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研究課題名（和文）Restoring tissue homeostasis by regulating TGF-beta signaling by using macrocyclic peptides

研究課題名（英文）Restoring tissue homeostasis by regulating TGF-beta signaling by using macrocyclic peptides

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　1,700,000円

研究成果の概要（和文）：シグナルは癌の進展に関与すると考えられている。本研究は、細胞内シグナル伝達を遮断することで、TGF-betaシグナルを制御することを目的とした。私たちは、I型受容体であるALK1に結合する特殊環状ペプチドを同定し、I型受容体とBMPもしくはII型受容体との結合を阻害することで、シグナル伝達を遮断できないかと考えた。In vitroセレクション後、ALK1に結合する有望な特殊環状ペプチドとして、10＾12種類以上を含むライブラリーより、ALK1-3R6-5を同定した。現在ALK1-3R6-5誘導体の化学合成を行っている。

研究成果の概要（英文）：TGF-beta signaling is implicated in cancer progression. The goal of this project is to regulate TGF-beta signaling by preventing signal transduction into the cell. We aim to identify a macrocyclic peptide or macrocyclic peptides that bind to the type 1 receptor ALK1 and prevent its interaction with the cytokine BMP and/or the type 2 receptor, thereby blocking signal transduction. After in vitro selection, a promising macrocyclic peptide, ALK1-3R6-5, was identified from a diverse library of over 10 unique macrocyclic peptides. Chemical synthesis of ALK1-3R6-5 derivatives is underway.

研究分野：　Cancer signaling

キーワード：　macrocyclic peptides　in vitro selection　RaPID system　receptors

１．研究開始当初の背景

TGF-beta signaling is implicated in both normal development and cancer. Under a healthy cellular state, TGF-beta signaling serves as a cancer suppressor. In some cancers, however, TGF-beta signaling is abnormally upregulated and further promotes cancer progression. Careful down-regulation, but not complete suppression, of aberrant TGF-beta signaling could help reduce cancer growth.

The components of extracellular signal transduction include the TGF-beta cytokine and type 1 and type 2 receptors. Signal activation occurs when TGF-beta binds 2 constitutively active type 2 receptors. This binding event increases the affinity for the type 1 receptor. Two type 1 receptors join the complex, bringing the intracellular kinase subdomains of the two types of receptors into close proximity. The intracellular kinase domain of the type 2 receptor then phosphorylates the intracellular kinase domain of the type 1 receptor, thereby activating it for propagation of the signal to the cytosolic signaling components. An example of this process, is the binding of the TGF-beta cytokine Bone Morphogenetic Protein (BMP) 9 to type 2 receptor BMPR (BMP Receptor) 2 and Activin receptor-Like Kinase (ALK) 1 in the BMP signaling pathway (Figure 1). Activated ALK1, in turn, phosphorylates receptor-regulated signaling components SMAD1/5/8.

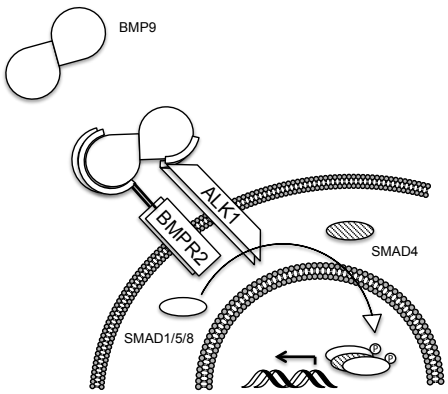

Figure 1. Extracellular and intracellular components of the BMP signaling pathway.

Both extracellular and intracellular components of TGF-beta signaling are valid targets for regulation of cancer progression. Targeting the extracellular components, however, avoids the technical difficulty of transporting a therapeutic through the cell membrane, which is one of the biggest challenges facing current drug discovery efforts. In our work, we attempt to disrupt TGF-beta signaling through the discovery of a macrocyclic peptide ligand that is specific for ALK1.

