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Receptors for Advanced Glycosylation Endproducts in Human Brain: Role in Brain Homeostasis

Jenny J. Li,[1] Dennis Dickson,[2] Patrick R. Hof,[3] and Helen Vlassara[1]

[1]Laboratory of Diabetes and Aging, The Picower Institute for Medical Research, Manhasset, New York, U.S.A.
[2]Mayo Clinic Jacksonville, Jacksonville, Florida, U.S.A.
[3]Department of Pathology, Division of Neuropathology, Albert Einstein College of Medicine, Bronx, New York, U.S.A.

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Abstract

Background: Advanced glycation end products (AGEs) are the reactive derivatives of nonenzymatic glucose-macromolecule condensation products. Aging human tissues accumulate AGEs in an age-dependent manner and contribute to age-related functional changes in vital organs. We have shown previously that AGE scavenger receptors are present on monocyte/macrophages, lymphocytes, and other cells. However, it remains unclear whether the human brain can efficiently eliminate AGE-modified proteins and whether excessive AGEs can contribute to inflammatory changes leading to brain injury in aging.

Materials and Methods: To explore the expression and characteristics of AGE-binding proteins on CNS glia components and their putative function, such as degradation of AGE-modified proteins, primary human astrocytes and human monocytes (as a microglial cell surrogate) and murine microglia (N9) cells and cell membrane extracts were used. Immunohistochemistry was used to examine the distribution of AGE-binding proteins in the human hippocampus; RT-PCR techniques were used to examine the biologic effects of AGEs and a model AGE compound, FFI, on AGE-binding protein modulation and cytokine responses of human astrocytes and monocytes.

Results: Our results showed that AGE-binding proteins AGE-R_1, -R_2, and -R_3 are present in glial cells. Western blot analyses and radiolabeled ligand binding studies show that AGE-R_1 and -R_3 from human astrocytes bind AGE-modified proteins; binding could be blocked by anti-AGE-R_1 and anti-AGE-R_3 antibodies, respectively. Immunohistochemistry showed that AGE-R_1 and -R_2 are expressed mainly in neurons; only some glial cells express these AGE-binding proteins. In contrast, AGE-R_3 was found only on those astrocytes whose positively stained foot processes extend and surround the sheath of microcapillaries. RT-PCR results showed that mRNAs of the three AGE-binding proteins are expressed constitutively in human astrocytes and monocytes, and receptor transcripts are not regulated by exogenous AGEs, the model AGE compound FFI, or phorbol ester. At the concentrations used, GM-CSF appears to be the only cytokine whose transcript and protein levels are regulated in human astrocytes by exogenous AGEs.

Conclusions: The selective presence of AGE-binding proteins in pyramidal neurons and glial cells and their roles in degrading AGE-modified protein in glial cells suggest that the human brain has a mechanism(s) to clear AGE-modified proteins. Without this capacity, accumulation of AGEs extracellularly could stimulate glial cells to produce the major inflammatory cytokine GM-CSF, which has been shown to be capable of up-regulating AGE-R_3. It remains to be determined whether AGE-binding proteins could be aberrant or down-regulated under certain pathological conditions, resulting in an insidious inflammatory state of the CNS in some aging humans.

Patrick R. Hof's present address: Neurobiology of Aging Laboratories, Fishberg Research Center for Neurobiology and Department of Geriatrics and Adult Development, Mount Sinai School of Medicine, New York, NY 10029, U.S.A.

Address correspondence and reprint requests to: Dr. Helen Vlassara, Laboratory of Diabetes and Aging, The Picower Institute for Medical Research, Manhasset, NY 11030, U.S.A. Phone: 516-562-9436; Fax: 516-562-9516; E-mail: hvlassara@picower.edu.

Introduction

Ambient reducing sugars such as glucose react nonenzymatically with a wide range of proteins and lipids to form advanced glycosylation end-products (AGEs) that accumulate in tissues with age (1–4). Tissue AGE accumulation is accelerated in diabetes (5–8) and in renal disease, as the kidneys are the distal excretory route for the byproducts of AGE catabolism (9). Increased tissue AGE deposition has been linked to adverse cellular responses, including activation of inflammatory cells to produce cytokines and growth factors (10), and oxidant stress (11–13), which exacerbates pathologic cascades that in turn lead to atherosclerosis and glomerulosclerosis (14–19). Recently, normal aging human brain was shown to accumulate AGEs within neurons and glial cells (20,21) as well as in senile plaques (22). The latter finding led to studies suggesting that AGE-modified β-amyloid peptide could serve as a "seed" to accelerate and stabilize the formation of amyloid plaques in the Alzheimer's brain (22). Because of the speculated role of ubiquitous AGEs in brain pathology (13,20,22), it has been challenging to understand the normal mechanisms of AGE turnover in the brain and whether they may affect cellular function and homeostasis during normal aging.

Several AGE-specific binding proteins have been associated with a cell-associated AGE-receptor system (AGE-R), including AGE-R_1 (OST/p48), AGE-R_2 (80K-H/p90) (23), AGE-R_3 (galectin-3) (24,25), scavenger receptor (class II) (26), and RAGE (27). These receptors are found widely distributed among human cells and tissues, including hemopoietic (24,27–29), endothelial (18,27,30), mesangial (26,31), and neuronal cells (27), and they are variously related to cellular activation, oxidant stress, and cytokine and growth factor regulation (11–13,28,32). Other plasma-soluble proteins with related functions include lysozyme (LZ) and lactoferrin (LF) (33,34). Results from this laboratory have demonstrated that AGE-R expression in normal monocyte/macrophages can be regulated by AGEs, cytokines, hormonal environment (28,32), and possibly genetic determinants (28,29). It is of interest to note that macrophages from older animals exhibit diminished AGE-receptor expression and function (35), thus, AGE receptors are responsive to environmental changes. It still remains unclear whether AGE-R partake in AGE turnover and whether AGE-receptor expression can be altered in connection with excessive AGE deposition in the human brain.

In this study, we examined the distribution of AGE-R relative to AGE deposition in the hippocampal region of the normal adult human brain and tested the interaction of AGEs with cultured neuroglia—in particular, with primary human astroglia. The findings point to the presence of a functional AGE-receptor system on brain cells that is possibly involved in the turnover of AGE tissue and in brain homeostasis.

Materials and Methods

Cells

For human fetal astrocytes, healthy human fetal brain tissue (18 weeks old) was minced into small pieces in cold Dulbecco's modified Eagle media (DMEM; GIBCO, Grand Island, NY) containing 20 mM HEPES, penicillin/streptomycin (100 U/100 μg/ml), and pressed gently through two different sizes of stainless steel mesh, 250 μm and 150 μm. The crude cell suspension was centrifuged at 250 $\times$ g for 10 min, and the pellet was resuspended in a trypsin (0.05%)-EDTA (2 mM) solution (GIBCO) and incubated at 37°C for 30 min. At the end of the incubation, fetal calf serum (FCS) was added to inhibit residual trypsin activity, and the cell suspension was triturated repeatedly with a pipette. Ten million of the resulting mixed glial cells were cultured in DMEM containing 10% FCS, 25 mM glucose, and 1 mM sodium pyruvate and penicillin/streptomycin at 37°C for 3 days; nonattached cells were then discarded. The mixed glial cultures were incubated for an additional week. At the end of the incubation, culture flasks were shaken using an orbital shaker at 220 rpm at 37°C overnight. The purity of the attached astrocytes was confirmed by immunoperoxidase staining using an anti-GFAP antibody (DAKO, Carpinteria, CA).

