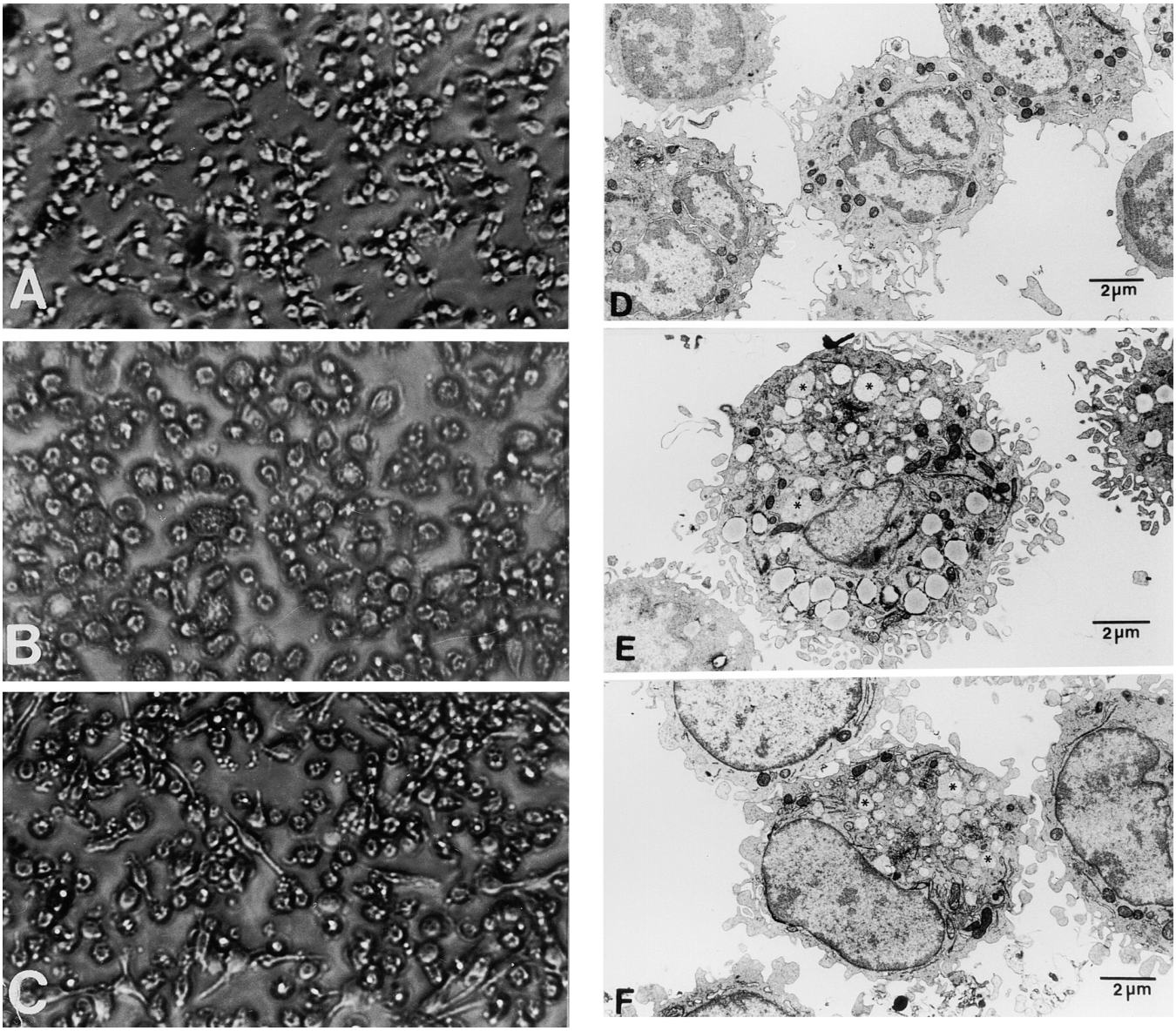

Fig. 6. Morphologic and ultrastructural characteristics of fresh isolated monocytes (*A* and *D*), monocytes cultured for four days with RPMI 1640–10% autologous serum (*B* and *E*), and with serum-free medium containing 100 μg/mL AGE-β_2m (*C* and *F*). All cell preparations, which were from the same donor, were photographed with a ×20 phase-contrast objective on a Nikon inverted microscopy (A–C) and then harvested and examined by transmission electron microscopy (D–F). Monocytes cultured with either AGE-β_2m or 10% autologous serum displayed morphology typical of mature macrophages. However, monocytes matured in AGE-β_2m (F) were smaller than those cultured with serum (E) and had fewer vacuoles.

nism for controlling the number of viable monocytes in an inflammatory lesion. Therefore, the delay of apoptosis by local AGE-β_2m may contribute to the accumulation of monocytes/macrophages observed in DRA.

