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Original article

Amyloid beta clearance and microglia: Effects of glycative stress and melatonin

Yoshikazu Yonei [1)], Toshio Taira [2)], Shino Otaka [2)], Shizuko Sekiguchi [2)], ANM Mamun-or-Rashid [1)]
Masayuki Yagi [1)], Hiroaki Masuzaki [3)]

1) Anti-Aging Medical Research Center/Glycative Stress Research Center,
Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, Japan
2) Sapporo Division, Cosmo Bio Co., Ltd., Otaru, Hokkaido, Japan
3) Division of Endocrinology, Diabetes and Metabolism, Hematology and Rheumatology,
(Second Department of Internal Medicine), Graduate School of Medicine,
University of the Ryukyus, Nishihara, Okinawa, Japan

Abstract

The physiological role of amyloid-β (Aβ) is unknown, while it plays an important role in the onset and progression of Alzheimer's disease (AD). Aβ polymerization leads to enhanced neurotoxicity, persistent degradation, and deposition in the brain, resulting in decreased Aβ clearance. Diabetes mellitus and poor sleep quality are representative risk factors for the development of AD. Methylglyoxal (MGO) and acrolein are increased in diabetic patients, a representative disease with high glycative stress, and melatonin secretion is decreased during poor sleep quality. In this study, we focused on the Aβ phagocytosis of microglia, which plays a role in Aβ clearance, and examined the effects of Aβ glycation and melatonin. Glycated Aβ was prepared by MGO or acrolein treatment. Fluorescently labeled TAMRA-Aβ and primary rat microglial cells (Cosmo Bio) were used in the experiments. Several new findings were obtained from this experiment. First, microglia phagocytose Aβ, while their phagocytic capacity for glycated Aβ was markedly reduced. Second, Aβ phagocytosis was enhanced by melatonin. Concurrently, spontaneous death of cultured microglia was greater when Aβ was not added than when Aβ was. These findings suggest that prevention of Aβ glycation by countermeasures against glycative stress and prevention of AD progression by lifestyle, *i.e.*, improvement of sleep quality, are important, rather than elimination of Aβ as has been conventionally practiced.

***KEY WORDS*:** Alzheimer's disease, microglia, amyloid beta, methylglyoxal, acrolein, melatonin, sleep quality

Introduction

Amyloid beta (Aβ) is a type of protein produced in the brain. It is also present in the brain of healthy individuals, but its physiological role is not well understood. Aβ is normally degraded and eliminated in a short period of time as a relic in the brain (Aβ clearance). However, oxidative stress or glycation stress modifies the structure of Aβ, resulting in the formation of abnormal Aβ [1, 2]. In particular, when proteins form cross-links due to glycation stress, Aβ binds to each other and forms abnormal Aβ polymers. The formation of abnormal Aβ polymers causes persistent degradation, increased neurotoxicity, and decreased Aβ clearance. When the resulting Aβ is not excreted and is deposited in the brain, it forms Aβ senile plaques (amyloid pathology). Neurotoxic abnormal Aβ can also affect neurons when it clings to the periphery of previously healthy neurons. This triggers the formation of neurofibrillary tangles, in which tau protein, a microtubule-binding protein, fibrillates and aggregates in the cytoplasm. This results in gradual brain atrophy and progression of Alzheimer's disease. This is the concept of the "amyloid cascade hypothesis" with the addition of glycation stress [3-5].

To date, there have been no reports on the effects on microglial phagocytosis when Aβ is subjected to translational modifications. In this study, we focused on

Correspondence to: Professor Yoshikazu Yonei, MD, PhD
Anti-Aging Medical Research Center,
Graduate School of Life and Medical Sciences, Doshisha University
1-3, Tatara Miyakodani, Kyotanabe, Kyoto, 610-0394 Japan
TEL & FAX: +81-774-65-6394 e-mail: yyonei@mail.doshisha.ac.jp
Co-authors: Taira T, primarycelltaira@gmail.com;
Otaka S, shino-otaka@cosmobio.co.jp; Sekiguchi S, shizuko-sekiguchi@cosmobio.co.jp;
Mamun-or-Rashid ANM, mamunbtgeiu@gmail.com; Yagi M, myagi@mail.doshisha.ac.jp;
Masuzaki H, hiroaki@med.u-ryukyu.ac.jp

microglia (MG), which play an important role in the Aβ clearance, and examined the uptake of Aβ into cells and the change in uptake capacity when Aβ is subjected to glycative modification. Concurrently, we examined the effect of melatonin (Mel), which is involved in sleep quality.

Methods

Preparation of test reagents

Fluorescently labeled TAMRA-Aβ (Cosmo Bio, Tokyo, Japan) was used as the experimental reagent (hereinafter referred to as T-Aβ). This reagent consists of Aβ(1-42) labeled with red fluorescent carboxytetramethylrhodamine (TAMRA) via a PEG spacer [6].

Preparation of glycated T-Aβ with methylglyoxal (MGO)

0.5 mg of T-Aβ was dissolved in 50 µL of trifluoroacetic acid (TFA) (Nacalai Tesque, Kyoto, Japan) and then 450 µL of ultrapure water was added. 100 µL each was dispensed into 5 microtubes and concentrated and dried overnight using a centrifugal evaporator. After drying, 200 µL/tube of 0.2 M phosphate buffer (pH 6.0) was added to two of the five tubes to dissolve them, and they were combined in one tube to make 400 µL of T-Aβ solution. 80 µL/well of T-Aβ solution was dispensed into four wells in 96 well black plates and MGO (Fujifilm Wako Pure Chemicals, Osaka, Japan) was added to final concentrations of 0 mM (Blank), 0.4 mM, 2 mM, and 10 mM. After addition, the plates were placed in an incubator at 37 °C for glycation. Fluorescence values at the wavelengths of fluorescent end glycation end products AGEs (advanced glycation end products), (excitation: 340 nm, fluorescence: 430 nm), were measured by a microplate reader before and 1 hour, 17 hours, and 65 hours after the addition, as an index of glycation.