Human astrocytoma cells (U87MG) were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained and propagated in DMEM medium with 10% FCS as described above.

The murine microglia cell line N9 was kindly provided by Dr. P. Ricciardi-Castagnoli, CNR, Milano, Italy and cultured identically as astroglica, above.

Human peripheral blood monocytes (PBM) were isolated from fresh human blood using a Percoll gradient, according to previously de-

scribed methods (23,24) and were cultured in RPMI 1640 containing penicillin/streptomycin and 10% FCS.

Immunohistochemistry

Paraffin-embedded (15 μm thickness) and 4% glutaraldehyde-fixed floating (40 μm thick) hippocampal sections from normal brain ($n = 14$, mean age, 56 years) and from Alzheimer's disease brain ($n = 9$, mean age, 75 years) were used in this study. Brain sections were processed as described previously (20) and were incubated with various concentrations of one of the following primary anti-receptor affinity-purified immunoglobulins (IgG): rabbit polyclonal anti-AGE-R_1 and anti-AGE-R_2, and rat monoclonal anti-AGE-R_3 (25). At the end of the incubation, brain sections were incubated with peroxidase-conjugated goat antibody and then with phosphate-buffered saline (PBS) containing 3, 3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma; St. Louis, MO) and 0.02% H_2O_2.

Ligand Preparation and Radiolabeling

AGE-modified bovine serum albumin (AGE-BSA) was prepared as previously described (25) and contained AGE unit/mg protein based on an AGE-ELISA (36). The synthetic AGE 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI) was also used after conjugation to BSA (FFI-BSA) (37) and used as a model AGE ligand. FFI conjugation was confirmed by measuring emission fluorescence at 440 nm (excitation at 370 nm). The amount of FFI conjugated onto BSA was quantitated by UV spectrophotometry to be 13–15 nmole FFI/nmole BSA. AGE-BSA, FFI-BSA, and BSA were iodinated using the IODO-GEN method with carrier-free ^{125}I (NEN, Boston, MA) (25). Greater than 93% of the protein was labeled with ^{125}I according to 10% precipitation by trichloroacetic acid (TCA). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Endotoxin levels of native BSA, AGE-BSA, and FFI-BSA were measured by the Limulus amebocyte lysate kit QCL-1000 (Bio-Whittaker, Walkersville, MD) (<0.03 EU/ml, 0.288 EU/ml, and 0.192 EU/ml, respectively).

Cell Membrane and Whole-Cell Extract Preparations

Human fetal astrocytes and astroglioma cells (U87MG) or microglia N9 were cultured in DMEM complete medium. Cell membrane–enriched fractions were prepared as previously described (25,38). Monolayers were detached with PBS containing EDTA (5 mM), centrifuged, and resuspended in the following lysis buffer: PBS containing 200 mM HEPES, 1 mM EDTA, 300 mM KCl, 3 mM $MgCl_2$, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride [PMSF], 5 μg/ml leupeptin, and 10 μg/ml aprotinin). Cells were homogenized at 4°C with a hand pestle and unbroken cells were removed after centrifugation for 10 min at 13,300 × g at 4°C. The supernatants were subjected to further centrifugation at 97,000 × g for 1 hr. The membrane-enriched pellets were resuspended in lysis buffer. Whole-cell extracts were prepared after PBS-washed cells were lysed with 0.1 N NaOH at 4°C. Protein concentration was quantitated by Bio-Rad assay.

Western and Ligand Blot Analysis

Cell membrane extracts (10–20 μg/lane) were electrophoresed under reducing conditions on a 12% SDS Tris-glycine gel (BioRad) and electroblotted onto nitrocellulose (BioRad) or Immobilon-P PVDF membrane (Millipore, Bedford, MA). Nonspecific binding sites were blocked with nonfat dry milk (5%). The primary anti-AGE receptor antibodies, anti-AGE-R_1 (1:50 dilution) and anti-AGE-R_2 (1:400 dilution), and monoclonal anti-AGE-R_3 (10 μg IgG/ml) in PBS containing 0.05% Tween-20 were added to blots and incubated at room temperature for 2 hr. Normal rabbit serum and rat IgG were used as negative controls. At the end of the incubation, respective peroxidase-conjugated secondary anti-rabbit and anti-rat antibodies were added, and blots were developed by fluorimage analysis, ECL (Amersham, Arlington Heights, IL). For ligand binding analysis, electroblotted membrane proteins or whole-cell extracts were incubated with radioligand (2–10 μg/ml; specific activity 15–30 × 10^6 cpm/μg protein) in the presence or absence of 100-fold excess of unlabeled ligand. Receptor–ligand binding specificity was studied after the nitrocellulose membrane was preincubated with each anti-AGE receptor antibody—rabbit anti-AGE-R_1 (1:25), rabbit anti-AGE-R_2 (1:200), or rat monoclonal anti-AGE-R_3 (40 μg IgG/ml)—at room temperature for 1 hr, before adding ^{125}I-AGE BSA for 2 hr, then the membranes were washed and exposed to X-ray film (Kodak) at −80°C for development of autoradiography.

Human Astrocyte ^{125}I-AGE-BSA Binding and Degradation Studies

Astrocytes (5.0 × 10^5 cells/well) were seeded onto 24-well plates and incubated overnight. Cells were then washed with binding buffer (HBSS containing 0.02% BSA and 25 mM HEPES) and incubated with varying amounts of ^{125}I-AGE-BSA in the presence or absence of 100× excess unlabeled competitors at 4°C for 2 hr. Monolayers were then washed, and cell-associated radioactivity was quantitated after cells were lysed with 0.1 N NaOH. To determine ^{125}I-AGE-BSA uptake and degradation by human astrocytes, monolayers were washed with DMEM containing 0.02% BSA and 25 mM HEPES (uptake buffer) and incubated with ^{125}I-AGE-BSA (25 μg) at 37°C for 6 or 22 hr in the presence or absence of competitors. Culture supernatants were collected and precipitated with an equal volume of 20% TCA. The amount of cell-degraded ^{125}I-AGE-BSA was calculated from the non-TCA-precipitable radioactivity as previously described (39).

AGE-$R_{1,2,3}$ Transcript Analysis

TOTAL RNA ISOLATION. After incubation of human fetal astroglia (1 × 10^6 cells/well) with the indicated ligand for 48 hr, cells were washed with PBS and lysed with RNAzol B buffer at 4°C for 15 min according to the manufacturer's instructions (Tel-Test Inc., Friendswood, TX). Cell lysate was then centrifuged at 12,000 × *g* for 15 min at 4°C; the aqueous phase was saved and the total RNA was precipitated and washed with 75% ethanol, dried, resuspended in RNase-free water containing diethylpyrocarbonate (DEPC) (Sigma), and quantitated at 260 nm/280 nm.

