学 位 論 文 の 要 旨

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学　位　論　文　名　A Carboxylated Zn-Phthalocyanine Inhibits Fibril Formation of Alzheimer's Amyloid β Peptide

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論 文 内 容 の 要 旨

INTRODUCTION:

Alzheimer's disease (AD) is a common dementia of the elderly. Evidences suggest that Aβ peptide has a critical role in AD pathogenesis. For example, gene mutations that increase Aβ burden in brains are found to be related to AD. Increased Aβ leads to aggregation, affording oligomers or polymeric fibrils. Aggregated Aβ species, such as oligomers, are more neurotoxic than monomers, suggesting that Aβ aggregation process in brains also have key roles in the disease pathogenesis by increasing neurodegeneration. Hence, Aβ accumulation and aggregation processes might be good targets for diagnosis and therapy of AD. Several compounds that show anti-amyloid activity *in vitro* or in animal models are already in clinical trials. However, an effective disease-modifying therapy remains elusive.

Diagnosis is also an important issue. Aβ in CSF or deposited in brain parenchyma could be a diagnostic marker. Deposited Aβ can be visualized by PET imaging with high-affinity compounds, but the equipment is expensive and not widely available. Conversely, near-infrared (NIR) spectroscopy is technically favorable for in vivo imaging due to its optical window from approximately 600 to 1000 nm, where the absorption coefficient of tissue is at a minimum, resulting in a low background. Recently, it was reported that phthalocyanines (Pcs), which are metal-containing, aromatic, macrocyclic NIR fluorophores, interact with α-synuclein and affect fibril formation. Further, Fe-containing Pc interacts with toxic $A\beta_{1\text{-}40}$ oligomers and converts them to an amyloid fibril meshwork. Thus, we hypothesized that Pcs might bind to Aβ and serve as amyloid fibril-modifying agents. For such property, they might be used for AD therapy, also be useful as amyloid-specific NIR imaging probes to visualize deposited Aβ.

Most Pc species are hydrophobic, and tend to aggregate in aqueous medium, resulting a self-quenching effect on their excited state. Therefore, we prepared water-soluble Zn-containing Pcs (ZnPcs) bearing sodium carboxylate groups as candidate for amyloid fibril-modifying agents

or *in vivo* NIR probes, and investigated their effects on Aβ during fibril formation process.

MATERIALS AND METHODS

In this study, 4 types of Pcs were used. $ZnPc(COONa)_8$ and $ZnPc(COONa)_{16}$ were dissolved in H_2O. $ZnPc(COOC_5H_{11})_8$ and PdPc dimer were dissolved in chloroform. These Pcs were prepared following described protocols. To check their effects on Aβ fibril formation, Aβ monomer was added to a fibril-forming buffer containing various concentrations of Pcs, and incubated for the indicated times at 37°C. The amount of fibrils in a sample was determined by ThT fluorescence assay using a spectrofluorimeter. To determine the effects of Pcs on the stability of Aβ fibrils, first fibrils were prepared by incubating the peptide in a fibril forming buffer without Pcs. Then $ZnPc(COONa)_8$ was added to Aβ fibrils, further incubated for 24 h, and fibril levels were determined by ThT fluorescence assay. Morphological analysis of Aβ fibrils was done by electron microscopy.

To understand the underlying mechanisms, we checked the binding of $ZnPc(COONa)_8$ with Aβ. $ZnPc(COONa)_8$ was incubated with Aβ. Then Aβ was immunoprecipitated with Aβ-specific monoclonal antibody, and $ZnPc(COONa)_8$ in the immunoprecipitate was detected by near infrared scanning. The changes in hydrophobicity during Aβ fibril formation were determined using 8-anilino-1-naphthalenesulfonic acid (ANS). This dye binds to exposed hydrophobic amino acids causing an increase in fluorescence with a blue shift. After incubation, ANS was added to the samples, and fluorescence intensity was measured with excitation at 360 nm, and emission was scanned from 380 to 600 nm. Next, we investigated whether $ZnPc(COONa)_8$ changes the aggregated species of the peptide. After fibril formation, Aβ samples were separated by SDS PAGE using 4–20% gradient tris-glycine gel in a non-reducing condition, and stained with Coomassie Blue to visualize different Aβ aggregated species. Moreover, high molecular weight aggregated oligomeric species of Aβ was analyzed by dot blot immunoassay. After fibril formation, the samples were spotted on a nitrocellulose membrane, and the oligomers on the membrane were detected by oligomer-specific antibody.