Since there was a possibility that the toxicity of residual MGO could adversely affect cells when glycated T-Aβ was added to cells, we performed two processes to remove MGO from the glycated T-Aβ solution: 1) crude purification by ether precipitation and 2) simple purification by reverse phase solid phase extraction.

1) Crude purification by ether precipitation

The 0.5 mg/mL T-Aβ solution glycated with 10 mM MGO was divided into microtubes in 30 µL portions, and 270 µL of TFA was added and stirred and mixed well. In addition, 1.5 mL of t-butyl methyl ether (Merck KGaA, Darmstadt, Germany) was added and the glycated T-Aβ was precipitated by standing at −80 °C for 3 hours and then at 4 °C overnight. The T-Aβ solution was centrifuged at 12,000 × g for 15 min to remove the supernatant containing residual MGO, 1 mL of t-butyl methyl ether was added again and stirred, then centrifuged at 12,000 × g for 15 min to remove the supernatant. After drying by nitrogen blowing, 500 µL of ACN was added, sonicated for 30 seconds, and 500 µL of 0.1% TFA was added to dissolve the glycated T-Aβ. The absorbance was measured at 556 nm using an ultra trace spectrophotometer. After measurement, the samples were lyophilized and stored under refrigeration.

2) Simple purification by reversed-phase solid-phase extraction

The SPE column (HyperSep™ C18 cartridge; Thermo Fisher Scientific, Tokyo, Japan) was conditioned by washing with 0.75 mL of 95% ACN/0.1% TFA and equilibrated three times with 2.25 mL of 0.1% TFA. ACN was obtained from Merck KgaA. 40 µL of 0.5 mg/mL T-Aβ solution glycated with 2 mM MGO and 100 µL of 0.1% TFA were applied to the SPE column, washed 5 times with 2.25 mL of 0.1% TFA and once more with 0.35 mL of 95% ACN/0.1% TFA. After washing, glycated T-Aβ was eluted with 0.6 mL of 95% ACN/ 0.1% TFA, and the eluate was collected. To the collected eluate, 0.6 mL of 0.1% TFA was added, and the absorbance at 556 nm was measured using an ultra-trace spectrophotometer. Then, the samples were lyophilized and stored under refrigeration.

Preparation of glycated T-Aβ with acrolein

0.5 mg of T-Aβ was dissolved in 50 µL of TFA and then 450 µL of ultrapure water was added; 100 µL each was dispensed into 5 microtubes and concentrated and dried overnight using a centrifugal evaporator. After drying, 200 µL/tube of 0.2 M phosphate buffer (pH 6.0) was added to two of the five tubes to dissolve and combined in one tube to make 400 µL of T-Aβ solution. T-Aβ solution was dispensed in 4 wells at 80 µL/well into 96 well black plates, and acrolein (Fujifilm Wako Pure Chemicals) was added to final concentrations of 0 mM (Blank), 0.4 mM, 2 mM and 10 mM. The plates were placed in an incubator at 37 °C for glycation. Fluorescence values at the wavelengths of fluorescent AGEs (excitation: 340 nm, fluorescence: 430 nm) were measured in a microplate reader before addition, 14 hours after addition, and 62 hours after addition, and were used as indicators of glycation.

MG Primary Cultured Cells

Cultured microglial cell strains that have been used for experiments generally exhibit macrophage-like properties, and their functions, *i.e.*, response to inflammation and neuroprotection, are distinct from those of microglia *in vivo*. Whereas, primary microglia are prepared from the neonatal brain, and are grown in a mixed culture system that includes microglia and other cells, mainly astrocytes and neurocytes, thereby maintaining the original functions of microglia in part [7]. In this study, we used a primary microglia culture kit (Cosmo Bio) to verify the function of microglia in recognizing and eliminating Aβ [8].

Effect of T-Aβ and glycated T-Aβ on primary microglial cells

Floating MG were collected from one 75 cm^2 flask of mixed culture of primary MG prepared from SD rat brain according to protocol [8] using the medium supplied with the kit and seeded onto 8 wells of 96 well black plates (the number of seedings was unknown because the number of seedings was not countable). Since MG cell numbers are reduced by adhesion to centrifuge tubes, measures were taken to prevent excessive adhesion by using instrumentation

coated with a macrophage anti-adhesion coating agent. Non-glycated T-Aβ and MGO-glycated T-Aβ were added to the medium at a final concentration of 1 μM the day after seeding, and the cells were exposed for 4 days to observe T-Aβ uptake into the cells by fluorescence microscopy.

After observation, the cells were washed twice with medium to remove T-Aβ and glycated T-Aβ. Changes in T-Aβ uptake into cells were observed on day one (culture day 5) and day 4 (culture day 8) after removal, and on day 8 (culture day 12), 1/10 of the medium was added with a cell proliferation/cytotoxicity assay reagent according to protocol [9], and a color reaction was performed in a 37 °C incubator for 2 hours. Cytotoxicity was evaluated by measuring absorbance at 450 nm (reference: 650 nm) in a microplate reader.