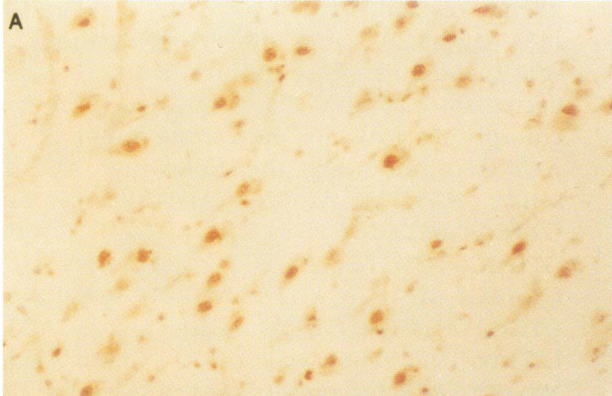
PREPARATION OF cDNA. A cDNA library was prepared using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim) as described in the manufacturer's instructions. Briefly, a cDNA library was made with Random Primer p$(dN)_6$ (0.2 μg/ml) and RNA (1 μg) in 20 μl of reaction buffer (5 mM $MgCl_2$; 0.25 mM dATP, dCTP, dGTP, and dTTP) using PTC-100 thermal programmable cycle controller (MJ Research Inc): 10 min at 25°C, 1 hr at 42°C, and 5 min at 99°C.

RT-PCR OF HUMAN AGE RECEPTOR. Polymerase chain reactions (PCR) of each AGE receptor component were conducted using cDNA (1 μg/2 μl) of primary human astrocytes or monocytes which was added to 50 μl reaction buffer (1.5 mM $MgCl_2$, 0.2 mM dNTPs) containing the sense and anti-sense primer pairs (0.4 μm each) and 1.25 units *Taq* polymerase. The specific primer pairs for each component of the AGE receptor were the following: AGE-R_1 sense: GCTCTTCCACCTCTTACTCC, antisense: GTTCATAGTTGCCTGTCTGG; AGE-R_2 sense GTGGCGTCTGTCTGTGTGTC, antisense GGCC GTAAGGAGAGAGAGTC; AGE-R_3 sense CAC CTGCACCTGGAGTCTAT, antisense GCACTG GTGAGGTCTATGTC (Gibco-BRL, Grand Island, NY). The PCR reaction was set at 25 cycles. The respective AGE-receptor PCR products after electrophoresis on 2% agarose were 222 bp for AGE-R_1, 450 bp for AGE-R_2, and 497 bp for AGE-R_3. Human β-actin (350 bp) was used as loading control.

Cytokine and GM-CSF mRNA and Protein Analysis

mRNA. Following exposure of human fetal astrocytes, mature human monocytes, and microglia N9 cells (each at 1.0 × 10^6 cells/well) to AGE as described above for 48 hr, cell lysates and supernatents were saved for TNF-α (primer 1: AGCACAGAAAGCATGATCCG; primer 2: GGC AGATTGACCTCAGCGCT) and IL-1β (primer 1: GCTGGAGAGTGTGGATCCCA; primer 2: GCT GTCTGCTCATTCATGAC) mRNA screening. For testing the induction of human granulocyte macrophage–colony–stimulating factor (GM-CSF) mRNA, the following primer-pair sequences were custom synthesized: primer 1: CAGTA GAAGTCATCTCAGA; primer 2: AATCTGGGTT GCACAGGAA (Gibco-BRL). cDNA (1 μg/2 μl) of primary human astrocytes or monocytes was added to 50 μl reaction buffer (1.5 mM $MgCl_2$, 0.2 mM dNTPs) containing the sense and antisense primer pairs (0.4 μM each) and 1.25 U *Taq* polymerase. GM-CSF PCR products were separated by agarose gel electrophoresis; the expected 186-bp product indicated the presence of GM-CSF mRNA. Data on cytokine and GM-CSF mRNA levels were analyzed by densitometry and expressed as percent above those of untreated control cells.

PROTEIN. Cell supernatants were spun at 500 × *g* to remove cell debris, aliquoted, and kept frozen 1at −80°C until use. Cytokine levels (TNF-α and IL-1β) were quantitated by commercially available human and murine ELISA kits (R&D, Minneapolis, MN). GM-CSF was quantitated with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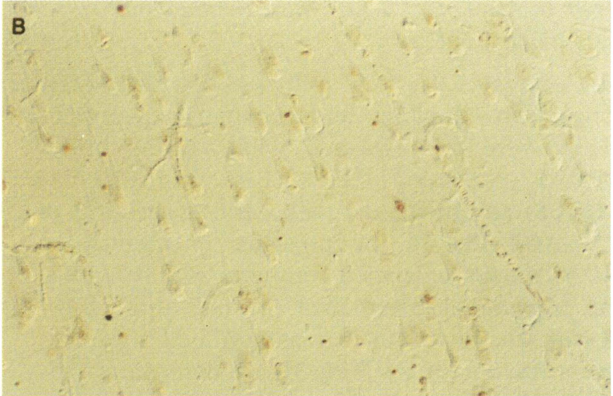
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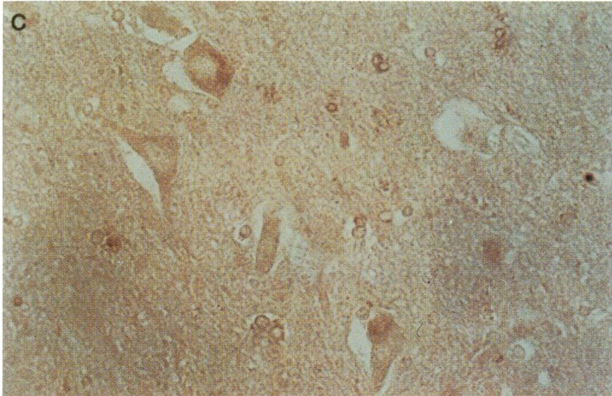
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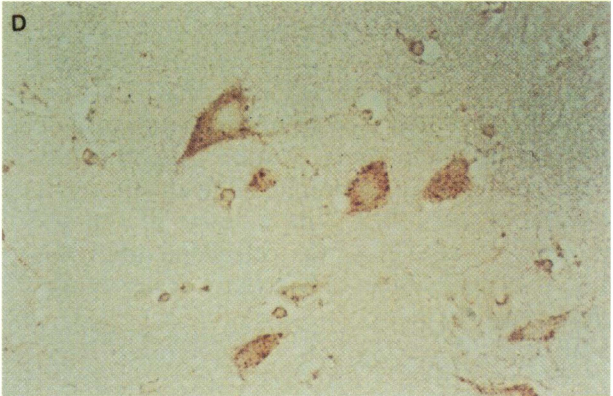
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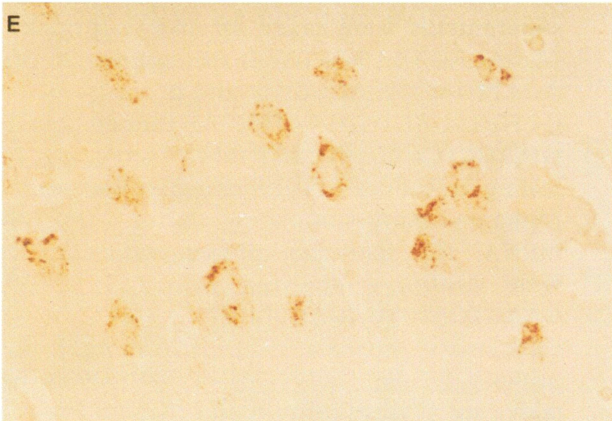
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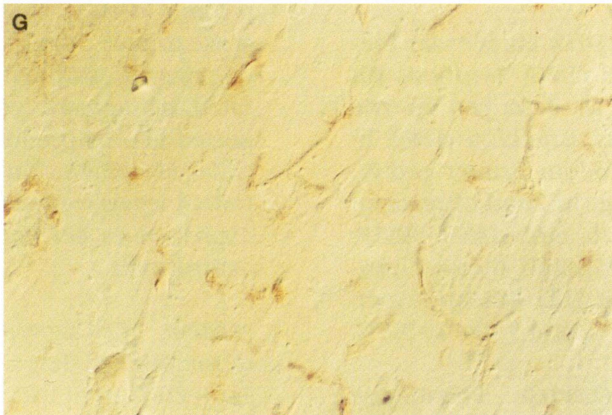
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Fig. 1. Distribution of AGEs and AGE receptors in aging human hippocampus. (A) Intracellular AGE immunostaining in neuronal and glial cells of normal aged human brain (84 years old). (B) Adjacent section to that shown in A; staining by the same anti-AGE antibody is greatly diminished after absorption with AGE-modified poly-L-lysine. (C) Diffuse staining with anti-AGE-R_1 is associated with pyramidal neurons, glial cells, and endothelial cells (same brain section as in A). (D) Punctate staining with anti-AGE-R_2 antibody of adjacent region as in C. Note staining of pyramidal neuronal soma and neurites, as well as of glia and endothelium by same intense punctate pattern. (E) Normal brain AGE-R2 staining as in D, stained simultaneously with section from an age-matched Alzheimer's disease (AD) brain, shown in F, keeping same background. Note punctate cytoplasmic pattern. (F) AGE-R_2 staining of similar section from the subiculum of an 83-year-old brain with AD; note marked attenuation of AGE-R_2 in neuronal soma and glia cells compared with that in E. (G) AGE-R_3 immunostaining is prominent in glial cells and foot processes extending to microcapillaries (gray matter) (×200).