The changes in the secondary structures were evaluated by circular dichroism (CD) spectral analysis. After fibril formation, CD spectra were acquired in the range of 190 – 250 nm, using a spectropolarimeter. The results were expressed as mean residue molar ellipticity, and the percentage of secondary structures in a sample was estimated using a computer program.

Finally, the effects of $ZnPc(COONa)_8$ on Aβ-induced neurotoxicity was evaluated using a neuronal cell (A1) culture. A1 cells were cultured on wells of a 96-well plate for 48 h. The cells were treated with $ZnPc(COONa)_8$ or $A\beta_{1-42}$, alone or in combination for 48 h. The viability of A1 cells was determined by MTT assay following established assay protocol.

RESULTS AND DISCUSSION:

Effects of Pcs on $A\beta_{1-40}$ and $A\beta_{1-42}$ fibril formation. $A\beta_{1-40}$ (50 μM) or $A\beta_{1-42}$ (12.5 μM) was each incubated with increasing concentrations of $ZnPc(COONa)_8$, $ZnPc(COONa)_{16}$, $ZnPc(COOC_5H_{11})_8$ and PdPc dimer for 48 h or 24 h. Measurement of fibrils by ThT fluorescence assay revealed that $ZnPc(COONa)_8$ dose-dependently inhibited fibril formation of both $A\beta_{1-40}$

and $A\beta_{1-42}$. $ZnPc(COONa)_{16}$ had similar but modest inhibitory effects. PdPc dimer inhibited, but $ZnPc(COOC_5H_{11})_8$ increased $A\beta_{1-40}$ fibril formation. Kinetics of Aβ fibril formation showed that $ZnPc(COONa)_8$ extended the lag time of fibril formation. However, it did not affect the overall morphology of the fibrils, as revealed by electron microscopic analysis. Moreover, our fibril stability experiments showed that it increased the breakdown of Aβ fibrils. These results are suggesting that $ZnPc(COONa)_8$ effectively interacted with Aβ and inhibited the fibril formation.

Possible mechanism of $ZnPc(COONa)_8$-mediated inhibition of Aβ fibril formation. Pcs are reported to produce singlet oxygen ($^1O_2$). Hence, it is possible that $^1O_2$ produced by $ZnPc(COONa)_8$ might cause the inhibitory effect. But our experiments with NaN_3 ($^1O_2$ scavenger) showed that $^1O_2$ had no role in the inhibitory effects. Next, we checked whether $ZnPc(COONa)_8$ directly binds to Aβ. After incubation of $A\beta_{1-42}$ monomers or pre-formed fibrils with $ZnPc(COONa)_8$ for 24 h in a fibril-forming environment, $A\beta_{1-42}$ was immunoprecipitated with Aβ-specific antibody, and near-infrared scanning was done for $ZnPc(COONa)_8$. We found ZnPc in the immunoprecipitate, suggesting its binding with the peptide. Such binding might affect the fibril formation process.

Hydrophobic amino acids in Aβ peptide as well as a hydrophobic microenvironment play a key role in fibril formation. To further explore the mechanism, we checked whether binding of $ZnPc(COONa)_8$ alters hydrophobic microenvironment. The change of hydrophobicity was evaluated using a hydrophobic fluorescent probe (ANS). The result showed that $ZnPc(COONa)_8$ decreased hydrophobicity of $A\beta_{1-42}$ during fibril formation process.

As hydrophobic interaction is important in secondary structure formation of Aβ, we investigated the effects of $ZnPc(COONa)_8$ on such process by CD spectral analysis. The results showed that $ZnPc(COONa)_8$ increased α helix, and decreased β sheet structures of $A\beta_{1-40}$ during fibril formation process. As a result, low molecular weight species (monomer, dimer or trimer) was increased and high molecular weight oligomeric species of Aβ was decreased, as shown by SDS PAGE, and dot blot immunoassay for oligomers, respectively.