２．研究の目的

Regulation of TGF-beta signaling is a promising means of controlling cancer progression. Although small molecules and biologic have been used to regulate TGF-beta signaling, both of these two therapeutic classes have their drawbacks. Small molecules can have off-target effects due to low affinity and non-specific binding. Biologics are expensive to produce and are not orally bioavailable. The goal of this project is to create a ligand that serves as an antagonist using a macrocyclic peptide scaffold. Macrocyclic peptides possess enough surface area to bind to their target biomolecule with high affinity and

specificity, thereby reducing the need for high concentrations and limiting off-target effects. The macrocyclic peptides are, however, small enough to potentially be membrane penetrating and capable of being orally administered. In addition, the macrocyclic scaffold enhances the peptides' resistance to degradation by proteases. For the aforementioned reasons, macrocyclic-peptide-based anti-cancer drugs would be extremely valuable.

３．研究の方法

(1) *In vitro selection for a macrocyclic-peptide-based ligand*

An in vitro selection system based on the Random non-standard Peptide Integrated Discovery (RaPID) system was used to identify macrocyclic peptides that bind with high affinity to ALK1. The RaPID system allows researchers to reprogram the genetic code for the exclusion of chosen standard amino acid and the inclusion of non-standard amino acids. For the construction of the macrocyclic peptide scaffold, only reprogramming of the first incorporation is required and can be accomplished using mainly commercial components. Identification of the active macrocyclic peptides is performed using mRNA display and iterative of screening and amplification (Figure 2). The target protein, ALK1, was provided by Wei Li (Cambridge, U.K.).

(2) *Clone assay*

After identification of the binding macrocyclic peptide or macrocyclic peptides through sequencing of the genetic material of the later rounds of selection, individual peptide clones are chosen for a single-clone assay using mRNA display and qPCR.

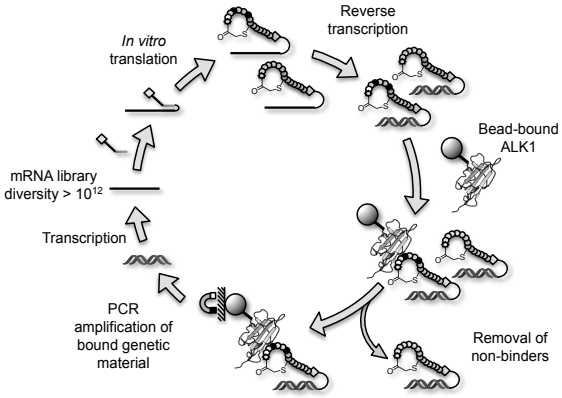

Figure 2. Schematic depicting a selection round for macrocyclic peptides that bind to ALK1.

(3) *Chemical synthesis*

ALK-3R6-5 was synthesized using standard FMOC chemistry with the aid of a Biotage Syro 1 automated peptide synthesizer. Chloroacetylation of the N-terminus was performed manually. *N*-chloroacetylated peptide was cleaved from the resin and deprotected using trifluoroacetic acid. Cleaved linear peptide was precipitated using 10 equivalent volumes of diethyl ether. The precipitate was washed 5 times with diethyl ether. The peptide pellet was dissolved in 49.95:49.95:0.1 water:acetonitrile:TFA. To promote macrocyclization, trimethylamine was added until the pH value was about 9-10. The macrocyclization reaction proceeded for an hour and was periodically checked to ensure the pH value stayed above 9. Macrocyclization was confirmed using MALDI-TOF mass spectrometry.

４．研究成果

(1) *Establishment of the new selection system*

A new in vitro selection system was established at the University of Tsukuba. Construction of the original RaPID system requires the laborious production and purification of well over twenty enzymes and factors for a customizable in vitro translation system. As time and personnel are limited, we established a new in vitro translation system based on components from a commercially available translation kit, New England Biolab's PURExpress. Using this in vitro translation system, we were able to produce macrocyclic peptides and combine that production with mRNA display for the selection of active macrocycle at the University of Tsukuba.

(2) *In vitro selection for ALK1-binding macrocyclic peptides*

The in vitro selection against ALK1 that resulted in the isolation of a binding macrocyclic peptide was carried out for 6 rounds (Figure 3). Although the overall cDNA recovery was low, the ratio of ALK1-binding to non-specific-binding cDNA recovery increased with the rounds prompting us to sequence after the 6th round of selection (Table 1).

