Cytotoxic role of advanced glycation end-products in PC12 cells treated with β-amyloid peptide

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Abstract. Alzheimer's disease (AD) is the most common type of dementia afflicting the elderly. Recent studies have increasingly suggested that a high concentration of advanced glycation end products (AGEs) may be important in AD pathogenesis. However, the mechanisms and pathways involved remain unknown. The aim of this study was to explore whether the mechanism of the effect of AGEs on Aβ-PC12 cells [PC12 cells treated with β-amyloid (Aβ) peptide] was associated with oxidative stress; and to study whether inhibiting the activity of the receptor for AGE (RAGE) attenuated the toxic effect of AGEs and Aβ on PC12 cells. Several PC12 cells were pretreated with Aβ, and were then treated with different concentrations of AGEs. Other PC12 cells were treated with trypsin, a pancreatic protein enzyme and an inhibitor of RAGE, and were then treated with Aβ and AGEs. Apoptosis was measured by flow cytometry (FCM) and cell viability was measured by MTT assay. RAGE and nuclear factor-κB (NF-κB) were measured by reverse transcription-polymerase chain reaction (RT-PCR) assay. With an increase in AGE concentration, the viability of Aβ-PC12 cells treated with AGEs decreased. However, the Aβ-PC12 cell viability was greater in the trypsin group than in the non-trypsin group. Cell apoptosis rates and mRNA expression of RAGE and NF-κB in Aβ-PC12 cells treated with AGEs were significantly higher than in the Aβ-PC12 cells. AGEs and Aβ were neurotoxic, and RAGE triggered the neural cytotoxic role of AGEs in Aβ-PC12 cells. The molecular mechanisms may be connected with the expression of NF-κB and apoptosis mediated by RAGE. Inhibiting the activity of RAGE may mitigate the toxic effect of AGEs and Aβ on neural cells.

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Introduction

Alzheimer's disease (AD) is one of the most common diseases among the elderly. AD is the primary cause of dementia in old age (1). Over the last two decades, several hypotheses have been proposed to explain AD pathogenesis. One such hypothesis is the amyloid hypothesis, which states that β-amyloid (Aβ) peptide deposits are the fundamental cause of the disease (2).

The accumulation of Aβ in cerebral senile plaques is a major pathological hallmark of AD. Therefore, Aβ peptides are central to the pathogenesis of AD. Despite the genetic and cell biological evidence that supports the amyloid hypothesis, it is becoming increasingly clear that AD etiology is complex, and that Aβ alone is unable to account for all aspects of AD. In 2000, evidence strongly suggested that advanced glycation end products (AGEs) have an important toxic role in AD pathogenesis (3). *In vitro* experiments demonstrated that AGEs and Aβ are co-localized in the core of senile plaques, and that they are able to attract and cause the aggregation of soluble Aβ.

Aβ is a pleiotropic peptide and is capable of binding to receptors at several different membrane locations (4). The receptor for AGEs (RAGE), a multi-ligand receptor of the immunoglobulin superfamily of cell surface molecules (5), possesses a cell surface binding site for Aβ peptides (4) and is expressed at higher levels when stimulated by excessive levels of Aβ (6). RAGE has been extensively studied for its roles in the migration and differentiation of neuronal cells during development, the perturbation of neuronal cells by Aβ and the inflammatory response (3,7).

Induced expression of RAGE is frequently correlated with pathological stages such as diabetic endothelial damage and AD (8,9). It has been proposed that RAGE is responsible for Aβ neurotoxicity (10). However, the mechanism whereby RAGE is able to recognize AGEs and Aβ, if these molecules are not glycated, remains unclear. Studies have suggested that AGEs are inducers of chronic inflammation and acute-phase responses in a variety of diseases (4,11). However, this hypothesis is not clear in the brains of patients with AD.

In the present study, we aimed to observe the effects of AGEs on PC12 cells pretreated with Aβ25-35, to explore whether the mechanism of action is associated with oxidative stress, and to study whether inhibiting the activity of RAGE attenuates the toxic effect of AGEs.

