Protective effect of ISO-1 against advanced glycation end product aggravation of PC12 cell injury induced by Aβ1-40

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Abstract. Advanced glycation end products (AGEs) are important pathogenic substances involved in diabetes mellitus (DM) and its complications. AGEs also serve important roles in promoting the development of Alzheimer’s disease (AD). Macrophage migration inhibitory factor (MIF), an inflammatory stimulant and a pathogenic factor involved in DM, was previously reported to be present at increased levels in the cerebrospinal fluid of patients with AD and mild cognitive impairment compared with age-matched healthy controls. By investigating the association between AGEs and MIF, and the effects of neuroinflammation on AD, the present study aimed to increase understanding of the specific molecular mechanisms involved in the pathogenesis of DM and AD, and the connection between these diseases. PC12 cells were cultured in vitro; the levels of MIF mRNA and protein were determined using reverse transcription-quantitative (RT-q)PCR and western blot analyses. The optimal concentrations of AGEs and amyloid β 1-40 (Aβ1-40) were also determined in the cell model of AD using Cell Counting Kit-8 and MTT assays. Cell numbers and morphological changes were observed following the treatment of Aβ1-40-stimulated PC12 cells with AGEs and the MIF inhibitor (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1). The mRNA expression levels of interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α) and MIF were determined via RT-qPCR analysis. The results showed that the levels of MIF mRNA and protein were significantly increased in cells treated with AGEs compared with the control group. In the AD model group, the inhibition of PC12 cell growth was significantly increased, and the mRNA expression levels of IL-1β, IL-6, TNF-α and MIF were also increased. Compared with treatment with AGEs alone, the combination of AGEs treatment with ISO-1 significantly improved the survival rate and resulted in the reduced expression of inflammatory mediators in the AD cell model. Thus, ISO-1 reduced AGEs-mediated damage in the AD cell model. This may be a consequence of AGEs-mediated MIF expression promoting neuritis in the AD cell model, whereas ISO-1 decreased the expression of neuroinflammatory mediators.

Introduction

Alzheimer’s disease (AD) is the most common neurodegenerative disorder of the central nervous system. Worldwide, >46.8 million patients have been diagnosed with AD, with 50% of those patients >85 years of age. The number of individuals with AD is expected to double by 2030 (1-3). Previous studies have reported a correlation between diabetes mellitus (DM) and the risk of developing AD (4,5). Certain scholars have even suggested that AD should be considered as a third type of DM (6-9).

Advanced glycation end products (AGEs) affect the functions of normal tissues through different pathways and play important roles in the occurrence and development of various diseases, including diabetic complications, osteoporosis, AD, tumorigenesis, aging and cardiovascular disease (10-13). AGEs can induce apoptosis in neurons, leading to the early onset of AD (14). Additionally, substantial quantities of carboxymethyllysine, pyrraline and pentosidine have been detected in the senile plaques and neurofibrillary tangles (NFTs) of patients with AD; these AGEs may promote the development of AD by affecting the metabolism of amyloid β (Aβ) (15).

Macrophage migration inhibitory factor (MIF) is a cytokine involved in the occurrence of various inflammatory and immune diseases (16,17). The inhibitor of MIF, (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1), can significantly inhibit the activity of MIF and affect its pathophysiological functions (18). MIF activity is one of the risk factors for type I diabetes and type II diabetes (T2D) (17,19). A previous clinical study by Bacher et al (20) reported significantly increased MIF levels in the cerebrospinal fluid of patients with AD and mild cognitive...
impairment (MCI) compared with controls of the same age. Oyama et al. (21) reported that MIF bound to Aβ in the brains of patients with AD, and thus, the toxicity of Aβ was directly attributed to the upregulation of MIF expression. MIF deficiency was also reported to attenuate the hyperphosphorylation of tau proteins (22).

Previous studies have identified a close link between DM and AD (4,5); however, the exact molecular link between these two diseases remains unclear. AGEs promote the deposition of Aβ and the hyperphosphorylation of tau protein (14,15). Whether MIF also serves a role in the AGEs-induced development of AD requires further investigation. The present study investigated the neuroinflammatory mechanisms underlying the pathogenesis of AD and assessed the effect of the pathogenic factor MIF on AD. It was hypothesized that AGEs would affect the levels of neuroinflammation observed in AD by inducing MIF expression, and that the levels of neuroinflammation could be reduced using ISO-1.