Quantikine Human GM-CSF Immunoassay kit (R&D).

Results

Distribution of AGE Proteins and AGE Receptors in the Human Hippocampal Region

Numerous AGE-immunoreactive (AGE-IR) epitopes were readily seen within pyramidal neurons and granular cells as well as in many glial cells of the normal human hippocampus (Fig. 1A), whereas AGE-IR microcapillaries appeared infrequently because of permaganate treatment. Immunoreactivity was AGE-specific in that all staining was abolished after the anti-AGE antibody was preabsorbed with AGE-modified poly-L-lysine beads (Fig. 1B) (20).

Most glial cells adjacent to neuronal soma were strongly positive for AGE-R_1 (Fig. 1C) and AGE-R_2 receptors (Fig. 1D), whereas AGE-R_3-positive glial cells appeared to constitute only a subpopulation of astrocytes (Fig. 1G), exhibiting anti-GFAP positivity (not shown). AGE-R_1 (Fig. 1C) and AGE-R_2-like epitopes (Fig. 1D, E) were both expressed strongly in the soma and neurites of pyramidal neurons; each had a distinct staining pattern, diffuse for AGE-R_1 and punctate for AGE-R_2. AGE-R_3-like epitopes were present within the glial cell and in foot processes surrounding the microcapillaries in both the gray (Fig. 1G) and white matter (not shown). It is noteworthy that in brain sections from patients with Alzheimer's disease (AD), AGE-R_2 immunoreactivity was markedly reduced within pyramidal neuronal cells of the subiculum (Fig. 1F) and granular cells of the dentate gyrus (not shown). Anti-R_3 recognizes only glutaraldehyde-fixed floating sections; therefore AGE-R_3 could not be detected in paraffin-fixed sections used here. Although AGE-R_1 and R_3 show no detectable differences in staining intensity, it is unclear whether similar suppression of immunoreactivity is present in normal aged and AD brain.

Cultured Fetal Human Astrocytes Exhibit Specific Binding and Degradation of ^{125}I-AGE-BSA

On the basis of human brain immunohistochemical results, the binding of AGEs to astrocytes was tested using both cultured primary fetal human astrocytes and astroglioma U87 cells. Fetal human astrocytes bound ^{125}I-AGE-BSA in a dose-dependent manner, which was saturable at 15 nM (Fig. 2A). Scatchard analysis of the binding data was consistent with a single class of binding sites with an apparent binding affinity of 0.84×10^6 M^{-1} (insert, Fig. 2A). Other well-established neuroglia cell lines, such as human astroglioma U87MG, and murine microglia N9 cells exhibited similar ^{125}I-AGE-BSA binding kinetics with a comparable K_a (not shown). Additional binding studies using U87MG cell membranes on dot blots (2.7 μg/dot) confirmed AGE specificity, in that the radioligand binding was inhibited in the presence of excess amounts of unlabeled FFI-BSA, a synthetic model AGE, but not by unmodified BSA, or a synthetically pure pre-AGE or Amadori product, (Nϵ-(1-deoxy-1-fructosyl)-lysine) (Fig. 2B). Astrocytes degraded ^{125}I-AGE-BSA shown as TCA-soluble material, but not ^{125}I-BSA (Fig. 2C). In the presence of excess unlabeled ligand, ^{125}I-AGE-BSA degradation by intact astrocytes was effectively inhibited (Fig. 2C), suggesting that degradation of AGE-modified proteins was a receptor-dependent event.

Human Astrocytes Express AGE-Specific Binding Species

Western analysis of primary astrocyte cell membrane extracts using the respective anti-AGE re-

ceptor antibodies demonstrated three dominant immunoreactive species: a 45- to 50-kD (AGE-R_1) (Fig. 3A, lane 1); a 70- to 90-kD (AGE-R_2) (lane 2); and a 28- to 32-kD species (AGE-R_3) (lane 3). Ligand-blot studies demonstrated ^{125}I-AGE-BSA binding by predominantly two species corresponding to the 50- and 30-kD sizes of AGE-R_1 and -R_3, respectively (lane 4). Anti-AGE-R_1 blocked ^{125}I-AGE-BSA binding to the ~50-kD species (Fig. 3B, lane 2, arrow), whereas anti-AGE-R_3 diminished markedly ligand binding to the 28- to 32-kDa species (Fig. 3C, lane 2, arrow). No band in the range of AGE-R_2 exhibited similar activity; we could not detect any binding of ^{125}I-BSA to primary human astrocytes under identical conditions (data not shown).

Fetal Astroglia AGE-Receptor Transcripts Are Not Up-regulated by Their Ligand

On the basis of previous evidence of enhanced receptor expression on monocyte/macrophages to AGE ligands (24,28,29), we compared the AGE-R_1 and R_3 mRNA responses of primary fetal glial cells with those of mature peripheral blood monocytes (PBM) after exposure to AGEs. As shown by RT-PCR (Fig. 4A), astrocyte AGE-R_1, -R_2, and -R_3 mRNA levels did not change in response to saturating amounts of AGE-BSA, FFI-BSA, or PMA, compared with untreated controls. In contrast, similar treatment of human monocytes markedly enhanced mRNA expression of all three components, AGE-R_1, -R_2, and -R_3 (Fig. 4B).