Materials and methods

Production of AGEs and fibrillar Aβ. AGEs were produced by incubation of 1 mM bovine serum albumin (BSA; Roce, Indianapolis, IN, USA) with 1 M glucose at 50˚C in phosphate-buffered saline (PBS) at pH 7.4 for 60 days. Samples were initially filtered through a 0.2-μm filter and kept sterile during the incubations. A slightly elevated temperature was used to accelerate the reaction and avoid bacterial contamination. Unbound sugars were removed through extensive dialysis using distilled water. AGEs were lyophilized and resuspended in PBS. The controlled BSA was incubated under the same conditions, except that glucose was omitted. AGE purity was assessed with fluorometry and chromatographic analysis exploiting the selective fluorescence of AGEs at an optical density of 400 nm (the excitation wavelength was 370 nm) (12). Fibrillar Aβ25-35 (13) was produced by incubating the peptide (1 mM) in 10 mM PBS under sterile conditions for 7 days at 37˚C.

Cell culture. PC-12 cells (ATCC, Manassas, VA, USA) were seeded into 96-well flat bottom tissue culture plates (Corning, USA) at a density of $3x10^5$ cells/ml for the MTT assay, and into 24-well plates at a density of $6x10^5$ cells/ml for the remaining assays. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, streptomycin and penicillin (100 mg/ml and 100 U/ml), and 2 mM L-glutamine at 37˚C in a humidified atmosphere containing 5% CO_2 and 95% air.

Grouping. Aβ-PC12 cells were PC-12 cells pretreated with Aβ25-35. Aβ-PC12 cells were treated with different levels of AGEs. PC-12 cells were divided into five groups: i) the control group: PC12 cells were cultured with 12.5 μl of 30% BSA; ii) the Aβ group: PC12 cells were cultured with 25 μmol/l Aβ25-35, and termed Aβ-PC12 cells; iii) the Aβ+L-AGE group: Aβ-PC12 cells were cultured with a low volume of AGEs (12.5 μl); iv) the Aβ+M-AGE group: Aβ-PC12 cells were cultured with a medium volume of AGEs (25 μl); and v) the Aβ+H-AGE group: Aβ-PC12 cells were cultured with a high volume of AGEs (50 μl).

RAGE was inhibited by trypsin (14) to determine whether RAGE was involved in AGE and Aβ toxicity. In the trypsin group, PC12 cells were pretreated with 1 mg/ml trypsin at 37˚C for 30 min, and washed three times with PBS. Subsequently, 25 μmol/l Aβ25-35 was added and cells were incubated at 37˚C for 24 h, before the addition of 50 μl AGE. In the non-trypsin group, PC12 cells were not pretreated with trypsin (Gibco, USA); however, 25 μmol/l Aβ25-35 was directly added and the cells were incubated at 37˚C for 24 h, which was followed by the addition of 50 μl AGE.

MTT assay. Following incubation of the cells with AGEs and Aβ, the medium was removed and the cells were washed with PBS. Subsequently, 100 ml DMEM, without phenol red, and 25 ml of an MTT solution (1.5 mg/ml in PBS) were added to each well, followed by incubation for 4 h. The MTT solution was carefully removed from the wells to avoid the loss of formazan crystals before they were dissolved with 100 ml dimethyl sulfoxide/ethanol (1:1). Absorbance was measured at 550 nm with the reference filter set to 630 nm. MTT assays were performed in triplicate for each experiment.

Reverse transcription-polymerase chain reaction (RT-PCR). PC-12 cells were grown under the same conditions as described previously. Following removal of the medium, cells were washed with PBS and lysed with 1 ml TRIzol (Takara, Japan) for 5 min. Following centrifugation at 12,000 x g at 4˚C for 10 min, the supernatant was mixed with 200 μl chloroform and shaken for 30 sec. Subsequently, 400 μl isopropanol was added to the aqueous phase, and the mixture was allowed to stand for 10 min. Following centrifugation at 12,000 x g at 4˚C for 10 min, the obtained pellet was rinsed with 75% ethanol, dried and then dissolved in diethyl pyrocarbonate-treated water. Further RNA purification was performed using the Qiagen RNeasy kit, according to the manufacturer's instructions (Qiagen, Germany). The Stratagene RT-PCR kit was used for reverse transcription of total RNA (Table I).

The PCR procedure was implemented as follows: RAGE: 1 cycle at 95˚C for 4 min; 35 cycles at 95˚C for 50 sec, 58˚C for 50 sec and 72˚C for 1 min; and 1 cycle at 72˚C for 7 min; and nuclear factor-κB (NF-κB): 1 cycle at 95˚C for 1 min; 35 cycles at 95˚C for 30 sec, 60˚C for 30 sec and 68˚C for 2 min; and 1 cycle at 65˚C for 10 min.

