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論文内容の要旨

Structure Activity Study on hA5G18 Peptide (DDFVFYYVGGYPS) from Laminin α5 Chain for Amyloid-like Fibril Formation and Cell Adhesion

Introduction

Laminins, consisting of three genetically distinct subunits, α, β, and γ chains, are multifunctional glycoproteins and locate in basement membranes. To date, five α chains (α1-α5), three β chains (β1-β3), and three γ chains (γ1-γ3) have been identified, and at least 19 different laminin isoforms have been discovered by various combination of each subunit. The α chains play a vital role in diverse biological functions of laminins and express in a tissue- and developmental stage-specific manner. Various active sequences from laminin molecules have been identified using synthetic peptides. The active peptides specifically interact with cellular receptors, including integrins and syndecans, a membrane-associated heparan sulfate proteoglycan (HSPG), and are useful to understand the biological function of laminins and to apply for biomaterials and drug delivery systems.

Biologically active sequences in the human laminin α5 chain G domain have been screened using 113 synthetic peptides by peptide-coated plate and peptide-conjugated chitosan matrix assays. In this screening, 17 peptides showed cell attachment activity in both or either peptide-coated plated and/or peptide-conjugated chitosan matrix assays, and 3 peptides, hA5G18 (DDFVFYYVGGYPS), hA5G26 (LDGTGFARISFD), and hA5G74 (GSLSSHLEFVGI), promoted integrin β1 mediated cell spreading. The hA5G74 peptide promoted cell attachment and spreading in both assays. In contrast, hA5G18 and hA5G26

showed cell attachment activity only in the peptide-coated plated assay. These results suggested that hA5G18 and hA5G26 aggregate on the plastic plates and promote cell attachment, but mechanism of the aggregation has not been identified.

Amyloid fibril formation of degraded proteins is often related to diseases, such as Alzheimer's disease, type II diabetes, Parkinson's disease, prion diseases, and systemic polyneuropathies. Identification of amyloidogenic peptides leads to understanding of the mechanism of disease. Detailed studies have also been conducted on the structural characteristics of amyloid β oligomers and fibrils in the neural tissue of Alzheimer's disease, and it has been reported that differences in morphology, physicochemical properties, and cytotoxicity occur due to their structural diversity. It is also known that peptides with amyloid fibrils are often arranged to form β-strands and specifically bind dyes such as thioflavin T and Congo red. Previously, diverse amyloidogenic peptides from laminin sequences have been identified. All of the peptides were stained with Congo red, and most of the peptides showed amyloid-like fibril formation in transmission electron microscope (TEM) analysis. Many of the amyloidogenic peptides contained basic amino acids, and their cellular effects, including promotion of cell attachment and spreading and neurite outgrowth, have been described. The amyloidogenic peptides are useful to understand the effect of the degraded proteins *in vivo*. Further, amyloidogenic peptides have been modified with biologically active peptides and applied for multifunctional biomaterials. Amyloidogenic peptides are useful to understand the function of degraded proteins and to use for biomaterials.

Chapter 1. Identification of amyloidogenic peptides from the human α5 chain G domain

The hA5G18 and hA5G26 peptides, derived from the human laminin α5 chain G domain, promoted integrin-mediated cell attachment and spreading only in a peptide-coated plated assay. I focused on hA5G18 and hA5G26 and evaluated their amyloidogenicity using Congo red assay and TEM analysis. The Congo red absorption peak at 490 nm was significantly shifted at 540 nm by hA5G18, similar to that by a control peptide B133 (DSITKYFQMSLE) from the laminin β1 chain but was not influenced by hA5G26. hA5G18 exhibited typical amyloid-like fibrils similar to those of B133 in a TEM, but hA5G26 did not show fibrils. These results indicate that hA5G18 forms amyloid-like fibrils.

Truncated hA5G18 peptides were synthesized to identify active core sequences for amyloid-like fibril formation and cell attachment (**Table 1, Figure 1**). AG73 (RKRLQVQLSIRT) from the laminin α1 chain G domain was used as a positive control for the cell attachment assay. First, the N-terminally truncated hA5G18 peptides were

examined by the Congo red staining, TEM analysis, and cell attachment assays. When the Congo red solution was incubated with the truncated peptides, the absorption peak was shifted to a long wavelength by hA5G18A and hA5G18B, but the hA5G18C peptide did not influence the absorption spectrum (**Figure 1A**). In addition, hA5G18A and hA5G18B showed fibrils in a TEM, but hA5G18C did not (**Figure 1D**). Further, hA5G18A and hA5G18B promoted cell attachment in a dose-dependent manner similar to that of hA5G18 and AG73, but hA5G18C did not (**Figure 1B**). These results suggest that hA5G18B has both amyloid-like fibril formation and cell attachment activity similar to those of hA5G18.