Composition of the experimental group

The composition of the experimental group is shown below.

	T-Aβ	Mel	Glycation
a) Non-T-Aβ group (no treatment)	–	–	–
b) T-Aβ group	+	–	–
c) T-Aβ + Mel group	+	+	–
d) Gly-T-Aβ group	+	–	+
e) Gly-T-Aβ + Mel group	+	+	+

Note that there are two types of glycated T-Aβ: MGO-treated and acrolein-treated. The final concentration of Mel (melatonin) in the culture medium was 100 μM. In this study, two MG seeding experiments were performed.

The first experiment was conducted as a, b, c, d, and e above, with MGO-treated Gly-T-Aβ used for d and e. In the second experiment, a, b, and d were used, and MGO-treated and acrolein-treated Gly-T-Aβ were used in d. Since the MG culture conditions in the two experiments were different, the analyses were performed separately, and no integrated analysis of T-Aβ phagocytosis was performed.

Statistical analysis

MG cell counts were measured as the number of nuclei per unit area using an inverted phase-contrast fluorescence microscope (BZ-X710, Keyence, Tokyo, Japan) with an image analyzer (BZ-H3CM). Analysis of T-Aβ phagocytosis by MG was performed on the area of T-Aβ-derived fluorescence (area of fluorescence intensity above the reference value) divided by the total cell area (μm^2). The figure showed the value adjusted for the area of nonspecific background fluorescence (nonspecific high intensity area in the non-T-Aβ group). Statistical analysis was performed using Excel statistics (Bell Curve, Tokyo, Japan) and evaluated by analysis of variance (ANOVA) followed by Fisher's post hoc test.

Results

Generation of glycated Aβ

During MGO treatment of Aβ, fluorescent AGEs tended to increase with increasing MGO concentration and glycation time (***Fig. 1***). 65 h after addition of 10 mM MGO, the fluorescence value decreased from 17 h. Therefore, the reaction time of one day and night at 10 mM was adopted for glycation conditions.

Similarly, during acrolein treatment, fluorescent AGEs tended to increase with increasing acrolein concentration and glycation time (***Fig. 2***). Since fluorescence values were lower at 62 h after acrolein addition than at 14 h, a reaction time of one day and night at 10 mM was adopted for glycation conditions.

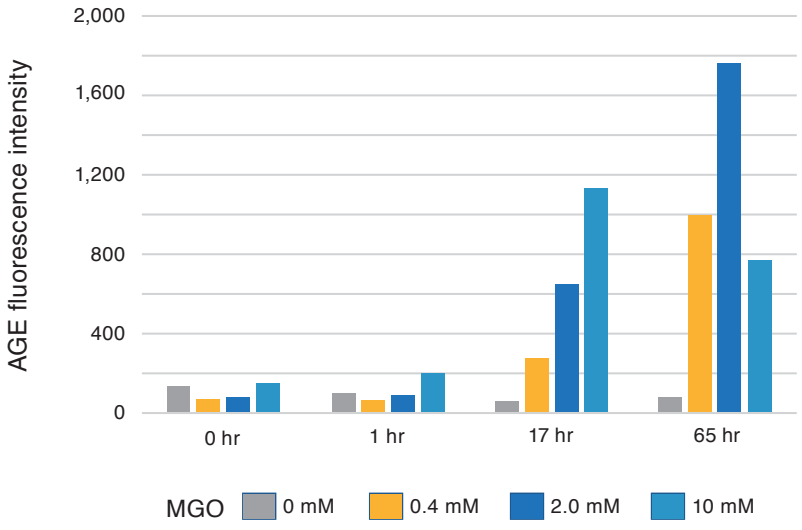

Fig. 1. **Glycation of T-Aβ by MGO treatment.**
Results are expressed as mean of duplicate values. Fluorescent intensity derived from AGEs were measured by Ex 340 nm/Em 430 nm. MGO concentration; 0 mM, 0.4 mM, 2.0 mM, 10 mM. T-Aβ, TAMRA-β-amyloid(1-42); MGO, methylglyoxal; AGEs, advanced glycation end products.

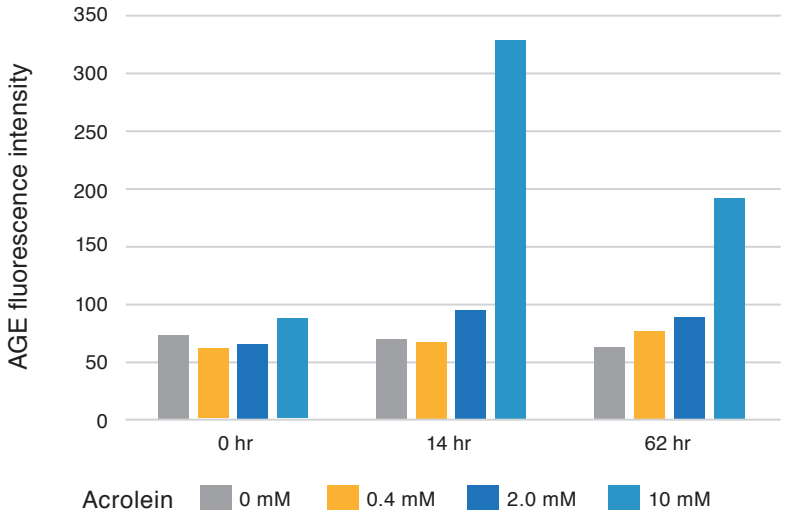

Fig. 2. **Glycation of T-Aβ by acrolein treatment.**
Results are expressed as mean of duplicate values. Fluorescent intensity derived from AGEs were measured by Ex 340 nm/Em 430 nm. Acrolein concentration; 0 mM, 0.4 mM, 2.0 mM, 10 mM. T-Aβ, TAMRA-β-amyloid(1-42); AGEs, advanced glycation end products.