Materials and methods

Cell culture and treatment. PC12 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences. PC12 cells were cultured in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (Zhejiang Tianhang Biotechnology Co., Ltd.) at 37˚C in a humidified atmosphere of 5% CO2/95% air. PC12 cells in the logarithmic phase of growth were plated in 6-well plates at the density of 5x10^5/ml and divided into three treatment groups: A blank control group, a BSA (300 µg/ml; Beijing ComWin Biotech Co., Ltd.) control group and an AGES (Biorbyt, Ltd.) group. The AGES groups were treated with different concentrations (100, 200 or 300 µg/ml) of AGES for 24 h at 37˚C, and then subsequent assays were performed. As AGES were prepared in a non-enzymatic glycosylation reaction using BSA as a substrate, BSA (300 µg/ml) was also used as a control.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from cells using TRIZol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the aforementioned treatments, according to the manufacturer's protocol. For mRNA analysis, cDNAs were synthesized using a TIANScript First Strand cDNA Synthesis kit (Tiangen Biotech Co., Ltd.) control group and an AGES (Biorbyt, Ltd.) group. The AGES groups were treated with different concentrations (100, 200 or 300 µg/ml) of AGES for 24 h at 37˚C, and then subsequent assays were performed. As AGES were prepared in a non-enzymatic glycosylation reaction using BSA and as a substrate, BSA (300 µg/ml) was also used as a control.

Screening for the optimum concentration of AGES and Aβ1-40 in the AD cell model. PC12 cells were plated in 96-well plates at the density of 5x10^3/ml, and the cells were exposed to various concentrations of AGES (100-400 µg/ml) at 37˚C for 24 h, with 5 replicates/group; equivalent concentrations were applied to the BSA control group. All groups were treated with 10 µl of Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc.) and incubated for 1-4 h at 37˚C before the supernatant was removed and the protein concentration was determined using the bicinchoninic acid method. Total protein (50 µg) was separated on 12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% dried skimmed milk/TBS 0.5% Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated overnight at 4˚C with a monoclonal rabbit anti-MIF antibody (1:100; cat. no. ab172730; Abcam) or anti-GAPDH antibody (1:1,000; cat. no. CW0101S; Beijing ComWin Biotech Co., Ltd.), and then washed three times with TBST for 10 min each. Subsequently, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:10,000; cat. no. D110058; BBI Life Sciences Corporation) secondary antibody was incubated with the membranes for 1 h at room temperature, which were then washed three times with TBST for 10 min each. The bands were visualized with an ECL reagent (Tanon Science and Technology Co., Ltd.) and detected with the Tanon Imaging System (Tanon Science and Technology Co., Ltd.). The relative intensity of the protein bands was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.). Experiments were repeated three times.

Western blot analysis. Protein levels in PC12 cells were determined after treatment using western blot analysis. Cells were lysed with RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Samples were incubated on ice for 30 min and then centrifuged at 16,000 x g for 15 min at 4˚C. The supernatant was removed and the protein concentration was determined using the bicinchoninic acid method. Total protein (50 µg) was separated on 12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% dried skimmed milk/TBS 0.5% Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated overnight at 4˚C with a monoclonal rabbit anti-MIF antibody (1:100; cat. no. ab172730; Abcam) or anti-GAPDH antibody (1:1,000; cat. no. CW0101S; Beijing ComWin Biotech Co., Ltd.), and then washed three times with TBST for 10 min each. The bands were visualized with an ECL reagent (Tanon Science and Technology Co., Ltd.) and detected with the Tanon Imaging System (Tanon Science and Technology Co., Ltd.). The relative intensity of the protein bands was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.). Experiments were repeated three times.

Table I. Oligonucleotide primer sets used for reverse transcription-quantitative PCR.

| Name  | Sequence (5'-3') | Length (bp) |
|-------|-----------------|-------------|
| MIF   | F: TACGACATGAAGTCAGCAAGCG | 22 |
|       | R: GAACAGCGGTCGAGTAAGTG | 23 |
| IL-1β | F: ATCTCAGACGCAATCTCGAAG | 24 |
|       | R: CAACATACGGCTCGATCATCC | 24 |
| IL-6  | F: ATGATGAGAAACAGCCACAATTG | 22 |
|       | R: GCCCGGCTCCCTTCTACGAG | 22 |
| TNF-α | F: TTGGGTTATGGCAAGAGTGTTG | 22 |
|       | R: GCTGTGTACGGCTTTTCTCAA | 22 |
| GAPDH | F: ACGGCAAGTTCACGCCGACAG | 21 |
|       | R: CGACATACTCGCAGGACCATC | 24 |

F, forward; R, reverse; MIF, macrophage migration inhibitory factor; IL, interleukin; TNF-α, tumor necrosis factor-α.
were plated in 96-well plates at the density of 5x10⁵/ml. The blank control group was treated with 100 µl of fresh culture medium and the AD model groups were incubated with 100 µl of medium containing different concentrations (10-40 µg/ml) of Aβ₁₋₄₀ at 37°C for 2, 6, 12 or 24 h, with 3 replicates/group (24). All groups were treated with 5 mg/ml MTT solution 10 µl at 37°C for 4 h, the medium was then discarded and 100 µl dimethyl sulfoxide was added to each well to dissolve the formazan crystal product. The absorbance was measured at 570 nm using a microplate reader.