The C-terminally truncated hA5G18B peptides were examined by the Congo red staining, TEM analysis, and cell attachment assays (**Table 1**). The hA5G18BTC5 peptide, C-terminal five amino acids deleted, still showed Congo red staining, but further shortened peptide hA5G18BTC6 eliminated the activity (**Figure 1C**). In addition, hA5G18BTC5 showed fibrils in a TEM analysis, but hA5G18BTC6 did not (**Figure 1D**). These results suggest that hA5G18BTC5 (FVFYV) is a minimum active sequence for amyloid-like fibril formation. In contrast, none of the C-terminally truncated hA5G18B peptides showed cell attachment activity, suggesting that hA5G18B (FVFYVGGYPS) is a minimum active sequence for cell attachment.

Table 1. Biological activities of hA5G18 and truncated hA5G18 peptides

Peptide	Sequence	Congo Red Staining [a]	Cell Attachment [b]	Cell Spreading [c]
hA5G18	DDFVFYVGGYPS	+	+	+
hA5G18A	DFVFYVGGYPS	+	+	+
hA5G18B	FVFYVGGYPS	+	+	+
hA5G18C	VFYVGGYPS	-	-	-
hA5G18BTC1	FVFYVGGYP	+	-	-
hA5G18BTC2	FVFYVGGY	+	-	-
hA5G18BTC3	FVFYVGG	+	-	-
hA5G18BTC4	FVFYVG	+	-	-
hA5G18BTC5	FVFYV	+	-	-
hA5G18BTC6	FVFY	-	-	-

[a] Peptides were incubated with a Congo red solution, and the absorption spectra measuring from 300 to 700 nm were evaluated on the following subjective scale: + showed Congo red activity; and - no shift in the absorption peak. [b] Cell attachment activity was scored on the following subjective scale: + showed cell attachment activity, and - no activity. [c] Cell spreading activity was scored on the following subjective scale: + showed cell spreading activity; and - no activity.

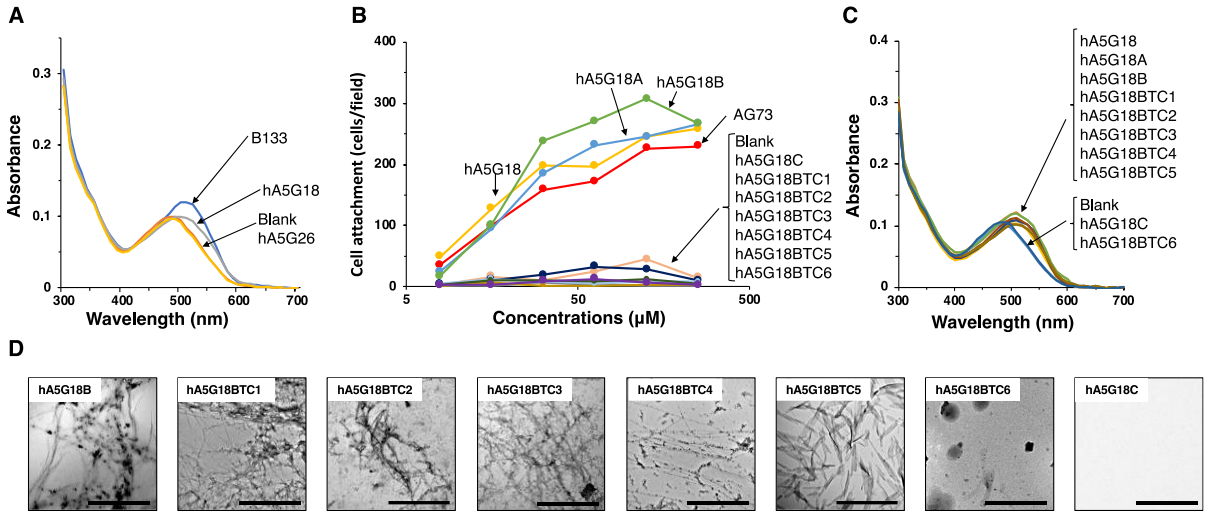

Figure 1. **Congo red staining, cell attachment activity, and electron micrographic analysis of peptides.** (**A**) Peptides were stained with Congo red, and absorption spectra were recorded from 300 to 700 nm. (**B**) Cell attachment of the truncated hA5G18 peptides in a peptide-coated plate assay. (**C**) Peptides were stained with Congo red, and absorption spectra were recorded from 300 to 700 nm. (**D**) Electron micrograph of peptides. Bars = 500 nm.