First culture experiment

MG cell count and Aβ phagocytosis

Cultures were fluorescently labeled T-Aβ added at day 2 after MG seeding and observed on day 10.

In the non-T-Aβ group, MG cell counts showed a decrease in the number of viable cells due to cell death on day 10 compared to day 2, experimental start date (***Fig. 3-a***, no photo on experimental start date). The cause of cell death was determined to be spontaneous death.

In the T-Aβ group, MG cell counts showed no decrease on day 10 compared to day 2 (***Fig. 3-b***); MG cell counts on day 10 were higher and spontaneous death was lower than in the non-T-Aβ group. T-Aβ labeled fluorescence was seen to be incorporated into the MG cytoplasm, confirming that Aβ is phagocytosed (taken into the cell) by MG. No specific morphological changes were observed.

In the T-Aβ + Mel group, the number of MG cells increased on day 10 compared to day 2 (***Fig. 3-c***) MG cell numbers were higher than in the T-Aβ group without Mel. It was not possible to determine whether there was less spontaneous death or enhanced MG proliferation. In the T-Aβ + Mel group, fluorescent uptake of MG cells was prominent and Aβ phagocytosis by MG was more active. No specific morphological changes were observed.

In contrast, in the MGO-treated Gly-T-Aβ group, the number of MG cells increased on day 10 compared to day 2 (***Fig. 3-d***). Little fluorescent uptake of MG cells was observed. No specific morphological changes were observed.

In the Gly-T-Aβ + Mel group, MG cell counts were similar on day 10 compared to day 2 (***Fig. 3-e***), and MG cell counts on day 10 were lower than those in the Mel-free Gly-T-Aβ group. The fluorescent uptake of MG cells was slight.

The image data of T-Aβ phagocytosis by MG was quantitatively evaluated by image analysis (***Fig. 4***), and the image data of the T-Aβ + Mel group showed a markedly higher trend intensity compared to the T-Aβ group, indicating that T-Aβ phagocytosis was increased by Mel. However, two of the three T-Aβ + Mel group data values were excluded from the analysis because of their abnormally high uptake fluorescence brightness; values that were not excluded are shown in the figure. Compared to the T-Aβ group, the fluorescent brightness in MG of the Gly- T-Aβ group was significantly lower ($p = 0.03$), indicating reduced phagocytosis.

Second culture experiment

Comparison of Aβ phagocytosis with MGO and acrolein

Fluorescently labeled T-Aβ was added at 2 days after MG seeding, and fluorescence microscopy images at 10 days are shown in ***Fig. 5***.

In the T-Aβ group, labeled fluorescence was seen in the MG cytoplasm, reconfirming that Aβ is phagocytosed by MG (***Fig. 5-b***). Whereas, in the MGO- and acrolein-treated Gly-T-Aβ groups, little labeled fluorescence was seen in the MG, and glycosylated Aβ phagocytosis by MG was virtually absent (***Fig. 5-c, d***). There was no difference between the MGO- and acrolein-treated groups.

Cell counts on day 10 of culture are shown in ***Fig. 6***; there were no significant differences in cell counts among the Non-T-Aβ, MGO-treated Gly-T-Aβ, acrolein-treated Gly-T-Aβ, and T-Aβ groups.

The results of Aβ phagocytosis by MG are summarized in ***Fig. 7***. When MG phagocytosis of T-Aβ was compared between the T-Aβ and Gly-T-Aβ groups, phagocytosis of glycated T-Aβ was markedly reduced ($p < 0.001$), with no difference between MGO and acrolein treatments.