Effect of AGEs on Fetal Astroglia TNF-α, IL-1β, and GM-CSF mRNA and Protein Levels

Inflammatory cytokines and growth factors have been detected in aging human brain (40,41), but it remains unknown whether brain deposits of AGE-modified proteins are capable of triggering inflammatory responses as a function of age (42). Using RT-PCR on cultured cells as above, we detected a 2- to 3-fold increase in GM-CSF mRNA level in astrocytes exposed to either AGE-BSA or FFI-BSA. This is above the GM-CSF mRNA levels in the untreated control cells and those cultured in the presence of FCS-derived unmodified albumin, and it is comparable to that induced by PMA (62.5 ng/ml) (Fig. 5A,C) or to the response seen in freshly prepared monocytes (Fig. 5B). This elevation of GM-CSF mRNA could be observed as early as 24 hr after exposure to AGEs, reaching a plateau by 48 hr, and it was associated with an increase in levels of GM-CSF protein in AGE-treated astrocytes and monocytes, but not in AGE-treated microglia cells (Table 1). Despite the absence of discernible changes in fetal astroglia TNF-α or IL-1β mRNA (not shown), a significant rise in IL-1β protein (>10-fold) was observed in AGE-treated astroglia (Table 1) and in TNF-α secreted by microglia N9 cells (Table 1). It is noteworthy that the TNF-α secretory response of AGE-treated monocytes and of N9 cells was greater than that of fetal astroglia.

Discussion

Increased levels of AGEs have been found in the normal aging brain and in Alzheimer's plaques and have been speculated as potential causative agents in these conditions (20,22). AGE-modified substances are subject to endocytosis by AGE-specific receptors present on a variety of cell systems—in particular, blood-derived monocyte/macrophages, which are thought to facilitate AGEs removal and elimination (26,28–31,37). In addition to serving in the scavenging of such senescent macromolecules, cellular interactions with AGEs—many of which are known to be reactive—have been shown to promote tissue damage via pathways involving chemical cross-linking and oxidatitive and proinflammatory reactions. The latter include abnormal production of cytokines and growth factors (10,43). Although links between the accumulation of AGE-modified substances in the brain and locally expressed AGE-receptors have been reported (27), the biological effects of AGEs in the human brain and their role in age-related cognitive functional decline remain obscure (11,12,20). The present study was focused mainly on the hippocampal area, a site involved principally in aging-related loss of cognitive function. Astrocytes, the most abundant cells in the central nervous system (CNS), are critical in the maintenance of the cellular and extracellular environment of the brain under both normal and pathological conditions. Glial elements are generally thought to mediate and/or contribute to the regulation of intercellular interactions, especially those between cells of vascular and of neuronal origin (44). We thus extended our investigation to cultured glial cells and in vitro glycated substances to establish in vitro evidence for a functional pathway analogous to the AGE-receptor system comprising the three components

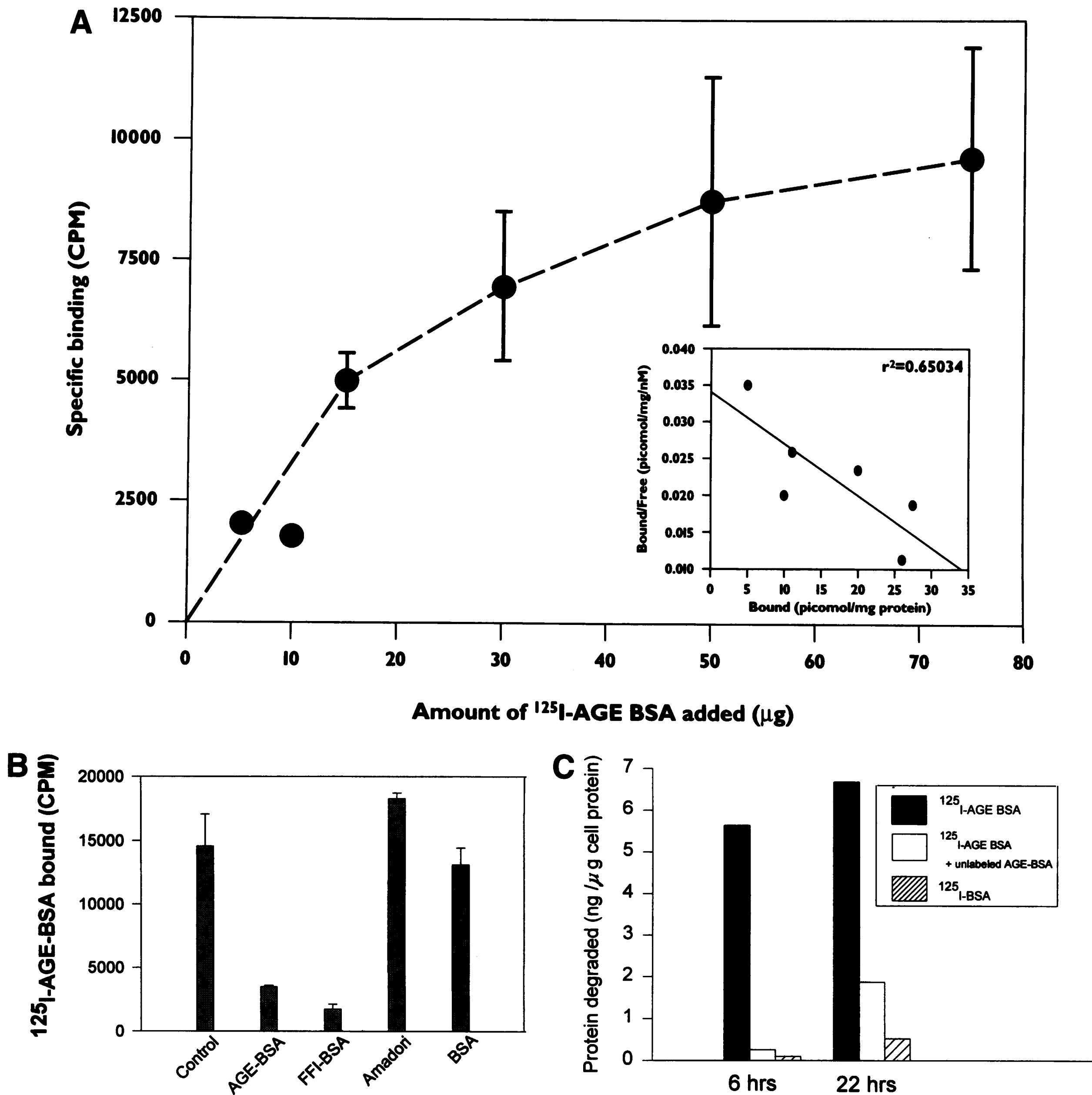

Fig. 2. AGE-specific binding by human fetal astrocytes. (A) ^{125}I-AGE-BSA specific binding kinetic analysis on human astrocytes incubated at the indicated ligand concentrations (2 hr, 4°C) in the presence or absence of excess amounts of unalabeled ligand, as described. Data represent the mean ± SD of four independent experiments, each done in triplicate; insert: Scatchard analysis of the binding data. (B) ^{125}I-AGE BSA (10 µg) binding specificity by U87 MG cell membrane fractions (2.7 µg) was tested by ligand blot analysis in the presence or absence of excess amount (×100) of unlabeled AGE-BSA, FFI-BSA, native BSA, and Amadori product. (C) Degradation of ^{125}I-AGE-BSA by fetal astrocytes is abolished in the presence of unlabeled AGE-BSA; no significant degradation of unmodified carrier BSA was noted. Cell cultures were incubated with the radiolabeled ligands (50 µg/ml each) as shown for 6 or 22 hr at 37°C; data represent the mean of three experiments, each in duplicate.