PCR products were loaded and run on a 1.8% agarose gel and visualized following ethidium bromide staining using a UV transilluminator.

Apoptosis rate and flow cytometry (FCM) analysis. PC12 cells were harvested by centrifugation at 12,000 x g for 5 min and then washed twice with cold PBS, before being resuspended in 100 μl binding buffer with ~10^6 cells. Subsequently, 5 μl fluorescein isothiocyanate (FITC)-annexin V and 5 μl propidium iodide (PI) were added. Cells were gently oscillated and incubated in the dark for 15 min at 25˚C. Following the addition of 400 μl binding buffer to each tube, the cells were analyzed by FCM within 1 h. Cells that stained positive for FITC-annexin V and negative for PI were considered to be in apoptosis. Cells that stained positive for FITC-annexin V and PI were considered to either be in necrosis or dead. Cells that stained negative for FITC-annexin V and PI were considered to be living. The FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to conduct the FCM. Data were analyzed using CellQuest software and the apoptosis rates were provided.

Statistical analysis. Statistical analysis of the results was carried out by analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS; SPSS, Inc., Chicago, IL, USA). Either the Student's t-test or the Wilcoxon rank sum test were used, depending on the normality of the data distribution. $P<0.05$ was considered to indicate a statistically significant result.

Results

Cell viability. The influence of Aβ and the different concentrations of AGEs on the viability of PC12 cells is presented in Table II. Optical density (OD) values were determined by MTT

Table I. Primers used in this study.

Type	Primer sets	Length (bp)
RAGE	5'-AGACCAAGTCCAACTACCGAG-3' 5'-CCTTCACAGATACTCCCTTCAT-3'	408
NF-κB	5'-AGCACAGATACCACCAAGACCC-3' 5'-CCCACGCTGCTCTTCTATAGGAAC-3'	198
β-actin	5'-CAATTCCATCATGAAGTGTGAC-3' 5'-CCACACAGAGTACTTGCGCTC-3'	260

RAGE, receptor for advanced glycation end products; NF-κB, nuclear factor-κB.

Table II. Changes in cell viability following incubation with Aβ and AGEs.

Group	OD	Viability (%)
Control	0.8109±0.1826	100.00
Aβ	0.5847±0.1044[a]	72.11
Aβ+L-AGE	0.3876±0.0781[a,b]	47.80
Aβ+M-AGE	0.2758±0.0593[a,b]	34.01
Aβ+H-AGE	0.0969±0.0800[a,b]	11.95

[a]P<0.01 vs. control group and [b]P<0.01 vs. Aβ group. Aβ, β-amyloid; AGE, advanced glycation end product; OD, optical density; L, low; M, medium; H, high.

Table III. Changes in cell viability following incubation with trypsin and AGEs.

Group	OD	Viability (%)
No trypsin	0.1103±0.0451	13.01
Trypsin	0.4414±0.0357[a]	52.05

[a]P<0.01 vs. the group without trypsin. AGE, advanced glycation end product; OD, optical density.

assay. The cell viability was calculated using the following formula: Cell viability = ($OD_{sample\ value}/OD_{control\ value}$ x 100%. Statistical analysis indicated that cell viability in the Aβ and Aβ+AGEs groups was lower than that of the control group (P<0.01). The cell viability decreased in the three groups of the different volumes of AGEs compared with the Aβ group (P<0.01). The effects of AGEs on Aβ-PC12 cell viability were dose dependent. As demonstrated in Table III, the cell viability in the trypsin group was greater than that of the non-trypsin group (P<0.01).

RT-PCR. The expression of RAGE mRNA in the PC12 cells was at 408 bp in all groups. The levels of RAGE mRNA in the Aβ+L-AGE, Aβ+M-AGE and Aβ+H-AGE groups were higher than in the Aβ group. The expression value of RAGE mRNA increased with increasing AGE concentration, as demonstrated in Table IV. The expression of NF-κB mRNA in the PC12 cells was at 198 bp in all groups. The levels of NF-κB mRNA in the Aβ+L-AGE, Aβ+M-AGE and Aβ+H-AGE groups were higher than in the Aβ group. The expression value of NF-κB mRNA increased with increasing AGE concentration, as presented in Table IV.