Critical amino acids for cell attachment and amyloid-like fibril formation of hA5G18B were evaluated using a set of alanine-substituted hA5G18B peptides. The alanine-substituted analysis of hA5G18B revealed that the Phe1, Val2, Phe3, and Tyr4 residues are critical and the Gly7 and Tyr8 residues partially contribute to the amyloid-like fibril formation, and the Phe1, Val2, Phe3, and Tyr4 residues are critical for the cell attachment activity.

Chapter 2. Application of FVFYV for functional amyloid-like fibrils as a biomaterial

FVFYV was used as an amyloid-like fibril template and modified with a cell adhesive peptide to apply for biomaterials. An integrin binding sequence Arg-Gly-Asp (RGD) was conjugated to FVFYV with Gly-Gly as a spacer. Arg-Gly-Glu (RGE), a negative control sequence of RGD, conjugated FVYFV was also synthesized as a control. FVFYVGGRGD and FVFYVGGRGE were stained with Congo red suggesting forming amyloid-like fibrils (**Figure 2A**). FVFYVGGRGD showed cell attachment activity in a dose-dependent manner. However, FVFYVGGRGE also promoted cell attachment similarly. These results suggest that the cell attachment activity of FVFYVGGRGD in the peptide coated-plate assay is not due to the integrin binding sequence RGD, and the Arg residue in amyloid-like fibrils may cause the activity.

To evaluate the effect of basic amino acid on the cell attachment activity of amyloid-

like fibrils, Arg, Lys, and His were conjugated to the C-terminus of FVFYV with Gly-Gly as a spacer. When the Congo red solution was incubated with the peptides, the absorption peak was shifted by all the peptides. These results suggest that the basic amino acid-conjugated FVFYV peptides form amyloid-like fibrils (**Figure 2A**). Cell attachment activity of the peptides was also evaluated (**Figure 2B**). FVFYVGGR and FVFYVGGK promoted cell attachment in a dose-dependent manner. In contrast, FVFYVGGH did not promote cell attachment similar to that of FVFYV. These results suggest that Arg and Lys residues contribute to the cell attachment activity in amyloid-like fibrils.

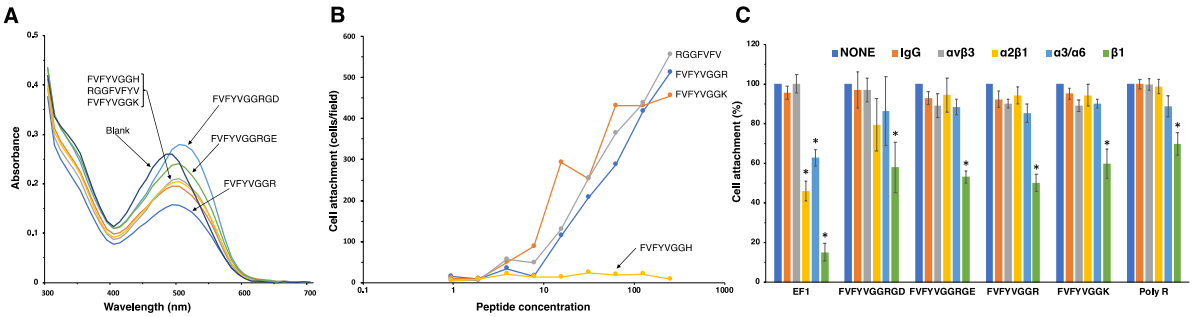

Figure 2. Amyloidogenicity, biological activity, and effect of anti-integrin antibodies on cell attachment of modified FVFYV peptides. (**A**) Peptides were stained with Congo red, and absorption spectra were recorded from 300 to 700 nm. (**B**) Cell attachment of the truncated hA5G18 peptides in a peptide-coated plate assay. (**C**) Effect of anti-integrin antibodies on cell attachment to peptides. Each value represents the mean of three separate determinations ± S.D. Triplicate experiments gave similar results. * $p < 0.05$.

Cell attachment of FVFYVGGRGD, FVFYVGGRGE, FVFYVGGR, FVFYVGGK, FVFYVGGH, and RGGFVFYV in a Sepharose bead assay was examined under disaggregated condition. The FVFYVGGRGD bead promoted cell attachment, but the other peptide beads did not show the activity.