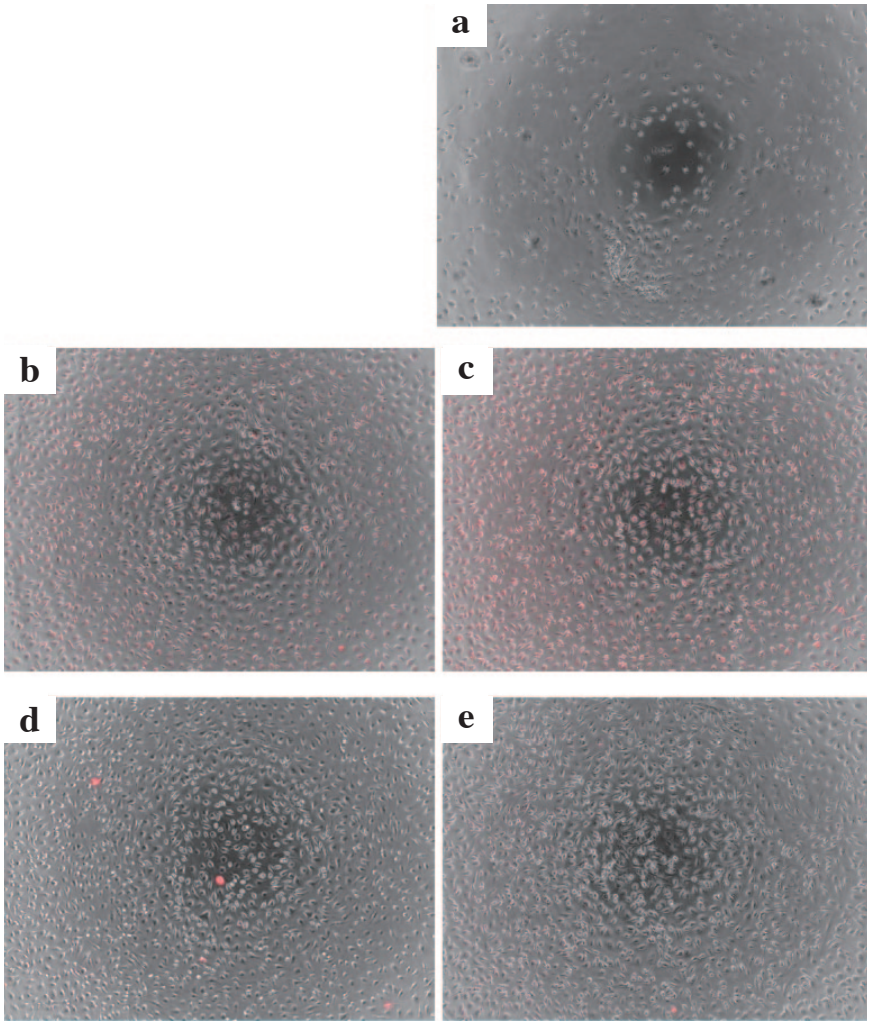

Fig. 3.

T-Aβ phagocytosis by microglia: Fluorescence microscopy image

a) Non-T-Aβ group (no treatment), **b)** T-Aβ group, **c)** T-Aβ + Mel group, **d)** Gly-T-Aβ group, **e)** Gly-T-Aβ + Mel group. Images (x 100) of cultured cells 10 days after seeding (8 days after addition of T-Aβ). Red color portions indicate T-Aβ-derived fluorescence uptaken by microglia. Note the enhanced fluorescence by Mel **(c)** compared with the T-Aβ group **(b)**. Gly-T-Aβ is prepared by MGO treatment (10 mM, one day). T-Aβ, TAMRA-β-amyloid(1-42); MGO, methylglyoxal; Mel, melatonin; Gly, glycated.

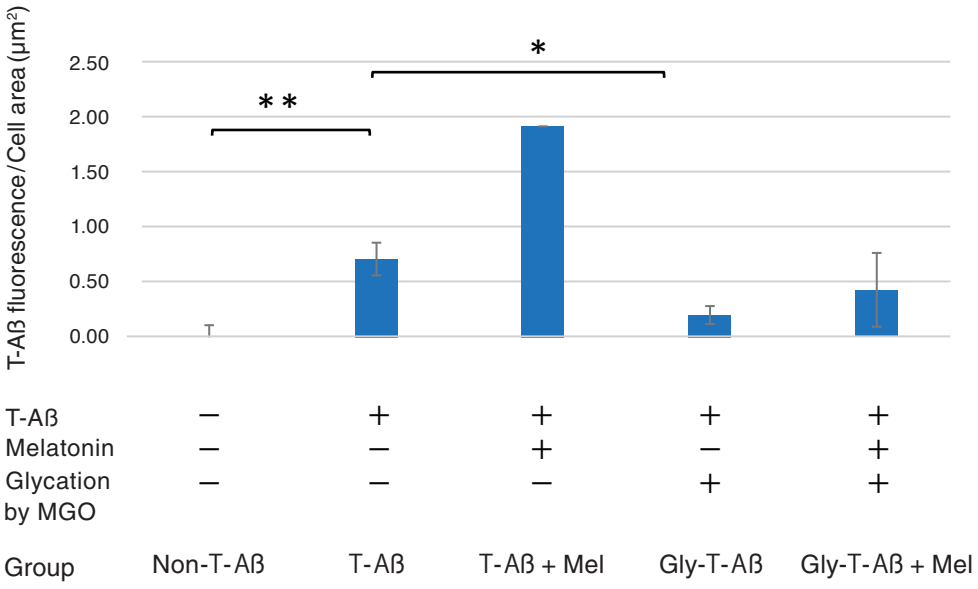

Fig. 4. **Comparison of T-Aβ phagocytosis by MG: The first experiment**

Y axis shows the values: T-Aβ fluorescence intensity / cell area (µm²) followed by adjustment for the area of nonspecific background fluorescence (nonspecific high intensity area in the non-T-Aβ group). Results are expressed as mean ± SD of triplet values (n = 4 except Non-T-Aβ group and T-Aβ + Mel group, n = 2), * $p < 0.05$, ** $p < 0.01$ by Fisher's post hoc test (T-Aβ + Mel group excluded). T-Aβ phagocytosis is inhibited by MGO-glycation while enhanced by Mel (100 µM). Gly-T-Aβ is prepared by MGO treatment (10 mM, one day). MG, microglia; T-Aβ, TAMRA-β-amyloid(1-42); MGO, methylglyoxal; Mel, melatonin; Gly, glycated; SD, standard deviation.