Apoptosis rate. Apoptosis was induced by Aβ and the different concentrations of AGEs, and was quantified as the percentage of apoptotic cells. The rate of apoptosis was calculated using the following formula: Apoptosis rate = (number of apoptotic cells/total number of cells) x 100%. Apoptosis rates in all groups of Aβ-PC12 cells (16.06, 24.57, 36.89 and 43.85% in the Aβ, Aβ+L-AGE, Aβ+M-AGE and Aβ+H-AGE groups, respectively) were higher than in PC12 cells (2.01% in the control group; P<0.01). The cell apoptosis rate significantly increased in the Aβ+M-AGE and Aβ+H-AGE groups compared with the Aβ group (P<0.01). In the Aβ+AGE groups, the cell apoptosis rate increased with increasing AGE concentration, as demonstrated in Fig. 1.

Discussion

AD is a neurodegenerative disorder characterized by progressive degeneration and loss of neurons in the brain, which has been correlated with the appearance of senile plaques, the neuropathological hallmarks of AD. As the major component of senile plaques, Aβ is considered to play a key role in the development and progression of AD (15).

A potential mechanism for the effect of Aβ is presented in Fig. 2. This mechanism accounts for the fact that the increasing concentrations of AGEs with age predispose to the injurious signal presented by Aβ.

Aβ-PC12 cells, PC12 cells treated with Aβ25-35, have been recognized as having the ability to mimic classical AD pathology, such as inhibited cell multiplication, induced cell metamorphosis, cell damage, functional loss and even cell death (12). *In vitro* studies have demonstrated that Aβ has nutritional value for cultured hippocampal neurons, as well as being toxic to these cells. The role that Aβ assumes depends on two factors, which include the maturity of neurons and the concentration of Aβ (14,16). A high concentration of Aβ triggers the toxic property, causing a loss of mature neurons and inhibition of axon growth. The working domain of Aβ has been confirmed

Table IV. Expression of RAGE mRNA and NF-κB mRNA in PC-12 cells.

Group	RAGE	β-Actin	RAGE/β-actin	NF-κB	β-Actin	NF-κB/β-actin
Aβ	113.27±4.16	290.83±8.47	38.94±0.60	121.20±8.21	278.80±15.33	43.47±5.02
Aβ+L-AGE	178.63±10.05	318.93±5.65	56.01±4.15[a]	176.90±2.69	311.07±6.89	56.87±1.08[a]
Aβ+M-AGE	308.80±7.89	362.60±12.11	85.16±2.11[a,b]	265.83±13.01	354.20±4.83	75.05±3.31[a]
Aβ+H-AGE	347.67±5.67	373.77±20.69	93.02±5.09[a,b]	316.87±10.05	371.17±10.18	85.37±6.63[a,b]

[a]P<0.01 vs. Aβ group and [b]P<0.01 vs. Aβ+L-AGE group. RAGE, receptor for advanced glycation end products; NF-κB, nuclear factor-κB; Aβ, β-amyloid; L, low; M, medium; H, high.