Observation of cell number and morphology under an inverted microscope, and detection of cell activity using the MTT method. PC12 cells were plated in 12-well plates at the density of 5x10⁵/ml. The cells were exposed to 300 µg/ml AGEs at 37°C for 24 h and then treated with 20 µg/ml Aβ₁₋₄₀ at 37°C for 12 h. To assess the effect of AGEs on the expression of inflammatory mediators through MIF and to determine the effect of ISO-1, cells were pre-treated with 7 µM ISO-1 (MedChemExpress LLC) at 37°C for 1 h. The blank control group and the Aβ₁₋₄₀ model group were used as control groups, with 3 replicates/group. Following treatment, the number and morphology of the cells were observed under an inverted microscope (magnification x100). Each sample was analyzed in five randomly selected fields. No significant differences were observed between the BSA control group and the blank control group; therefore, BSA was no longer used as a control.

PC12 cells were plated in 96-well plates at the density of 5x10⁵/ml. The cell groupings and treatments were the same as for the aforementioned microscopy assay, and the activity of cells was determined using the MTT method, according the aforementioned protocol.

Determining the expression of neuroinflammatory cytokines and MIF mRNA using the RT-qPCR assay. PC12 cells were plated in 12-well plates at the density of 5x10⁵/ml. Cells were divided into four groups and exposed to the aforementioned treatments. After treatment, total RNA was extracted and RT-qPCR was conducted using the primers presented in Table I, according the aforementioned protocol.

Kyoto Encyclopedia of Genes and Genomes (KEGG) database search for AGEs signal transduction pathways. KEGG (www.kegg.jp) is a database resource that integrates genomic, chemical and systemic functional information (25). By searching for AGEs-associated signaling pathways, map04933 was identified; AGEs-mediated proinflammatory signaling was investigated using this map.

Statistical analysis. Data are presented as the mean ± SD. Differences among groups were evaluated using one-way ANOVA followed by Tukey's post hoc test using SPSS version 19.0 software (IBM Corp.) and Prism version 5.0 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of AGEs on the expression of MIF mRNA and protein. A significant difference in MIF mRNA expression was not observed between the BSA control group and the blank control group; however, compared with the blank control group, the treatment of cells with 200 or 300 µg/ml AGEs produced statistically significant differences, with MIF mRNA expression significantly increased (Fig. 1A). Cells treated with 100 or 300 µg/ml AGEs exhibited significantly increased levels of MIF protein expression compared with the blank control group (Fig. 1B and C). It was hypothesized that a direct intermolecular relationship existed between AGEs and MIF, and...
that AGEs could induce the expression of MIF mRNA and protein.

Optimal concentration of AGEs and the viability of PC-12 cells following treatment with Aβ1-40. To screen for the optimum concentration of AGEs, PC12 cells were treated with different concentrations of AGEs, and the survival rate was determined using a CCK-8 assay. The results revealed that the viability of cells decreased with increasing concentrations of AGEs. The half-maximal effective concentration was calculated to be 335.0 μg/ml using GraphPad Prism 5 software (Fig. 2A). The BSA control group was administered an equivalent concentration of BSA to the optimal concentration of AGEs for PC12 cells (300 μg/ml); no significant difference was observed between the BSA control group and the blank control group (data not shown). The inhibition rate of cells was measured using an MTT assay. As presented in Fig. 2B, the time at which each concentration of Aβ1-40 inhibited cell survival to the greatest extent was 12-24 h; however, at 24 h, the cells were poorly adherent and a small number of cells were detached. Therefore, cells were treated with Aβ1-40 for 12 h. Using GraphPad Prism 5 software, it was calculated that the half-maximal inhibitory concentration of PC12 cells induced by Aβ1-40 was 22.17 μg/ml (Fig. 2C). The optimal concentration of Aβ1-40 treatment and exposure time to establish an AD model was determined to be 20 μg/ml for 12 h.