$R_{1,2,3}$, recently described in several peripheral tissues (18,24,26–31). This system could serve toward the turnover of in vivo deposited AGE-brain components (20,22), and it could serve as a trigger for the chronic inflammatory reactions associated with eventual neuronal death, gliosis, and cognitive decline.

In probing normal adult human brain histo-

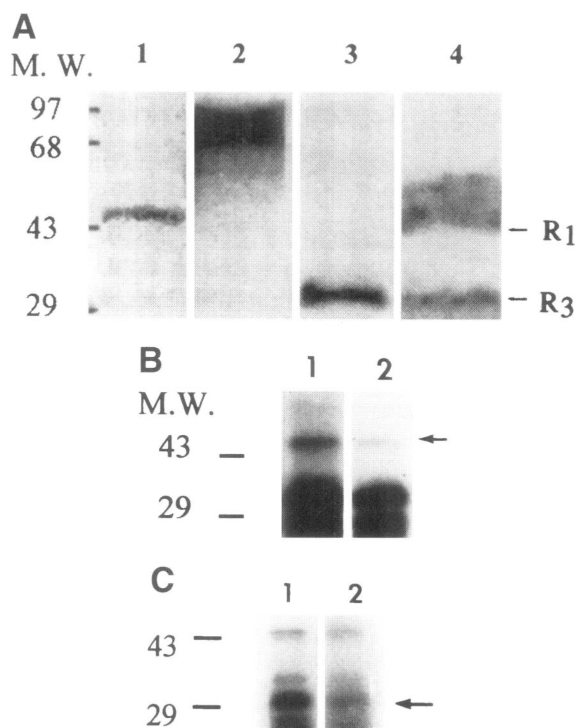

Fig. 3. Cell surface AGE-specific binding proteins on human astrocytic U87MG cells. (A) Western blot of U87 MG cell membrane fractions using anti-AGE-R_1, -R_2, and -R_3 antibodies (lanes 1–3, respectively). Lane 4 is a ^{125}I-AGE-ligand blot on an identical cell membrane fraction as in lanes 1–3. Two main AGE-binding species of ~50 kD and ~30 kD, respectively, correspond to the size of AGE-R_1 and AGE-R_3. (B) ^{125}I-AGE-BSA binding to the ~50 kD-species (lane 1) is inhibitable by anti-AGE-R_1 (lane 2, arrow). (C) ^{125}I-AGE-BSA binding to the ~30 kD (lane 1) is attenuated by anti-AGE-R_3 antibody (lane 2, arrow).

logical sections, AGE-receptor expression was found to be abundant and widespread within the hippocampal area: AGE-R_1 and -R_2 were associated with most glial cells as well as pyramidal neurons and dentate gyrus granular cells, whereas AGE-R_3 was mainly present in perivascular glial cells. Distinct patterns of neuronal immunoreactivity were exhibited by anti-AGE-R_1 (diffuse staining) and anti-AGE-R_2 antibodies (punctate staining); within the pyramidal neuronal population, the expression of AGE-R_2 was also heterogeneous in that pyramidal neurons in the subiculum and layer II of the entorhinal cortex stained more intensely compared with those in CA_1 and CA_3 of the hippocampus (data not shown). Of particular interest was the marked reduction of AGE-R_2 staining in the corresponding segments of AD brains. Although we are unable to provide a satisfactory interpretation of this finding at this time, it is tempting to speculate that, artifact aside, in the face of increased AGE aggregates in AD brains (22), the absence of this receptor component, which was previously linked to signaling and cell activation, is genetic in origin. Underexpression of other AGE-R components, e.g. R_1, has been observed in association with diabetic vascular and renal complications in nonobese diabetic mice (NOD) mice and humans, both species exhibiting elevated AGE tissue and plasma levels (45,46). However, further studies are required to confirm and explore this interesting possibility.

In addition, it was readily apparent that the overall receptor distribution pattern colocalized with AGE-immunoreactive substances within pyramidal neurons, dentate gyrus granular cells, and within glial cells of normally aging human brain. In addition, AGE-R_1- and -R_3-positive immunoreactivity epitopes were both detected in glial cells located elsewhere, e.g., at the subdural area and in ependymal cells surrounding the choroid pluxes of the brain (data not shown). It is of particular interest that AGE-R_3 localization was strikingly prominent on perivascular glial cells; the staining appeared restricted to a particular subgroup of glial cells, with extended foot processes ensheathing regional brain capillaries. Given the proximity of perivascular glia to blood supply and therefore to systemic AGEs, this intense AGE-R_3 surface expression may be indicative of cellular activation related to glial cell exposure to these substances (10,17,26,43). This could be due to direct exposure of local glia to the time-dependent AGE formation within the vascular matrix (30,47–49), or it may reflect recruitment of such cells from surrounding areas generally sparse in extracellular matrix. In either case, the strategic location of AGE-R_3-positive foot processes of astrocytes adjacent to microcapillaries suggests that these cells could play a pivotal role in the protection of brain parenchyma against AGE transfer from the systemic pool, or they may assist in the disposal of brain AGE byproducts in the direction of the systemic circulation, as it may occur in areas adjacent to the choroid plexus.

AGE-modified proteins are shown to be endocytosed by endothelial cells and deposited at the luminal surface or subendothelial space (30), or they leak through a disrupted intercellular barrier (50), while secretion of degradation products also occurs by the apical surface of the endothelium. However, no studies have focused on