AGE-β_2m, but not unmodified β_2m, delayed apoptosis of monocytes. Furthermore, this effect was no different from that of AGE-HSA, suggesting that the inhibition of apoptosis was due to AGE modification of the protein and not a specific property of AGE-β_2m. The capacity of AGE-β_2m to prevent monocyte apoptosis was significantly diminished by an antibody that blocks the interaction of AGE protein and RAGE both in vitro and in vivo [14, 33], indicating that the protective effect of AGE-β_2m on monocyte apoptosis is AGE receptor mediated. RAGE is a 35 kD member of the immunoglobulin superfamily of receptors, which is present on various types of cells including human mononuclear phagocytes [14, 34, 35]. Human AGE-β_2m binds to the extracellular domain of RAGE with a kD ≈83.5 nmol/L [14]. The findings reported herein suggest that AGE-modified pro-

Table 2. Membrane antigens expression on monocytes in AGE-β_2m containing cultures

Treatment	MFI (indicated mAb/isotype control)			
	HLA-DR	CD11b	CD11c	FSC (size)
Day 0	3.3	2.8	2.2	240.0
Day 4				
10% serum[a]	5.7	3.6	4.9	405.0
AGE-β_2m[a]	4.2	3.2	3.4	310.0

Monocytes were cultured for 2 hrs (day 0) and four days with RPMI 1640 containing 10% autologuous serum or with serum-free medium containing 100 μg/mL of AGE-β_2m. The results are the average of two experiments. Abbreviations are: MFI, mean fluorescence intensity; FSC, forward light scatter.

[a] Cells were more than 95% viable in cultures containing 10% autologous serum and more than 68% viable in cultures containing AGE-β_2m when analyzed. Only viable cells were analyzed for fluorescence.

teins such as AGE-β_2m have multiple effects mediated through RAGE. Another example is offered by the observation that AGE-β_2m–induced chemotaxis by human mononuclear phagocytes is abrogated by anti-RAGE IgG [14].

Advanced glycation end products are heterogeneous structures containing a variety of specific epitopes. Clarification of the chemical nature of the experimental AGE responsible for the effect on apoptosis is indeed of particular interest. Although pentosidine and CML are well-characterized surrogate markers of AGE [18, 19] and were identified in our AGE-β_2m sample, neither of these structures, either artificially protein bound or in free form, appears to protect monocytes from apoptosis. It remains unknown whether protein bound forms of pentosidine or CML, naturally present in vivo, have such biological effects, due to the difficulty in purifying protein bound forms of pentosidine or CML to homogeneity. Another as yet unidentified AGE structure might be responsible for the activity.

Because of increased production, AGE and their precursors accumulate with diabetes mellitus and aging. Therefore, patients with diabetes and hyperglycemia have higher blood concentrations of AGE than healthy euglycemic individuals [36]. We are unaware of information about differences in blood levels of AGE-β_2m between diabetics and nondiabetics with end-stage renal disease (ESRD), but there is poor correlation between serum levels of β_2m and DRA [1]. If AGE are involved in the pathogenesis of both diabetic complications and DRA, diabetic patients with ESRD might be expected to manifest signs and symptoms of DRA earlier than nondiabetic patients with renal failure. However, the five-year survival of diabetic patients with ESRD is approximately one half that of nondiabetic patients [37]. Hence, ESRD patients with diabetes may not survive sufficiently long to clinically manifest DRA, that is, prevalence skewed by survival bias. In a cross sectional, case controlled study of ESRD patients with and without diabetes mellitus, and receiving renal replacement therapy for more than five years, the prevalence of radiolucent cysts in the carpal bones, and carpal tunnel syndrome, were equivalent [38]. Because these are only surrogates for β_2m deposition and DRA, and other conditions can result in similar radiographic and clinical findings, additional study is needed to clarify the relationship between diabetes mellitus and the rate of progression of DRA.