$ZnPc(COONa)_8$ on $A\beta_{1-42}$-induced cytotoxicity. Oligomers of Aβ peptides are considered to be more cytotoxic than the monomers. As $ZnPc(COONa)_8$ inhibited oligomer formation, we explored whether it affected $A\beta_{1-42}$-mediated cytotoxicity to a neuronal cell line (A1). Both morphological analysis and MTT cell viability assay showed that $ZnPc(COONa)_8$ had no cytotoxic effect on A1 cells, rather it protected the cells from $A\beta_{1-42}$-mediated cytotoxicity.

CONCLUSION

In conclusion, our results indicate that water-soluble $ZnPc(COONa)_8$ binds to Aβ peptides and inhibits the oligomerization and subsequent fibril formation processes. It also destabilizes pre-formed fibrils. We consider that $ZnPc(COONa)_8$ has potential value as a diagnostic probe for near-infrared imaging of fibrils in AD brains, and it may also be a candidate for therapy of AD.

論文審査及び最終試験又は学力の確認の結果の要旨

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論文審査の結果の要旨

アルツハイマー病(AD)は認知症の代表的疾患で、病理学的にアミロイドβタンパク(Aβ)が線維形成し、脳内へ沈着することが特徴である。その確定診断は困難で、アミロイドを標識した positron emission tomography が用いられるが高価で汎用性に乏しい。また、Aβ沈着を改善させる治療法は確立されていない。本研究では AD の診断や治療を目的とし、近赤外(near infrared:NIR)域に吸収波長を有する phthalocyanine(Pc)誘導体を複数作製し、Aβとの相互作用を検討した。Thioflavin T アッセイによる解析から、亜鉛(Zn)錯体である $ZnPc(COONa)_8$ が量依存性に $Aβ_{1-40}$ および $Aβ_{1-42}$ の線維形成を抑制した。Dot blot 分析では、神経細胞毒性を示すAβオリゴマーの形成を遅延させた。さらに、線維形成した $Aβ_{1-40}$、$Aβ_{1-42}$ に作用させたところ、その凝集を抑制することにより、モノマー状態にとどめることが SDS-PAGE で明らかとなった。作用機序として CD スペクトロスコピーなどによる解析から、$ZnPc(COONa)_8$ はAβの疎水性を減少させることによりβシート構造の形成を阻害し、結果としてAβ凝集を抑制することが示唆された。また培養神経細胞を用いた実験から、$ZnPc(COONa)_8$ は $Aβ_{1-42}$ による神経毒性を軽減した。以上より、$ZnPc(COONa)_8$ はその Aβ結合性を生かすことにより Aβイメージング用の NIR プローブ開発への応用や、Aβ凝集抑制作用を基にした AD 治療への応用につながることが示唆された。故に本研究は基礎的研究として高い価値を有しており、博士(医学)の学位授与に値すると判断した。

最終試験又は学力の確認の結果の要旨

申請者は複数のPc誘導体を用いて、ADで重要なAβに対する凝集抑制作用および神経毒性軽減作用について検討した。その結果、$ZnPc(COONa)_8$が最も作用が強く、その特性を生かしてAD病変のイメージングや治療への応用につながる可能性が示唆された。質疑応答も的確であることから、学位授与に値すると判断した。

(主査: 和田孝一郎)

申請者はADの原因となるAβに対するPc誘導体の作用を検討し、Aβの重合過程に抑制作用を示すことを明らかにした。本研究は考察も適切になされ、Pc誘導体のADへの治療薬としての可能性を示すもので学位授与に値すると判断した。

(副査 森田 栄伸)

申請者は4種類のPc誘導体のAβに対する作用を検討し、特に$ZnPc(COONa)_8$がAβ線維形成抑制作用と神経細胞毒性の原因となるAβオリゴマー形成遅延作用、さらには形成されたAβ線維の凝集抑制作用を有することを明らかにしてADの治療への新たな可能性を示した。関連知識も豊富で学位授与に値すると判断した。

(副査 猪俣 泰典)

(備考) 要旨は、それぞれ４００字程度とする。