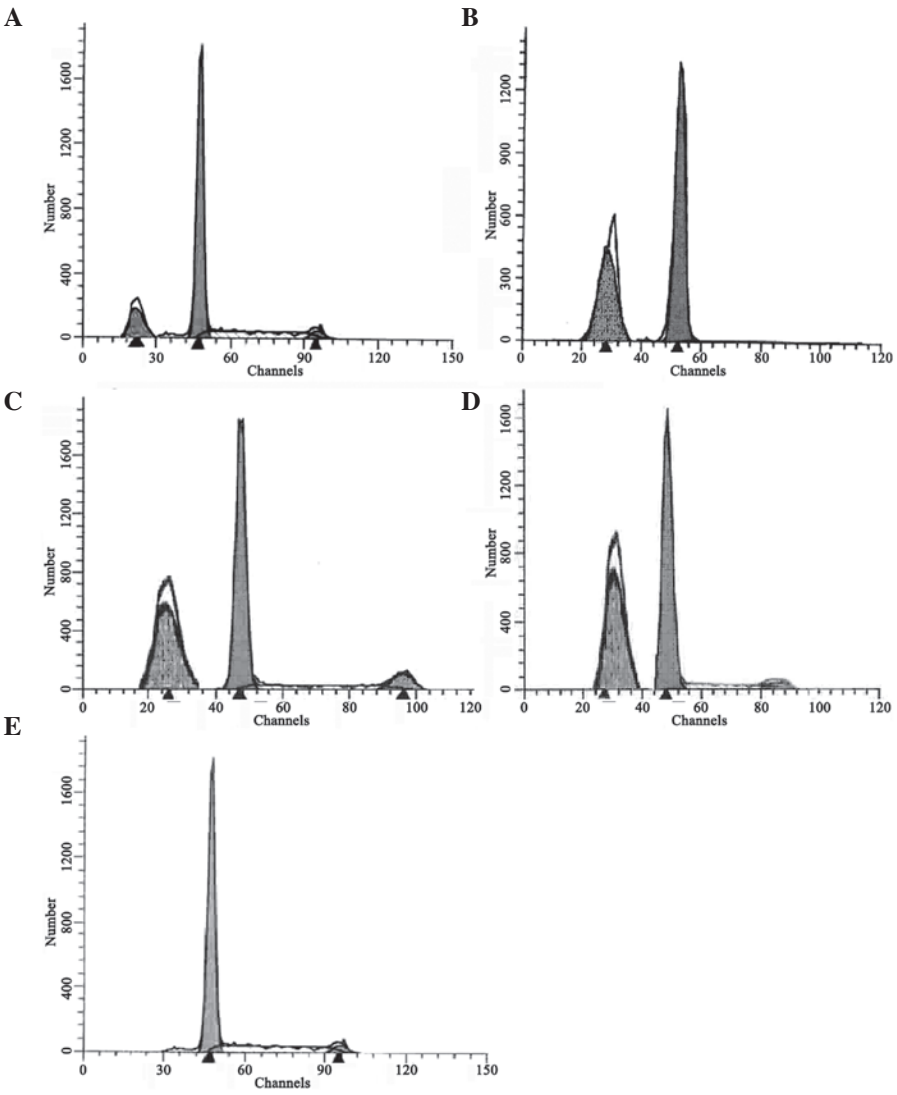

Figure 1. Apoptosis of PC12 cells.

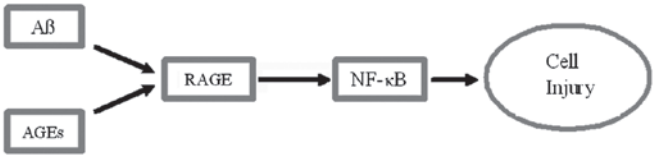

Figure 2. Potential mechanism for the effect of β-amyloid (Aβ). AGE, advanced glycation end product; RAGE, receptor for AGE; NF-κB, nuclear factor-κB.

to be the amino acid residues in 25-35 sites. In the present study, Aβ25-35 was added to PC12 cells as a neurotoxin. This addition inhibited PC12 cell multiplication and induced cell death, which was consistent with previous studies (12,17).

AGEs are important agents in the proposed mechanism of Aβ-mediated cell injury in AD. AGEs are a series of irreversible polymers produced by the Maillard reaction, a non-enzymatic glycation and oxidation reaction between carbohydrate-derived

carbonyl compounds and the free N-terminal of proteins, forming brown fluorescent reaction end products (18). As these products auto-fluoresce, we can use the fluorescence chromatogram to identify them at an excitation wavelength of 350-399 nm and an emission wavelength of 440-470 nm.

RAGE is a 404-amino acid protein, which belongs to a family of cell surface molecules with immunoglobulin folds. RAGE is expressed in endothelial cells, mononuclear phagocytes (monocytes, macrophages and mesangial cells), neurons and muscle cells. The protein is expressed at a high level in the nervous system during development. Induced expression is frequently associated with pathological stages, such as diabetic endothelial damage and AD (8,9,19).

Proteins modified by AGEs may lose their normal functions. Studies have proposed that AGEs transmit their cell toxicity signals through RAGE. When AGEs are combined with RAGE, located at the cytomembrane of macrophages, these proteins may be degraded and cleared. Thus, the modification of proteins by AGEs is considered to be a signal participating in the procedure of rebuilding and clearing aging tissues. The production of AGEs is enhanced due to the glycometabolic disorder in patients with AD, while the clearance of AGEs is inhibited.