The Fas–FasL interaction has been found to play an important role in the induction of spontaneous apoptosis of human monocytes. Blocking this interaction by a Fas-immunoglobulin fusion protein or an anti-FasL antibody greatly but not completely reduces the onset of apoptosis [28]. In the AGE-β_2m culture system, treatment of monocytes with AGE-β_2m at concentrations that protect the cells from apoptosis did not markedly alter the expression of either Fas or FasL. These findings indicate that the protective effects on monocytes may occur at a site downstream of the receptor–ligand interaction, such as through direct caspase activation [39]. Alternatively, Fas-FasL–independent mechanism(s) may be induced; two possibilities exist. AGE-β_2m may have direct effects on the monocytes by inducing intracellular signals that delay apoptosis or might indirectly enhance monocyte survival via induction of cytokines that act in an autocrine fashion. Interestingly, it has been found that the production of TNF-α and IL-1β, cytokines that down-regulate monocyte apoptosis [10], is significantly increased in monocytes/macrophages stimulated by AGE-β_2m [15]. Caspace-dependent and -independent pathways by which AGE-modified proteins, such as β_2m, rescues monocytes from apoptosis are currently under investigation.

Human peripheral monocytes differentiate into macrophage-like cells when cultured with human serum for more than three days [24]. In our culture system, 60% of monocytes incubated with AGE-β_2m were viable after three days of culture and became resistant to apoptosis, a characteristic of in vitro–derived macrophages [30]. Cells cultured with AGE-β_2m exhibited an increase in size and similar ultrastructural changes as monocytes cultured with 10% autologous serum. Similar phenotypic alterations, observed when adherent culture of monocytes are cultured in medium containing human serum, may represent events occurring in vivo as monocytes migrate out of the blood vessels and differentiate into tissue macrophages in the absence of a specific modifying stimulus [24, 26]. The activity of the lysosomal enzymes increased with time in cells cultured with AGE-β_2m.

Previous studies have demonstrated that membrane surface antigens are expressed at a lower amount on monocytes than on macrophages due to their smaller size and surface area. Some antigens such as CD11c are therefore directly related to cell size, whereas others like HLA-DR can be modulated by different biologic factors [24, 40]. The three antigens investigated, HLA-DR, CD11b, and

Table 3. TNF-α, IL-1β, and PGE_2 production in monocytes cultured with serum or AGE-β_2m

Treatment	Day 0[a]			Day 4[a]			Day 6[a]		
	TNF-α	IL-1β	PGE_2	TNF-α	IL-1β	PGE_2	TNF-α	IL-1β	PGE_2
Cultured with serum									
Medium alone	7.9 ± 1.0	2.0 ± 0.4	107.3 ± 6.5	3.2 ± 0.5	1.1 ± 0.2	79.8 ± 4.5	2.9 ± 0.8	0.8 ± 0.2	75.5 ± 4.9
LPS *1 μg/mL*	203.3 ± 13.3	57.2 ± 7.7	514.9 ± 48.5	193.0 ± 12.4	4.8 ± 0.8	495.3 ± 69.1	185.1 ± 23.7	3.0 ± 0.7	480.0 ± 29.8
AGE-β_2m *100 μg/mL*	24.5 ± 3.4	14.4 ± 2.3	118.2 ± 10.8	23.8 ± 4.1	4.0 ± 0.8	126.2 ± 12.8	21.1 ± 3.2	3.2 ± 0.7	128.8 ± 14.2
Cultured with AGE-β_2m									
Medium alone	7.8 ± 1.3	2.1 ± 0.5	109.9 ± 8.1	26.2 ± 1.1	4.2 ± 0.5	221.6 ± 28.9	28.4 ± 1.8	4.1 ± 0.5	228.8 ± 36.9
LPS *1 μg/mL*	207.2 ± 26.3	56.7 ± 5.7	524.6 ± 51.2	285.3 ± 22.8	5.4 ± 0.4	673.7 ± 42.5	294.4 ± 31.2	5.1 ± 0.4	679.5 ± 75.9
AGE-β_2m *100 μg/mL*	25.6 ± 1.5	13.7 ± 2.5	124.0 ± 11.2	27.3 ± 3.5	4.3 ± 0.3	218.7 ± 18.6	29.2 ± 4.1	4.2 ± 0.4	232.6 ± 26.8

