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Glyceraldehyde-3-phosphate dehydrogenase aggregation inhibitor peptide: A potential therapeutic strategy against oxidative stress-induced cell death

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ABSTRACT

The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has multiple functions, including mediating oxidative stress-induced neuronal cell death. This process is associated with disulfide-bonded GAPDH aggregation. Some reports suggest a link between GAPDH and the pathogenesis of several oxidative stress-related diseases. However, the pathological significance of GAPDH aggregation in disease pathogenesis remains unclear due to the lack of an effective GAPDH aggregation inhibitor. In this study, we identified a GAPDH aggregation inhibitor (GAI) peptide and evaluated its biological profile. The decapeptide GAI specifically inhibited GAPDH aggregation in a concentration-dependent manner. Additionally, the GAI peptide did not affect GAPDH glycolytic activity or cell viability. The GAI peptide also exerted a protective effect against oxidative stress-induced cell death in SH-SY5Y cells. This peptide could potentially serve as a tool to investigate GAPDH aggregation-related neurodegenerative and neuropsychiatric disorders and as a possible therapy for diseases associated with oxidative stress-induced cell death.

1. Introduction

Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was first identified as a glycolytic enzyme [1]. However, GAPDH also has non-glycolytic functions, such as DNA repair [2], membrane fusion and transport [3], transcriptional regulation [4], and oxidative stress-induced cell death [5]. In cells, GAPDH acts as an oxidant-stress sensor. Under oxidative stress conditions, GAPDH translocates to the nucleus [6], which induces p53-dependent transcription of apoptotic genes [7] and activates poly(ADP-ribose) polymerase-1 [8]. We previously reported that oxidative stress leads to disulfide-bonded GAPDH formation, which results in amyloid-like GAPDH aggregation and contributes to cell death [9]. We also reported the critical role of the GAPDH active site cysteine-152 in aggregation. Cysteine-152 is easily oxidized, and substituting the residue with alanine abolishes GAPDH aggregation and cell death [10].

Several neurodegenerative disorders occur under oxidative stress conditions, such as stroke, Alzheimer's disease, and Parkinson's disease. Protein aggregation and subsequent accumulation have been identified as key determinants in the pathogenesis of these diseases [11,12]. Several studies have indicated a relationship between GAPDH aggregation and oxidative stress-induced brain damage [10,13,14]. More recently, we reported that GAPDH/amyloid-beta coaggregates, which plays a pathogenic role in Alzheimer's disease [15]. However, the pathological significance of GAPDH aggregation is not fully understood, largely due to lack of an effective GAPDH aggregation inhibitor.

Here, we tested a theoretically designed GAPDH aggregation inhibitor (GAI) peptide *in vitro* to evaluate its characteristics, including potency, specificity, and pharmacological activity. We

Abbreviations: ANOVA, one-way analysis of variance; DMEM/F12, Dulbecco's modified Eagle medium/Ham's F12; EDTA, ethylenediaminetetraacetic acid; GAI, GAPDH aggregation inhibitor; GAP, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; IC_{50}, 50% inhibitory concentration; LC-MS, liquid chromatography-mass spectrometry; NAD^+, nicotinamide adenine dinucleotide.

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also discuss potential therapeutic applications.

2. Materials and methods

2.1. Compounds and peptide synthesis

All chemicals were analytical grade. Dopamine was obtained from Wako pure chemicals (Osaka, Japan). The GAI peptide (SNASCTTNAL) and the inverse peptide (LANTTCSANS) were synthesized by Sigma-Aldrich-Japan-Genosys (Hokkaido, Japan). The purity was >95%, as assessed by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS).

2.2. Cloning, expression, and purification of human GAPDH

Human GAPDH cDNA was generated as reported previously [9]. For bacterial expression, cDNA was cloned into the pBAD-HisA vector (Invitrogen, Carlsbad, CA) using *Sac*I and *Kpn*I sites. The construct coding region sequence was identical to that of human GAPDH (GenBank accession No. M33197). The construct was transformed into the *gap*(−) *Escherichia coli* strain W3CG [16]. Recombinant GAPDH proteins were expressed and purified as described previously [9]. Briefly, transformants were cultured for 2 h at 37 °C in M63 medium containing 50 μg/ml ampicillin, 15 μg/ml tetracycline, and 0.2% (w/v) L-(+)-arabinose. After 24 h, the cells were pelleted using centrifugation (3000× g for 15 min at 4 °C) and resuspended in lysis buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 30 mM imidazole, 10% glycerol, and 2 mM 2-mercaptethanol. The supernatants were incubated with Ni-NTA agarose resin (50% slurry, QIAGEN Japan, Tokyo, Japan) for 2 h at room temperature with rocking. The resin was washed with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, 50 mM imidazole, 10% glycerol, and 2 mM 2-mercaptethanol. The proteins bound to the resin were eluted with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, 300 mM imidazole, 10% glycerol, and 2 mM 2-mercaptethanol and the eluates were immediately mixed with 1 mM NAD^+, 1 mM dithiothreitol and 1 mM EDTA/2Na, followed by incubation at 4 °C overnight. The reduced proteins were loaded directly onto a PD-10 column (GE Health Care Bioscience Japan, Tokyo, Japan) equilibrated with G2′ buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA/2Na, and 5% glycerol).