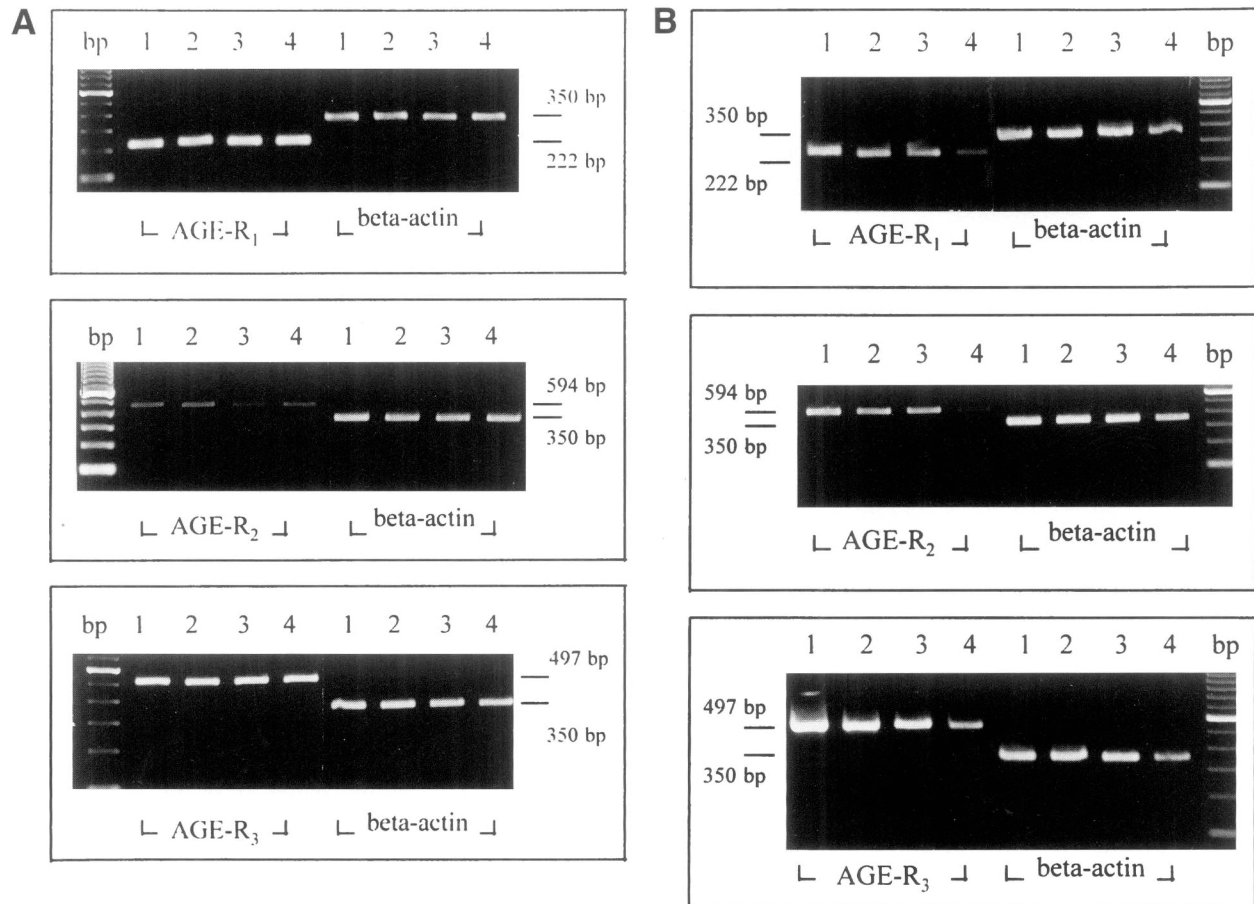

Fig. 4. Fetal astroglia AGE-receptor transcript level is resistant to regulation by AGEs. (A) RT-PCR of AGE-R_1, -R_2, and -R_3 mRNA from primary fetal human astrocytes (1×10^6/well). No detectable changes were observed following incubation for 48 hr at 37°C in the presence of 300 μg/ml FFI-BSA (lanes 1), AGE-BSA (lanes 2), PMA (lanes 3), or medium alone (lanes 4). (B) RT-PCR of AGE-R_{1-3} mRNA from peripheral blood monocytes cultured under the same density and conditions in the presence of FFI-BSA (lanes 1), AGE-BSA (lanes 2), PMA (lanes 3), showing significant up-regulation of all three transcripts, compared with control cells kept in medium alone (lanes 4). β-actin mRNA was used as an internal control.

brain-specific endothelial cells, and such important issues remain to be elucidated, although AGE-mediated disruption of the blood-brain barrier was observed following the intravenous infusion of AGEs to normal rats (51). Furthermore, given the evidence that AGEs are chemotatic to peripheral monocytes and macrophages (43), the presence of AGE-R_3-positive cells around microcapillaries suggests that astrocytes and other glia cells may be actively recruited to areas of extravasated AGE for their subsequent removal. However, the mechanisms involved in such responses in the brain are largely speculative. As a host of perivascularly deposited proteins, β-amyloid, and lipids undergo complex glycoxidation reactions and free-radical generation, increased prooxidant activity is likely to trigger proinflammatory cascades (52,53). Certain reports have already implicated receptor-mediated pathways, which include AGE-recognizing molecules, such as RAGE (54) and class A scavenger receptor (26,55,56), both of which exhibit a broad specificity for non-AGE ligands, namely β-amyloid peptide, or modified lipoproteins (16). Such interactions could take place in normal aging and much more so in Alzheimer's disease, contributing to neuronal damage as well as to glial cell activation—two processes that may or may not be directly connected to each other nor specifically to glycation. On the basis of previous evidence of time-dependent AGE deposition in the brain (20,52), especially in connection with re-