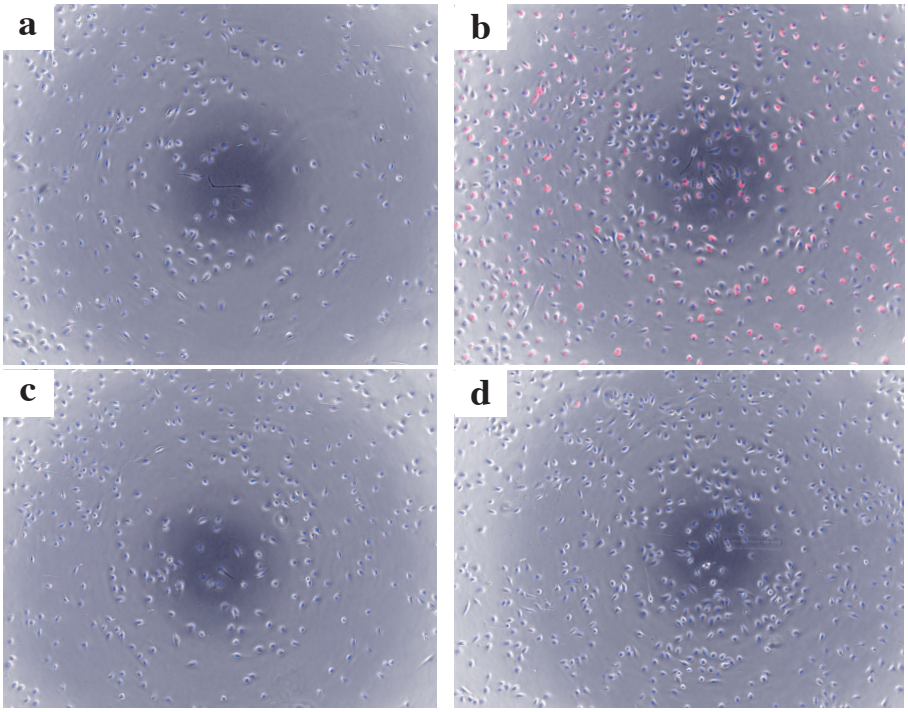

Fig. 5. **T-Aβ phagocytosis by MG: Comparison between MGO and acrolein treatment.**

a) Non-T-Aβ group (no treatment), **b)** T-Aβ group, **c)** MGO-Gly-T-Aβ group, **d)** Acrolein-Gly-T-Aβ group. Images (x 400) of cultured cells 10 days after seeding (8 days after addition of T-Aβ). Red color portions indicate T-Aβ-derived fluorescence uptaken by MG. No difference between MGO **(c)** and acrolein **(d)** treatment. Note the much less fluorescence in **c)** and **d)** than that in **b)**. Gly-T-Aβ is prepared by MGO or acrolein treatment (10 mM, one day). MG, microglia; T-Aβ, TAMRA-β-amyloid(1-42); MGO, methylglyoxal; Gly, glycated.

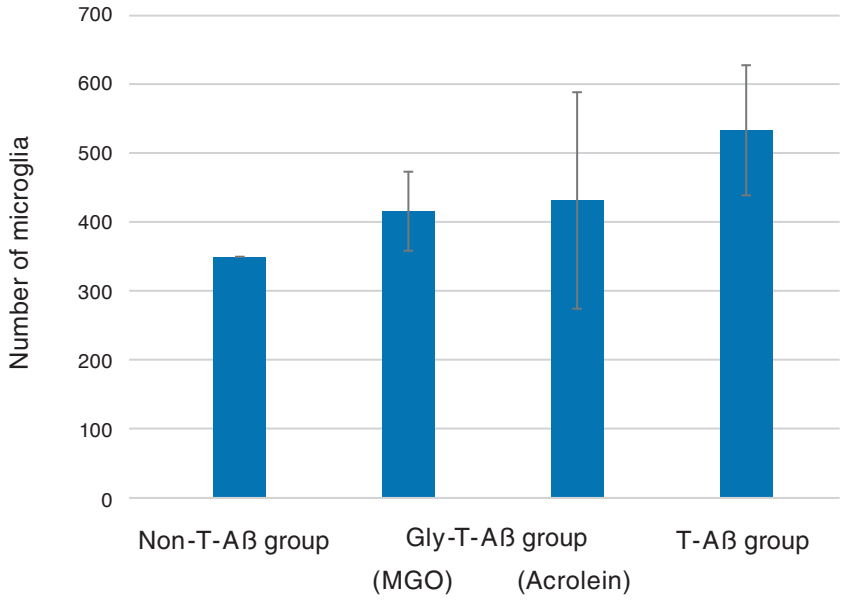

Fig. 6. **Live cell counts of MG 10 days after seeding.**

Results are expressed as mean ± SD of triplet values, counted the number of nuclei by image analyzer (n = 4, except Non-T-Aβ group, n = 2). Gly-T-Aβ is prepared by MGO or acrolein treatment (10 mM, one day). MG, microglia; T-Aβ, TAMRA-β-amyloid(1-42); MGO, methylglyoxal; Gly, glycated; SD, standard deviation.