2.3. Evaluation of GAI peptide for GAPDH aggregation inhibitory activity

Human recombinant GAPDH solutions (5 μM) containing 0, 1, 3, 10, 30, 60, 100, or 300 μM GAI peptide were added to individual wells in a 96-well plate. The plates were incubated for 48 h at 37 °C. The turbidity was measured spectrophotometrically using the absorbance at 405 nm on a VERSAmax micro-plate reader (Molecular Devices Japan), and IC_{50} was calculated using GraphPad Prism (ver. 6, GraphPad Software, Inc.). To examine the selective activities of the GAI peptide, the effect of 50 μM cysteine or 50 μM inverse peptide on these turbidities of GAPDH aggregation was investigated by the same procedures.

2.4. The effect of GAI peptide on GAPDH enzyme activity

An assay mixture containing 50 mM triethanolamine (pH 8.9), 0.2 mM EDTA/2Na, and 50 mM K_2PO_4 was mixed with 10 nM GAPDH, 10 μM GAI peptide, and 1 mM NAD^+. The enzyme reaction was initiated with the addition of 2 mM GAP. GAPDH enzyme activity was measured for 1 min at 25 °C via the change in absorbance at 340 nm using a VERSAmax micro-plate reader.

2.5. Cytotoxicity of GAI peptide on PC12 cells

PC12 cells in Dulbecco's modified Eagle medium (DMEM) were plated at a cell density of 3.13×10^5 cells/cm^2 into a 96-well plate coated with 0.5 mg/ml poly-D-lysine hydrobromide. The cells were incubated for 72 h at 37 °C with 1 mM of GAI peptide (20-fold molar concentration against the IC_{50} value). The cell viability was measured using a Cell Titer Glo Luminescent Cell Viability Assay kit (Promega, WI) according to the manufacture's protocol.

2.6. The effect of GAI peptide on dopamine-induced cell death

SH-SY5Y neuroblastoma cells in Dulbecco's modified Eagle medium/Ham's F12 medium (DMEM/F12, 1:1) were plated at a density of 3.2×10^4 cells/cm^2 in a 96-well plate and immediately treated with a single dose of GAI peptide (10, 30, or 100 μM) or 100 μM cysteine. Cells were incubated for 30 min at 37 °C. The cells were then treated with either 200 μM dopamine or vehicle (DMEM/F12) and incubated at 37 °C for 48 h. The cell viability was measured as described above.

2.7. Statistical analysis

All data are represented the mean ± SEM of independent experiments as indicated the numbers (N) in each figure legend. For statistical analysis, two groups and multiple groups were compared with unpaired Student's *t* tests or Dunnet's multiple tests after one-way analysis of variance (ANOVA), respectively.

3. Results and discussion

In order to develop a GAPDH aggregation inhibitor (GAI) peptide, we designed a decapeptide that is 90% identical to the GAPDH sequence of the active site, which contains the aggregation-associated cysteine-152. This design was based on the previous observation that cysteine-152 oxidation triggers GAPDH aggregation (Fig. 1A). Further, substituting the peptide equivalent of cysteine-156 with an alanine prevented the formation of intramolecular-disulfide bonds (Fig. 1B).

We evaluated the GAI peptide for GAPDH aggregation inhibition. The GAI peptide inhibited aggregation in a concentration-dependent manner (IC_{50} = 53.2 μM, Fig. 2A). Because the GAI peptide contains a cysteine residue, this inhibition could potentially be attributed to the antioxidative property of the cysteine. To examine this possibility, we compared the GAPDH aggregation-inhibition effect of the GAI peptide with that of cysteine alone. Additionally, we assessed the sequence-specificity of the GAI peptide using an inverse peptide. Treatment with cysteine or the inverse peptide did not attenuate GAPDH aggregation (Fig. 2B). These results indicate that the GAI peptide inhibits GAPDH aggregation, both in an antioxidative activity-independent and a sequence-specific manner.