Monocytes were cultured with RPMI 1640 containing 10% autologous serum or serum-free medium containing 100 μg/mL of AGE-β_2m for indicated period. The cells were washed and incubated in fresh medium with or without LPS for 18 hours, or cultured in medium with or without of AGE-β_2m for 24 hours. TNF-α, IL-1β, and PGE_2 levels in the supernatants were quantitated. The data are expressed as mean ± SD of 3 cultures. ANOVA, $P < 0.0001$; duration of incubation, $P = 0.02$; culture condition, $P < 0.0001$; stimulation, $P < 0.0001$; duration of incubation × culture condition interaction, $P < 0.0001$; culture condition × stimulation interaction, $P = 0.007$.

[a] TNF-α, IL-1β, and PGE_2 levels were expressed as pg/10^4 viable cells

CD11c, are expressed on freshly isolated monocytes. Their expression was higher on cells cultured for four days with serum or AGE-β_2m. These findings further support that AGE-β_2m was capable of inducing phenotypic changes representative of monocyte maturation.

Although exhibiting phenotypic characteristics of macrophages, monocytes maintained with AGE-β_2m showed different functional behavior than macrophages cultured with serum. The ability of cultured monocytes to produce the reactive oxygen species, O_2^- and H_2O_2, decreases as the cells mature into macrophages in serum-containing medium [32]. In contrast, when monocytes were cultured with AGE-β_2m, this activity was maintained. Constitutive synthesis of TNF-α, IL-1β and PGE_2 also decreased as monocytes were cultured with autologous serum. In contrast, the constitutive production of these cytokines and PGE_2 increased in monocytes maintained by AGE-β_2m. This finding corresponds to reports that AGE-β_2m stimulates mononuclear cells to produce TNF-α, IL-1β, and IL-6 [14, 15, 41] and that mononuclear cells obtained from carpal tunnel tenosynovium of patients undergoing long-term hemodialysis have spontaneously produced IL-1β and IL-6 in vitro [6]. Therefore, although phenotypically similar, the AGE-β_2m–derived macrophages are functionally different.

The effect of AGE-β_2m on monocyte apoptosis and phenotype are consistent with prior observations that the cells present in deposits of β_2m amyloid are predominantly monocytes/macrophages and that these cells are strongly histochemically positive for TNF-α and IL-1β [6, 7]. Furthermore, monocyte production of autocrine proinflammatory cytokines, such as TNF-α and IL-1β, may increase monocyte survival by their effect to delay apoptosis [10]. PGE_2 and IL-1β have been suggested to play a critical role in bone resorption [42, 43]. Both PGE_2 and IL-1β production increased in monocytes cultured with AGE-β_2m. This effect of AGE-β_2m may contribute to the development and expansion of bone cysts at the site of β_2m amyloid deposits.

In summary, AGE-β_2m, a major component of amyloid in DRA, delays spontaneous monocytes apoptosis in vitro via a pathway mediated by RAGE. Monocyte survival in an AGE-β_2m–containing microenviroment is associated with their alteration into macrophage-like cells that generate more reactive oxygen species and elaborate greater quantities of proinflammatory cytokines and PGE_2. Future in vitro studies will more fully characterize if all the effects described herein on monocyte are due to AGE modification of the protein and not a specific property of AGE-β_2m, as suggested. If this model is applicable in vivo, the effect of AGE-β_2m on monocyte survival and differentiation may represent an important mechanism contributing to the accumulation of monocytes/macrophages and the inflammatory process in DRA.

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APPENDIX

Abbreviations used in this article are: AGE, advanced glycation end products; AGE-β_2m, advanced glycation end product-modified β_2-microglobulin; BSA, bovine serum albumin; CML, carboxymethyllysine; DRA, dialysis-related amyloidosis; FACs, FACScan flow cy-

tometry; HLA, human lymphocyte antigen; HSA, human serum albumin; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MCP-1, monocyte chemoattractant protein-1; PGE_2, prostaglandin 2; PMA, phorbol myristate acetate; RAGE, advanced glycation end product receptor; TNF-α, tumor necrosis factor-alpha; TUNEL, nick end-labeling technique.

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