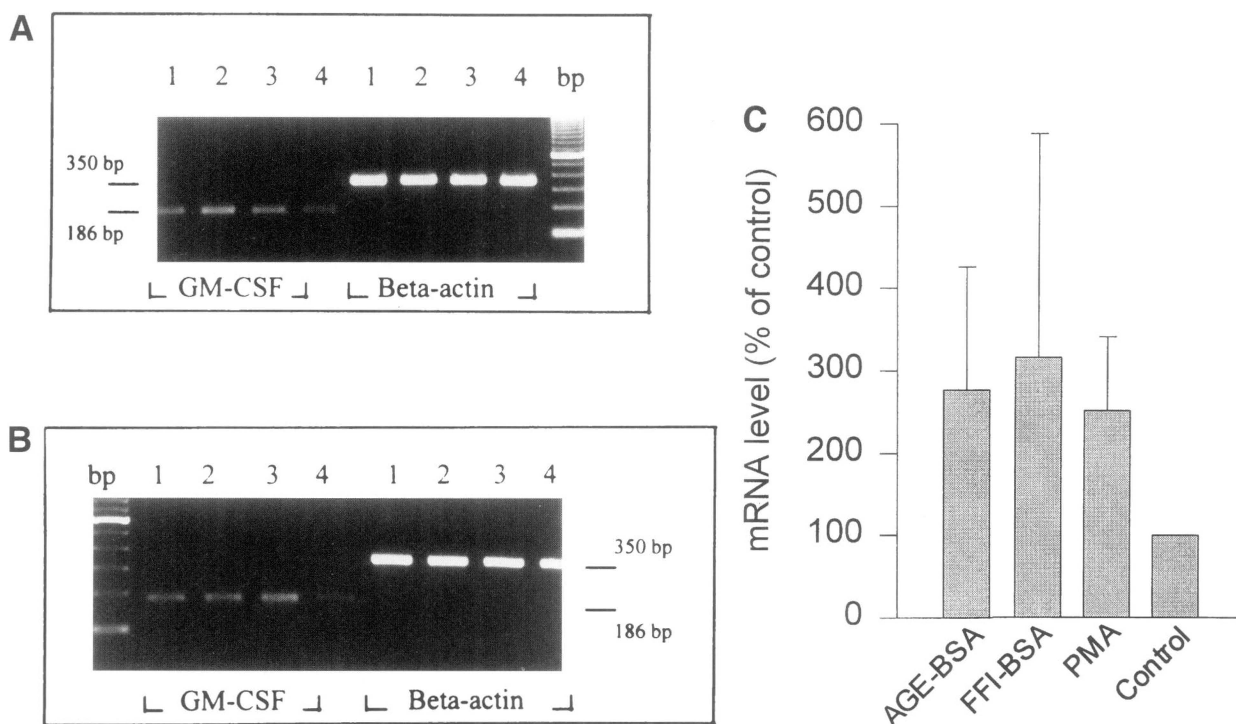

Fig. 5. AGEs modulate human fetal astroglia GM-CSF transcription and protein secretion. (A) RT-PCR of GM-CSF mRNA from primary fetal astroglia incubated with 300 μg/ml of FFI-BSA (lanes 1), AGE-BSA (lanes 2), or PMA (62.5 ng/ml) (lanes 3) or medium alone (lanes 4) for 48 hr at 37°C. (B) GM-CSF mRNA levels in peripheral human monocytes cultured under similar conditions with 300 μg/ml FFI-BSA (lanes 1), AGE-BSA (lanes 2), PMA (62.5 ng/ml) (lanes 3), or medium alone (lanes 4). β-actin mRNA was used as internal control. (C) Densitometric analysis of astrocyte GM-CSF mRNA levels representing four independent experiments (data expressed as % mean ± SD above control cells).

active glia, as determined by class II MHC antigen expression (52,57–59), the identification of an AGE-specific cellular pathway is of obvious importance in both normal homeostasis and in the pathogenesis of brain damage with aging.

The presence of functional AGE-specific receptors in human brain glia was confirmed using cultured primary fetal astroglia with in vitro prepared model AGE-modified proteins and specific immunological and molecular probes corresponding to the three previously characterized AGE-receptor components, AGE-$R_{1,2,3}$ (23–25).

That human astrocytes can, in fact, process AGE-modified macromolecules was supported by the active endocytosis and the degradation of AGE proteins, which suggests that human glia may be capable of modulating in vivo formed AGE-modified brain constituents. Thus, intra- or extracellular AGE-modified CNS components forming over a lifetime can be removed by an AGE-receptor system based on glial cells. It can be further speculated that the breakdown derivatives of AGE turnover are exported to the CSF, then to the systemic circulation, and ultimately through the kidney to the urine (9). This mechanism may help maintain low local CNS AGE levels and thus protect against the harmful effects of reactive AGE deposits in a manner analogous to the model proposed for the AGE turnover in peripheral tissues (29). Such a system may exhibit a wide range of flexibility to meet the local needs of glycoxidant burden.

Such ability of peripheral AGE receptors to respond to various metabolic regulatory factors, e.g., glycation levels, insulin (28), glycoxidative products, cytokines (32), and ultimately, senescence (35), has been previously reported. Whether the specific AGE-R_1, -R_2, and -R_3 receptor components of glial cells are subject to modulation by their ligands under normal or pathological conditions remains unclear. In the studies presented, human fetal astrocyte AGE-R_1, -R_2, and -R_3 transcript levels were not responsive to either the mixture of in vitro pre-

Table 1. Effect of AGE-modified albumin on GM-CSF, TNF-α, and IL-1β secretion by human astroglia, human monocytes, or murine microglia cells (N9)

	GM-CSF (pg/ml)	TNF-α (pg/ml)	IL-1β (pg/ml)
Astrocytes			
Control[a]	<8	<1	49 ± 2
AGE-BSA	40 ± 4	<1	660 ± 13
PMA	63 ± 8	53 ± 4	11499 ± 232
Monocytes			
Control	8 ± 2	<1	N/T[b]
AGE-BSA	52 ± 4	378 ± 28	N/T[b]
PMA	64 ± 9	518 ± 18	N/T[b]
Microglial cells (N9)			
Control	<8	22 ± 3	<23
AGE-BSA	<8	61 ± 2	<23
PMA	<8	85 ± 7	<23

The cell culture conditions and cytokine and growth factor quantitation methods are described in Materials and Methods. Data from three independent experiments, each done in duplicate, are expressed as mean ± SEM pg/ml.
[a]Control, unmodified BSA; [b]N/T, not tested.

pared AGEs or to the chemically pure AGE, FFI, a compound known to alter mature peripheral monocyte AGE-R expression (49). This may suggest that astrocytes, although constitutively expressing AGE-binding components, may be resistant to activation by these substances, as they are to other mitogens, such as PMA. This is largely consistent with the well-described low level of CNS immune cell responsiveness to inflammatory stimuli (59). Alternatively, it may be that fetal glial cells are lacking these properties, potentially acquired later in development.

The AGE-mediated mRNA up-regulation observed on all three AGE-receptor components of human peripheral monocytes may be extrapolated as reflecting human microglial cell responses to AGEs, assuming that monocytes represent a precursor of, and thus a model for, human microglial cells (60,61), and an inference may be made from the findings shown regarding the possible effect of time-dependent AGE accumulation in the aging brain on glial cell AGE-receptor expression. However, given our histological data, a dominant in vivo role could not be readily attributed to these cells, as only small numbers were seen, compared with astroglial populations. This may be physiologically sound, given the sheer abundance of astrocytes present in the human brain; however, in aging, the rate of cumulative deposition of AGEs may exceed the rate of their removal. In the latter case, the paucity of reactive microglia in our study may also reflect differences in postmortem tissue preparation methods (62). Nevertheless, it is thought that AGE aggregates may act as a nidus and a trigger for glial cells to initiate a subinflammatory network of brain cytokine and growth-promoting stimuli, culminating in the inevitable neuronal injury. The critical role of microglia-to-astroglia interactions is increasingly accepted (57). Although we were unable to utilize human microglia cells in this study, we observed the ability of murine microglial cell line N9 to produce TNF-α in response to AGE and mitogens, a finding that cannot be extrapolated to humans at the present time. However, some of the astroglia responses have been tracked to microglial effects and vice versa (57). Astrocytes can synthesize, among other potent molecules, IL-1β and GM-CSF (57), although the in vivo regulators of such responses are still to be determined, especially in normal aging brain (57,60). A key link between such responses and AGE toxicity was obtained through the observation that exposure of cultured glial cells to AGEs can induce one or the other of these mediators at either the transcriptional or the mature protein level, albeit to a far lesser extent than mature blood monocytes. These findings are consistent with reports showing variable results or the need for very high doses of a combination of stimulants, e.g., LPS/IL-1β or IFN-γ/IL-1β to produce TNF-α (59).

Age-associated gliosis that results in excessive proliferation of glial cells is a complex process of chronic and subacute inflammatory responses to cellular and environmental changes in normal aging brain. However, its mechanism remains unknown. Our results, although preliminary, suggest that accumulation of AGEs in the extracellular milieu may act to enhance GM-CSF overproduction, a response that could facilitate gliotic changes (58). GM-CSF is shown to upregulate AGE-R_3, also called galectin-3, expression in monocytes as well as in Schwann cells (32). Thus, AGE-mediated GM-CSF production could potentially be responsible for modulating the level of AGE-receptor expression in the CNS and/or in the peripheral nervous system, although this will require further investigation. AGE-associated GM-CSF production by cultured

macrophages has been reported (63), as have other cytokines and growth factors. Thus, AGEs could act as the fuel for a chronic subacute inflammatory cytotoxic process contributing to the age-related decline in cognitive function in humans.

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