Next, to determine whether treatment with the peptide might cause adverse effects, we assessed the effects of the GAI peptide on GAPDH enzyme activity and cell viability. GAPDH's glycolytic activity depends on the aggregation-associated cysteine-152 and disrupting it may reduce catalysis. However, the GAI peptide did not affect GAPDH enzyme activity even at a 1000-fold higher concentration of GAI peptide (Fig. 3A). Additionally, treatment with 1 mM GAI peptide did not affect cell viability (Fig. 3B). These results suggest that the GAI peptide does not disturb cellular homeostasis, inhibit irreversibly, or covalently bind to GAPDH.

Finally, we examined the cytoprotective effect of the GAI peptide against dopamine-induced cell death. High concentrations of dopamine are known to cause robust oxidative stress [17]. Similar

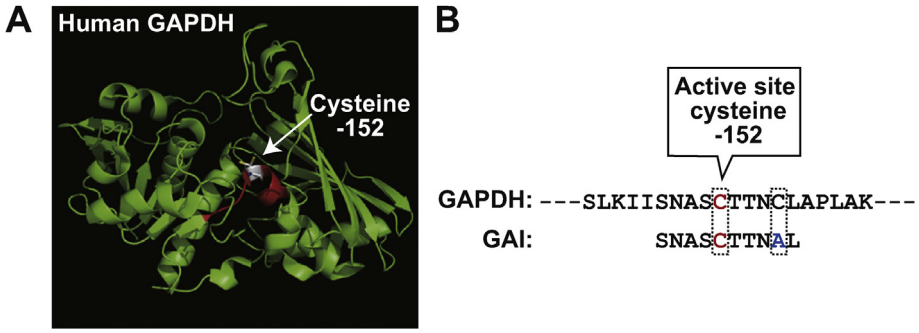

Fig. 1. Design of the GAPDH aggregation inhibitor (GAI) peptide. (A) A ribbon diagram of human GAPDH (PDB code 1U8F) and the location of the active site cysteine-152 (white). The ten amino acids used for the design of the GAI peptide are shown in red. (B) The sequence of the GAI decapeptide, which is identical to the corresponding GAPDH sequence except for an alanine substitution at the position equivalent to GAPDH cysteine-156.

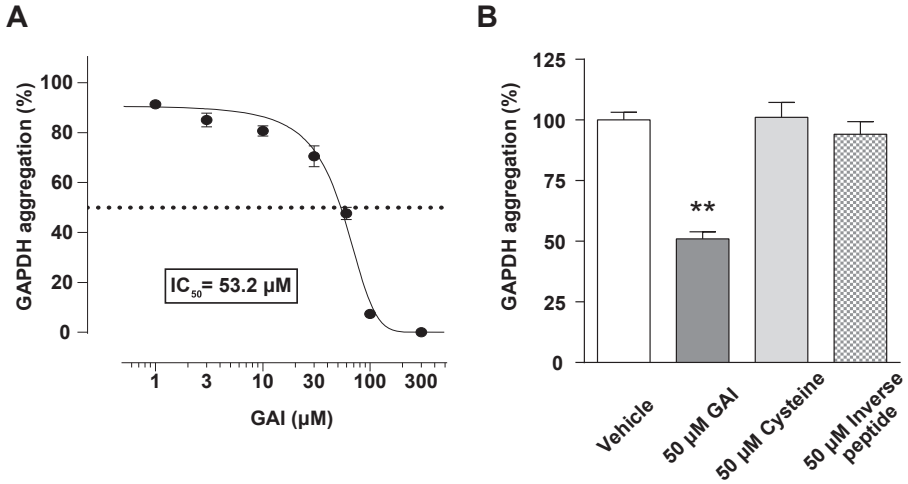

Fig. 2. Characterization of the GAPDH aggregation inhibition of the GAI peptide. (A) GAPDH (5 μM) was incubated at 37 °C for 48 h in the presence of the GAI peptide (1, 3, 10, 30, 60, 100, or 300 μM), the absorbance at 405 nm was measured spectrophotometrically, and the IC_{50} was calculated. (B) The GAPDH aggregation inhibition of 50 μM GAI peptide, 50 μM cysteine, and 50 μM inverse peptide was compared. Data represent means ± SD (N = 3, $^{**}p < 0.01$, relative to vehicle, as determined by Student's t-test).