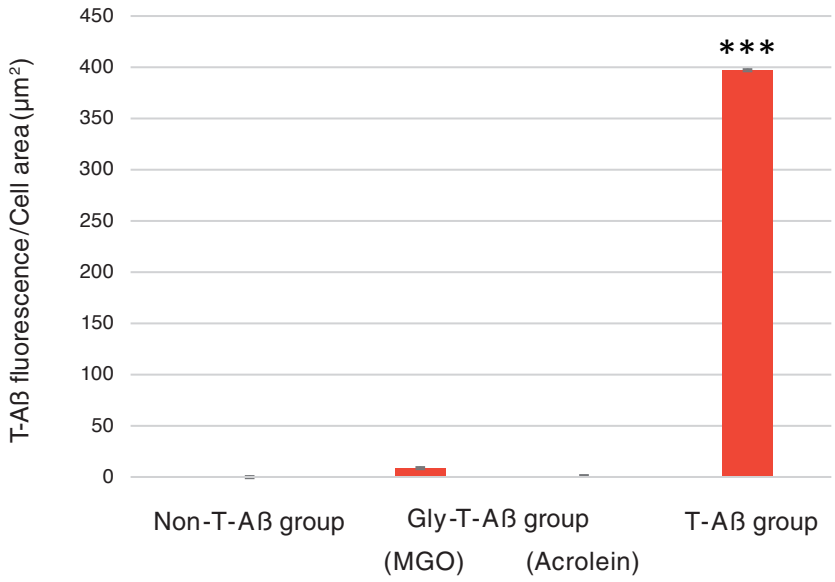

Fig. 7. **Comparison of T-Aβ phagocytosis by MG: The second experiment**

Y axis shows the values: T-Aβ fluorescence intensity/cell area (μm²). Results are expressed as mean ± SD, n = 3, *** $p < 0.001$ vs other groups, by Fisher's post hoc test. T-Aβ phagocytosis is inhibited by MGO- and acrolein-treatment (10 mM, one day). MG, microglia; T-Aβ, TAMRA-β-amyloid(1-42); MGO, methylglyoxal; Gly, glycated; SD, standard deviation.

Discussion

Alzheimer's disease (AD) is a leading cause of progressive dementia; autopsy brains of AD patients have been found to have an accumulation of activated MG around senile plaques, suggesting the involvement of impaired Aβ clearance. However, the reason for the presence of Aβ in the healthy state and its physiological role are not clear.

Aβ is produced in vivo under physiological conditions. γ-secretase is a membrane protein complex composed of presenilin, nicastrin, Aph-1, and Pen-2 with an active center. The substrate (APP or Notch) is cleaved by γ-secretase after cleavage at the lumenal side, and the resulting Aβ is secreted extracellularly [3,4]. γ-secretase is involved in carboxy-terminal cleavage in the Aβ production pathway and determines the amount of Aβ and its aggregation. Therefore, it is considered an important AD drug target molecule, and various small molecule compounds have been developed as γ-secretase inhibitors. However, therapies that inhibit Aβ production have repeatedly failed in clinical trials, suggesting that Aβ has some physiological significance and that lowering Aβ levels in the brain may not lead to AD treatment [5]. Aβ production occurs regardless of age or presence of AD, and in the normal brain, Aβ is properly removed and does not often accumulate in the brain [10,11].

These findings indicate that homeostasis of Aβ clearance is important for controlling AD progression. Aβ deposition, which is pathognomonic at autopsy, reflects the balance of Aβ production, deposition, and removal at that time. Activated MG accumulate around senile plaques and play an important role in Aβ clearance. Phagocytosis of Aβ by cultured rat MG is regulated by the low molecular weight G protein Rac1 and the Wiskott-Aldrich syndrome protein family verprolinhomologous protein (WAVE) that acts downstream actin fiber reorganization [12].

When Aβ is taken up by MG, it is processed intracellularly and the N-terminal fragment is lost [11]. Activated MG in advanced AD model mice express the nicotinic acetylcholine receptor α7 nAChR, and CD14/TLR complexes are expressed to perform clearance functions [13]. Regarding the morphology of MG, it has been reported that the morphology of MG prepared from rat neonates before Aβ42 treatment was rod-type, but immediately after treatment, they changed their morphology to ameboid-type and began phagocytosis [14]. The morphology of the cultured MG used in this experiment was ameboid type from the second day of culture.

Factors affecting Aβ clearance

There are few reports on factors affecting Aβ clearance in MG. Galantamine, an acetylcholinesterase (AChE) inhibitor, binds to microglial nAChR as a ligand, promoting the expression of the CD14/TLR complex and facilitating Aβ clearance [15].

Microglial Aβ phagocytosis is enhanced by heat shock proteins (Hsp) and, conversely, inhibited by high mobility groupbox protein-1 (HMGB 1), a known nuclear protein [16]. Rutin (quercetin-3-rutinoside) is a natural flavonoid with antioxidant and AGE formation inhibitory properties. It has been suggested that sodium rutin (NaR) may increase MG and promote Aβ clearance by increasing the expression level of phagocytosis-related receptors in MG [17]. With the results

of this experiment, Mel was newly added as a factor that promotes microglial Aβ phagocytosis. Since Mel secretion increases as "sleep quality" improves, it is important to aim for good sleep to maintain Aβ clearance homeostasis in order to prevent the progression of AD.

In an experiment comparing mouse brain MG divided into two groups, a subset that produces high levels of reactive oxygen species (ROS) and a subset that does not produce ROS, the former showed neurotoxicity and the latter neuroprotection when co-cultured with neurons[18]. In culture experiments in which HIV-1 Nef protein was introduced to increase NADPH oxidase activity, neuroprotective microglial clones were transformed into toxic microglial clones. These findings suggest that MG may change from a neuroprotective subset to a neurotoxic subset in response to the environment. It is a subject for future study how MG in contact with Aβ and glycated Aβ undergo changes and whether there are differences between the two types of Aβ.

Involvement of Aldehydes in AD

Diabetes mellitus, a typical disease with high glycation stress, is said to be the source of all diseases and increases the risk of developing various age-related diseases. The fundamental factor is the tendency to produce aldehydes (compounds with -CHO groups), open-ring glucose and open-ring fructose, as well as lipid peroxidation products such as 4-hydroxynonenal (HNE), acrolein, MGO, glyoxal (GO), glyceraldehyde, 3 deoxyglucosone (3DG) are increased. As a carbonyl stress, these aldehydes induce protein carbonylation and further produce AGEs, which contribute to neurodegeneration in AD[19-22]. Aβ amyloid protein glycation by aldehydes is a potentially toxic morphological change with enhanced toxicity[23].