(3) *Chemical synthesis of ALK1-3R6-5 derivatives*

We chemically synthesized a cysteine to serine mutant version of ALK1-3R6-5 (ALK1-3R6-5-Ser) to avoid oxidation of the peptide (Figure 4). It is known that the macrocyclization reaction occurs between the chloroacetyl and the closest

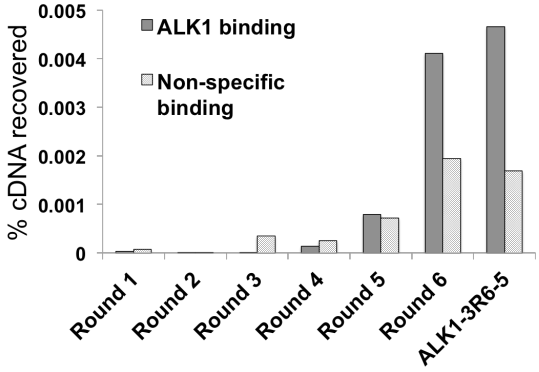

Figure 3. Selection progress and single-clone assay of ALK-3R6-5

Table 1. ALK1-3R6-5 and related peptide sequences identified after 6 round of *in vitro* selection. The position of the chloroacetyl-D-tryptophan is indicated by a w.

ALK1 3R6 top sequences	Amino acid sequence	out of 39851 sequences	% of the population
ALK1-3R6-5	wAYSNATKNACWAVHQC	1102	2.73%
ALK1-3R6-24	wAYSNAT**R**NACWAVHQC	169	0.42%
ALK1-3R6-120	wA**S**SNATKNACWAVHQC	20	0.05%
ALK1-3R6-211	wAYSNATKNA**R**WAVHQC	12	0.03%

cysteine, meaning that the scaffold of ALK-3R6-5 is a lariat, not a macrocycle. Since the contribution of the linear peptide tail to binding is unknown, a tail-less version (ALK1-3R6-5-minimal) was also synthesized. Biotin- and fluorescein-modified versions of ALK1-3R6-5-Ser are also being synthesized for on-cell ALK1-staining experiments. Purification is underway.

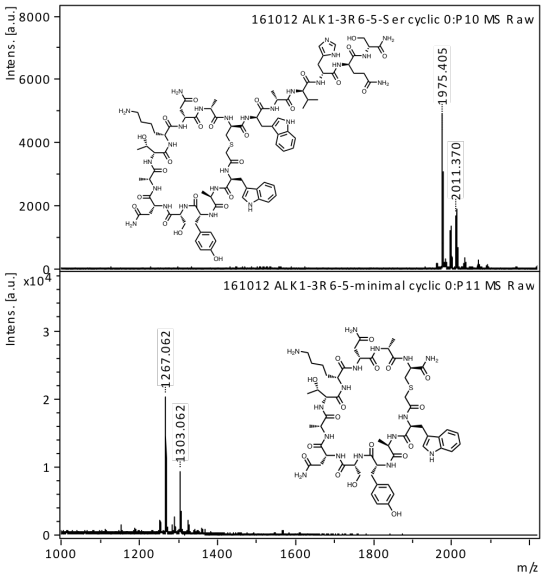

Figure 4. Chemical structures and MALDI mass spectra of chemically synthesized ALK-3R6-5-Ser (top, expected m/z: 1975.887) and ALK-3R6-5-minimal (bottom, expected m/z: 1267.552).

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　1　件）Peer reviewed

1. Leenheer, D.; ten Dijke, P.; Hipolito C. J. A current perspective on applications of macrocyclic-peptide-based high-affinity ligands. *Biopolymers: Peptide Science.* **2016**, *106*(6), 889-900. DOI: 10.1002/bip.22900

〔学会発表〕（計　2　件）

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2. Hipolito, C.; Janson, S.; Li, W.; ten Dijke. Restoring tissue homeostasis by using macrocyclic peptides. TGF-beta Meeting. August 21-23 2016. Leiden, the Netherlands.

〔図書〕（計　0　件）

〔産業財産権〕

○出願状況（計　0　件）

名称：
発明者：
権利者：
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番号：
出願年月日：
国内外の別：

○取得状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
取得年月日：
国内外の別：

〔その他〕
ホームページ等

http://www.md.tsukuba.ac.jp/tmrc/foundation_core/peptide/peptide_en.html

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