AGE formation is normally slow. In humans, AGEs normally accumulate with increasing age. In AD, AGEs accumulate on β-amyloid plaques near microglia and astrocytes. Aβ, derived by proteolytic cleavage of the amyloid precursor protein, is the major protein component of senile plaques. *In vitro* experiments have demonstrated that AGEs and Aβ, co-localized in the core of senile plaques, attract additional Aβ to form aggregates (20). Several studies have proposed that once AGEs have accumulated on β-amyloid plaques in the brains of patients with AD, they may aggravate Aβ-mediated oxidative stress, cell damage, functional loss and even neuronal cell death in the AD brain via RAGE-dependent mechanisms.

Several different theories have been proposed for the initial interaction between Aβ and the cell, as Aβ has a direct toxic effect on neuronal cells. RAGE has been demonstrated to be responsible for Aβ neurotoxicity (10). However, the mechanism whereby RAGE is able recognize AGEs, if these synthetic peptides are not glycated, remains unclear. In the present study, different concentrations of AGEs were added to cultures of Aβ-PC12 cells to determine whether RAGE was involved in Aβ toxicity. High concentrations of AGEs accelerated the Aβ toxicity in PC12 cells. The toxicity of AGEs and Aβ was decreased when the RAGE of the cultured cell was inactivated by treatment with trypsin. Glycated albumin bound to RAGE on the cell activated the Aβ toxic response and increased the toxicity induced by Aβ in neural cells. Inactivating RAGE may block neurocytotoxicity induced by Aβ. RAGE mRNA was detected by PCR analysis when AGEs were added to cultures of Aβ-PC12 cells. These results strongly suggested that RAGE was involved in mediating the toxic effect of AGEs and Aβ on nerve cells.

AGEs have been suggested to be inducers of chronic inflammation and acute-phase responses in a variety of diseases (4,11). However, this is not clear in brains of patients with AD. Recent studies have determined that microglia activated by AGEs are co-localized with AGE-modified β-amyloid plaques (21,22). The present study and previous studies have demonstrated that AGEs activate NF-κB and upregulate NF-κB mRNA expression in Aβ-PC12 cells, and that NF-κB mRNA expression increased with an elevation in AGE concentration.

Binding of Aβ to RAGE on neurons may cause cellular perturbation due to the induction of oxidative stress and the activation of the transcription factor, NF-κB. One of the consequences of this interaction is the production of microglia/macrophage growth factor and macrophage colony-stimulating factor (M-CSF) (23). The promoter of the RAGE gene contains two functional NF-κB binding sites, which provide a mechanism whereby RAGE activation by Aβ results in increased expression of the RAGE gene. Furthermore, RAGE-dependent NF-κB activation by Aβ has other pro-inflammatory effects. For example, RAGE-Aβ interactions lead to increased TNF-α secretion, and increased M-CSF and vascular cell adhesion molecule expression by neuroblastoma cells. Other RAGE ligands have also been determined to induce NF-κB activation and contribute to inflammatory responses (24).

In the present study, we inferred that an internal cycle between Aβ, AGEs, oxidative stress and NF-κB exists; and that this cycle may form a morbigenous network in AD pathogenesis. In our findings, Aβ cooperated with AGEs, resulting in concentration-dependent expression of NF-κB. However, the effects of AGEs and Aβ were no longer evident if RAGE was blocked by trypsin. This indicated that one of the pathways of cytotoxicity caused by Aβ was dependent on RAGE on the surface of neurons and glial cells for stimulating the release of reactive oxygen species (ROS), as well as the expression of NF-κB and the series of subsequent pathological processes.

Aβ may be involved in the etiology of AD through oxidative stress. Aβ generates free radicals in a metal-catalyzed reaction, which is able to induce neuronal cell death by a ROS-mediated process, and is able to damage neuronal membrane lipids, proteins and nucleic acids. Several studies have demonstrated that necrotic and apoptotic mechanisms are implicated in Aβ-mediated neurotoxicity (25). Apoptosis is induced by micromolar concentrations of Aβ in cultured neurons (26,27). Our experiment demonstrated that the rate of PC12 cell apoptosis was higher in the Aβ+AGEs groups than in the Aβ group, and that the apoptosis rate increased with increasing AGE concentration. Additionally, RAGE may be involved in the pathogenesis of AD cells as one of the surface receptors mediating apoptosis. Further research is required to clarify this mechanism.

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