Cell morphology and activity. Under an inverted microscope (magnification x100), PC12 cells in the blank control group displayed numerous long dendrites. The blank control cells adhered tightly to the bottom of the culture plate, with dense, clear and visible nucleoli, and exhibited a rapid rate of proliferation (Fig. 3A). Compared with the blank control group, the proliferation of cells in the Aβ1-40 model group was inhibited, as determined by the decreased number of cells, relaxed intercellular junctions and partial retraction of dendrites (Fig. 3B). Following AGEs treatment, cell proliferation was further inhibited, a large number of cells were detached and more cell fragments were observed; the remaining adherent cells lost their characteristic neuronal features (Fig. 3C). Following pre-treatment with ISO-1, the decrease in cell proliferation was notably attenuated. The number of adherent cells increased, the number of detached cells decreased, cells were strongly adherent, and formed more intercellular junctions with longer and more numerous dendrites (Fig. 3D). Compared with the blank control group, the viability of cells in the Aβ1-40 model group significantly decreased; the addition of AGEs further decreased cell viability. Following pre-treatment with ISO-1, the survival rate was significantly improved compared with AGEs and Aβ1-40 alone (Fig. 4). Based on these results, Aβ1-40 successfully established an AD cell model. AGEs were found to aggravate the cytotoxicity of Aβ1-40 in the AD cell model and this effect was significantly alleviated by exposure to the MIF inhibitor ISO-1.

Expression of neuroinflammatory cytokines and MIF. Compared with the blank control group, the expression levels of interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α) and MIF mRNA in cells were significantly increased following treatment with Aβ1-40. The effects of Aβ1-40 on MIF expression were markedly smaller compared with the effects on IL expression; it is possible that other factors in the AD cell

Figure 2. Finding the optimal concentration of AGEs and determining the inhibition of PC12 cell survival induced by Aβ1-40. (A) PC12 cells were treated with different concentrations of AGEs, and the percentage of surviving cells was determined using a Cell Counting Kit-8 assay; the percentage of surviving cells decreased as the AGE concentration increased. The half-maximal effective concentration was calculated to be 335.0 μg/ml using GraphPad Prism 5 software; therefore, the optimal concentration of AGEs for PC12 cell treatment was determined to be 300 μg/ml. (B) PC12 cells were treated with different concentrations of Aβ1-40 for various periods of time. The percent inhibition of cell growth was measured using an MTT assay to determine the most appropriate interval and concentration to create a cell model of AD. Compared with Aβ1-40 (0 μg/ml), the growth of a greater percentage of cells was inhibited as the Aβ1-40 concentration increased. (C) Using GraphPad Prism 5 software, it was calculated that the IC50 of Aβ1-40 in PC12 cells was 22.17 μg/ml. The optimal concentration and exposure time of Aβ1-40 to establish the AD model was determined to be 20 μg/ml for 12 h. Data are presented as the mean ± SD; n=3. *P<0.05, **P<0.01, ***P<0.001 vs. 0 μg/ml AGEs/Aβ1-40. AGEs, advanced glycation end products; Aβ1-40, amyloid β 1-40; IC50, half-maximal inhibitory concentration; AD, Alzheimer's disease.
model may also regulate the expression of MIF. The expression levels of IL-1β and TNF-α were notably further increased by treatment with aGes, and the expression of MIF and IL-6 was significantly increased. Compared with the group treated with aGes, pre-treatment of cells with ISO-1 resulted in a significant decrease in the mRNA expression levels of IL-1β, IL-6, TNF-α and MIF (Fig. 5). Based on these results, aGes aggravated neuroinflammation in the AD model by inducing MIF expression, whereas ISO-1 attenuated this damage.

Discussion

Diabetes has been reported to promote the occurrence of AD by disrupting the insulin signal transduction pathway. Insulin resistance and T2D may be associated with an increased incidence of AD (6). AGEs, important pathogenic factors involved in DM and its complications, induce neurotoxicity by promoting the deposition of Aβ, the hyperphosphorylation of tau protein and the expression of proinflammatory cytokines in glial cells (14,15). MIF inhibits macrophage migration and chemotaxis, and promotes the aggregation of white blood cells at inflammatory sites, serving an important role in a number of diseases, including rheumatoid arthritis, septic shock, inflammatory lung diseases, cancer and delayed-type

Figure 3. AGEs promote apoptosis by inducing MIF expression. Changes to the number and morphology of cells in the (A) blank group, (B) AD cell model group, (C) AGEs-treated AD model group and (D) ISO-1-pretreated group were observed under an inverted microscope (magnification, x100). AGEs inhibited the proliferation of cells in the AD model; following pre-treatment with the MIF inhibitor ISO-1, the decrease in cell proliferation was notably attenuated. Scale bar, 100 μm. AGEs, advanced glycation end products; Aβ1-40, amyloid β 1-40; AD, Alzheimer's disease; MIF, macrophage migration inhibitory factor; ISO-1, (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester.