AGE formation by Aβ glycation alters Aβ aggregation capacity[24]. Aβ glycation meaningfully delays β conversion to mature fibers, and toxic forms. *i.e.,* oligomers, are more likely to remain[25,26]. In the central nervous system, glycation of Aβ as well as tau protein and α-synuclein is thought to be involved in the onset and progression of cognitive impairment.

MGO increases the size of Aβ aggregates[26]; MGO concentrations in cerebrospinal fluid (CSF) of AD patients are higher than in healthy subjects[27] and MGO-derived hydroimidazolone compounds are more abundant[28]. Regarding the development of cognitive impairment, it has been reported that higher serum MGO levels are associated with greater cognitive decline[29].

Acrolein, on the other hand, is produced by lipid peroxidation and polyamine metabolism and is highly nucleophilic and reactive. Acrolein undergoes Michael addition reactions with nucleophilic amino acids, *i.e.,* cysteine, lysine, and histidine, to induce protein carbonylation. Oral administration of acrolein to rats (2.5 mg/kg/day, for 8 weeks) resulted in cognitive decline and hippocampal atrophy[30]. Clinically, acrolein is increased in the hippocampus and temporal lobe of AD patients[31] and acrolein-conjugated protein in plasma and spinal fluid is higher than in normal subjects[32,33]. It is assumed that acrolein is involved in the onset and progression of AD[34].

There are only a limited number of studies on the effects of Aβ morphological changes; glycated Aβ formed by MGO has been reported to be highly toxic, inducing increased RAGE expression and glycogen synthase kinase-3 (GSK-3) activation[35]. Therefore, in this study, we prepared glycated Aβ by MGO and acrolein and verified the effect on microglial phagocytosis, and found that phagocytosis was decreased for glycated Aβ.

Involvement of Mel

Mel is a hormone secreted by the pineal gland that has effects of antioxidation[36] and glucose metabolism homeostasis[37]. Concurrently, poor sleep quality and circadian rhythm disturbances are known to increase the risk of developing AD[38], and Mel has attracted attention as a factor linking these events[39]. Mel acts in an inhibitory manner on amyloid precursor protein (APP) processing and Aβ generation by modulating the regulatory network of secretase expression[40].

Mel inhibits Aβ aggregation[41-44], ameliorates neurotoxicity[45,46], improves lymphatic drainage through lymphatic vessels[47], and promotes Aβ clearance through BBB transport and degradation pathways.

Since Aβ40 is highly aggregative and is easily deposited in the brain, and Aβ42 is easily excreted, the plasma Aβ40/42 ratio is significant as an indicator of impaired Aβ clearance, and a high ratio is associated with a high risk of developing AD[48]. We have reported that Aβ40/42 increases with age[49] and that Aβ40/42 is higher in populations with poor sleep quality[50]. This finding suggests that Aβ clearance may be decreased in populations with low Mel secretion, which is compatible with the results of the present study.

Mel also has a positive effect on memory[51,52]. In OXYS rats, which exhibit impaired Mel secretion, Mel administration suppresses increased anxiety and deterioration of reference memory[53]. Mel has also been shown to improve memory function in D-galactose-treated mice by suppressing the elevation of ROS and RAGE (receptor for advanced glycation end products)[51]. It was reported that Mel crosses the blood-brain barrier and is converted to N1-acetyl-5-methoxykynuramine (AMK) in brain tissue, which promotes long-term object memory in mice[54].

When Aβ is taken up by neurons, the ionic gradient in the inner mitochondrial membrane is altered, inducing mitochondrial damage and reducing respiratory control ratio and ATPase hydrolytic activity[55]. Furthermore, mitophagy is impaired, resulting in mitochondrial repair[56]. In AD, mitochondrial aldehyde dehydrogenase (ALDH2)[57] and RAGE[51] have been implicated in mitochondrial and mitophagic disturbances.

We have studied Mel from the viewpoint of underlying glycation stress, and reported that Mel suppresses postprandial hyperglycemia (blood glucose spike)[58] and promotes AGEs degradation[59]. Furthermore, in the present study, we found that Mel promotes microglial Aβ phagocytosis. These findings indicate that improving sleep quality and enhancing one's own Mel secretion are important for primary prevention in order to delay the progression of AD as much as possible, which may be called a paradigm shift in AD prevention measures.

Conclusion

MG have previously been shown to play an important role in brain homeostasis as cells involved in the regulation of synaptic function, removal of foreign substances from the brain, or immunity. The present experiments provide several new findings. First, MG not only phagocytose and degrade Aβ, but also act more protectively toward MG than without Aβ, suggesting that Aβ may contribute to microglial homeostasis. Second, Aβ phagocytosis by MG is facilitated by Mel. This is consistent with epidemiological findings that AD incidence is higher in individuals with poor "sleep quality" who have decreased Mel secretion. Third, MG may have difficulty phagocytosing glycated Aβ, resulting in decreased Aβ clearance. This is consistent with the epidemiological finding of a higher incidence of AD in diabetic patients with high glycation stress and increased glycated Aβ. Based on the above findings, it is considered important to prevent the progression of AD by preventing Aβ glycation through measures against glycative stress and lifestyle habits, i.e., improving sleep quality, rather than by eliminating Aβ as has been done in the past. From the viewpoint of new drug discovery, it is expected to search for substances that process glycation-modified Aβ accumulated in the brain and substances that promote phagocytosis of MG.

Conflict of Interest Statement

The authors claim no conflict of interest in this study.

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