Effect of EDTA and heparin on the cell attachment to FVFYVGGRGD, FVFYVGGRGE, FVFYVGGR, and FVFYVGGK were examined in a peptide-coated plate assay. Additionally, poly arginine (poly-R) was also examined. EF1 (ATLQLQEGRLHFMDFLGKR) from the laminin α1 chain G domain was used as a control to interact with integrin α2β1 and promote divalent cation-dependent cell attachment. AG73 was used as a control to interact with syndecans and promote heparin-dependent cell attachment. Cell attachment to EF1 was inhibited by EDTA but not by heparin, and that to AG73 was inhibited by heparin but not by EDTA, as shown previously. Cell attachment to FVFYVGGRGD, FVFYVGGRGE, FVFYVGGR, FVFYVGGK, and poly-R was inhibited by EDTA and heparin. These results suggest that the cell attachment of FVFYVGGR, FVFYVGGK, FVFYVGGRGD, FVFYVGGRGE, and poly-R is mediated by

integrins and HSPGs.

Next, effect of antibodies against integrin αvβ3, α2β1, α3/α6, and β1 on the cell attachment was examined (**Figure 2C**). Cell attachment to FVFYVGGRGD, FVFYVGGRGE, FVFYVGGR, FVFYVGGK, and poly-R was significantly inhibited by the anti-integrin β1 antibody, while the other anti-integrin antibodies did not influence. These results suggest that integrin β1 is involved in the cell attachment to FVFYVGGRGD, FVFYVGGRGE, FVFYVGGR, FVFYVGGK, and poly-R.

Conclusion

The hA5G18 peptide was demonstrated to form amyloid-like fibrils and promote cell attachment. A deletion analysis of hA5G18 revealed that hA5G18B (FVFYVGGYPS) was a minimum active sequence for the cell attachment, and FVFYV was a minimum active sequence for the amyloid-like fibril formation. Functional amyloid-like fibrils were prepared by conjugation of FVFYV and cell adhesive peptides. FVFYV is used to apply as a core sequence for designing functional amyloid-like fibrils and has the potential for use as a unique platform for a cell scaffold material.

Publication

1. **Zhang G**, Yamada Y, Kumai J, Hamada K, Kikkawa Y, Nomizu M. *Molecules*, **27 (19)**, 6610 (2022).

【論文審査の結果の要旨】

本論文は、ラミニン α5 鎖の G ドメイン由来の活性ペプチドに注目し、アミロイド線維を形成することにより細胞接着活性を示すメカニズムを解明し、そのアミロイド線維形成のコア配列を同定するとともに、そのコア配列を用いて機能性アミロイド線維を創製しバイオマテリアルとしての有用性を示したものである。

ヒトラミニン α5 鎖 G ドメインの生物学的に活性な配列が、113 種類のペプチドを用い、細胞接着活性をペプチドをプレートにコートする方法（ペプチドコート法）とペプチドを多糖マトリックスに結合させる方法（ペプチドマトリックス法）で評価することにより同定されてきた。その中で、ペプチドコート法のみで細胞接着活性を示す 2 種類のペプチド、hA5G18 (DDFVFYVGGYPS)と hA5G26 (LDGTGFARISFD)に注目し、コンゴレッド評価や透過電子顕微鏡で解析し、hA5G18 がアミロイド様線維を形成し、細胞接着を促進することを見出した。次に、hA5G18 の deletion 実験やアラニン置換実験から、FVFYVGGYPS が細胞接着活性の、FVFYV がアミロイド線維形成の最小配列であることを見出した。FVFYV はアミロイド線維を形成するが細胞接着活性を示さない。次に、FVFYV に細胞接着活性ペプチドや塩基性アミノ酸を修飾し、機能性アミロイド線維のデザインを行ったところ、アルギニンやリジンなどの塩基性アミノ酸を修飾することによりインテグリン β1 を介した細胞接着活性を有するアミロイド線維の創製に成功した。FVFYV は、機能的なアミロイド様線維を設計するためのプラットホームともいえるコア配列として用いることが可能で、細胞の足場材料のバイオマテリアルの開発に有用であることが示された。

本研究において見出されたアミロイド繊維を形成することによりインテグリンを介した細胞接着を示すメカニズムは細胞接着機構の解析に新たな知見を与えるものであるとともに、hA5G18 ペプチドはラミニンの機能解明に有用である。また、FVFYV ペプチドは、アミロイド繊維のコア配列として用いることができ、新たなバイオマテリアルの開発研究に寄与できるものである。

以上より、本論文は博士（薬学）の学位論文に相応しいと判断する。