Figure 4. AGEs decrease cell activity by inducing MIF expression. Cell viability in the blank group, AD cell model group, AGEs-treated AD model group and the ISO-1-pretreated group was detected using an MTT assay. ISO-1 attenuated the AGEs-mediated decrease in cellular viability in the AD cell model. Data are presented as the mean ± SD; n=3. "P<0.01, ""P<0.001 vs. Blank control; "P<0.05. AGEs, advanced glycation end products; Aβ1-40, amyloid β 1-40; AD, Alzheimer's disease; ISO-1, (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester.
Yu et al.: Protective effect of ISO-1 on age-mediated stress in PC12 cell injury induced by Aβ1-40 hypersensitivity (16). MIF promotes the development of diabetes by disrupting the mechanism that regulates the production of inflammatory cytokines, causing insulin resistance and inflammatory injury to islet cells (26). In the present study, the association between AGEs and MIF, and its effects on neuroinflammation in AD, was investigated. AGEs induced the expression of MIF mRNA and protein in PC12 cells, and further aggravated the occurrence of neuroinflammation in the AD cell model. The results showed that the effects of AGEs on the expression of MIF mRNA and protein were not in parallel; the maximal expression of MIF mRNA was observed following treatment with 200 μg/ml AGEs; whereas the expression of MIF protein further increased following treatment with 300 μg/ml AGEs. This phenomenon of non-parallel expression requires further investigation.

AD is a neurodegenerative disease. Hippocampal neuronal injury is an important component of AD pathology and is closely related to the occurrence of neuritis, the deposition of Aβ and the hyperphosphorylation of tau proteins (27). A previous study into the causal relationship between neuroinflammation and AD reported that neuroinflammation may be involved in the pathogenesis of AD, and that an increase in the levels of neuroinflammatory mediators was observed in AD (28). Thus, neuroinflammation serves an important role in the development of AD. Inflammatory mediators cause cognitive impairment through cytokine-mediated glial cell activation and neuronal toxicity (29). In addition, AD has been associated with the upregulation of proinflammatory cytokines, which promote neuronal degeneration (30). These cytokines include IL-1β, IL-6 and TNF-α, which are regarded as important mediators of neuroinflammation and leukocyte infiltration (31). Notable roles of these cytokines include the promotion of Aβ deposition and activation of glial cells (32). In the present study, cells were pre-treated with the MIF inhibitor
ISO-1 to determine if the increased expression of IL-1β, IL-6 and TNF-α was a result of AGE-induced MIF expression. Cells in each group were observed under an inverted microscope, and the effects of a MIF inhibitor on the expression of mRNAs encoding inflammatory mediators were analyzed. AGEs were revealed to mediate neuroinflammation in the AD cell model by inducing MIF expression. MIF inhibitors block the hyperactivation of microglia, and exert anti-inflammatory and neuroprotective effects (33). It was observed that ISO-1 protected the AD cell model from a Ges-mediated damage. The results of the present study may have important theoretical and practical significance in understanding the pathogenesis and prevention of neuroinflammation in AD, and improving treatment.

Previous studies have shown that MIF promotes the development of AD by activating glial cells, initiating cascade reactions, and promoting the occurrence of neuroinflammation, and the formation of senile plaques and NFTs (19-21,33). In the present study, it was demonstrated that AGES promoted the expression of MIF and aggravated the neuroinflammatory response at the cell level. Future studies should investigate the significance of AGES in animal models and at the clinical level. Whether AGES promote MIF expression in glial cells, not only in nerve cells, should also be addressed in future studies using animal models. It should also be investigated as to whether AGES induce the activation of glial cells by promoting MIF expression, and if AGES then aggregate around nerve cells, leading to neuroinflammation and the toxic effects of neuronal phagocytosis.

In the present study of the interaction between AGES and MIF, the molecular signaling pathways affected by AGES were not discussed in detail. The KEGG database (map04933) revealed that AGES promoted inflammation via a number of signalling pathways, including the NF-κB and PI3K pathways (map not shown). The signaling pathways induced by AGES that promote the expression of MIF will be investigated in subsequent studies. Novel ideas and methods for the treatment of AD may be discovered with a more complete understanding of the molecular mechanisms underlying AD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MY and DZ conceived and designed the study, and drafted the manuscript. DZ conducted the experimental protocols and analyzed the experimental results. YX, JM and SQ contributed to the analysis of data and revised the manuscript critically. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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