to our previous work demonstrating that GAPDH aggregation contributes to dopamine-induced cell death [10], treatment with 200 μM dopamine for 48 h decreased the viability of SH-SY5Y cells substantially. Treatment with the GAI peptide restored cell viability

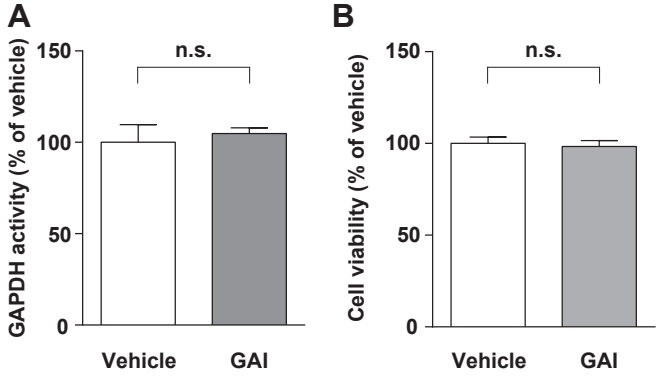

Fig. 3. Assessment of potential adverse effects with GAI peptide treatment. (A) GAPDH (10 nM) enzyme activity was measured spectrophotometrically in the presence of vehicle or the GAI peptide (10 μM). (B) PC12 cells were incubated with vehicle or GAI peptide (1 mM) for 72 h and cell viability was measured. Data represent means ± SD (N = 3) and were analyzed using Student's t-test.

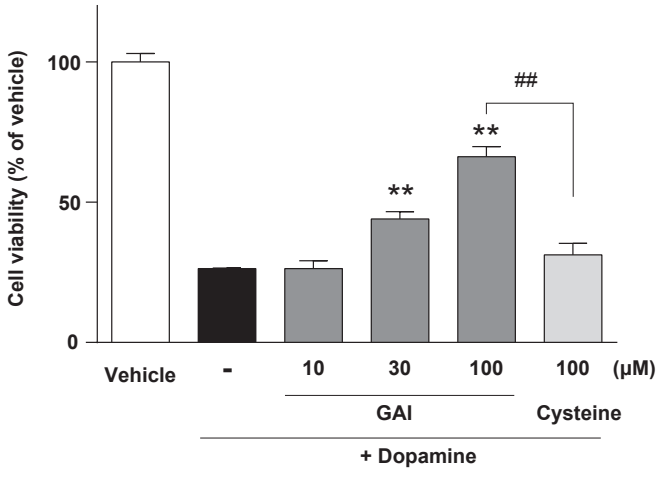

Fig. 4. Protective effect of GAI peptide against dopamine-induced neuronal cell death. SH-SY5Y cells were incubated for 30 min with or without GAI peptide (10, 30, and 100 μM) or with cysteine (100 μM) and treated with dopamine (200 μM) or vehicle for 48 h. Cell viability was measured. Data represent means ± SD (N = 4–5, $^{**}p < 0.01$, relative to dopamine alone, determined by one-way analysis of variance followed by Dunnett's multiple range test; $^{\#\#}p < 0.01$, relative to the treatment with dopamine in the presence of 100 μM GAI peptide, determined by Student's t-test).

in a concentration-dependent manner (Fig. 4). However, treatment with 100 μM cysteine failed to restore cell viability compared with that of 100 μM GAI peptide (Fig. 4), similar to the GAPDH aggregation inhibition results (Fig. 2B). These results indicate that the GAI peptide inhibits dopamine-induced GAPDH aggregation, providing a protective effect against cell death.

In the present study, we developed a novel GAI peptide and demonstrated its GAPDH aggregation inhibitory activity. In addition, the GAI peptide has no overt adverse effects such as the GAPDH activity inhibition and cytotoxicity. Furthermore, GAI peptide treatment attenuated dopamine-induced neuronal cell death. While these results demonstrated the efficacy of the GAI peptide, the potency ($IC_{50} = 53.2$ μM) is not sufficient for most applications, and the peptide may not be stable enough due to potential endopeptidase cleavage. Therefore, further investigations trying to optimize the peptide for potency or developing peptide-mimic compound are needed.

Overall, these results indicate that a GAI peptide or peptide-mimic compound could potentially be a tool to investigate GAPDH aggregation-associated neurodegenerative or neuropsychiatric disorders and may provide a new therapeutic strategy to combat oxidative stress-induced cell death in these disorders.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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