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科学研究費助成事業　　研究成果報告書

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課題番号：２０Ｋ１６２４６

研究課題名（和文）コレラ菌が産生するコリックス毒素の受容体解析

研究課題名（英文）Analysis to identify the receptor for cholix toxin from Vibrio cholerae

研究代表者
アワスティ　シャルダ（AWASTHI, SHARDA）

大阪公立大学・大学院獣医学研究科　・特任講師

研究者番号：３０７５１８８８

交付決定額（研究期間全体）：（直接経費）　　3,100,000円

研究成果の概要（和文）：ChxA がさまざまな細胞株で細胞毒性を誘発することが確認されましたが、CHO 細胞では増殖阻害のみが見られた。In-silico ドッキング研究では、ChxA と cRP の間に安定した結合界面が示されており、相互作用の可能性を示唆しています。これは、野生型 CHO 細胞とは異なり、ChxA およびその後の細胞死に対する感受性を示した cRP を発現する形質転換 CHO 細胞によって実験的に裏付けられました。これらの成果は、毒素生物学の研究を進めるために不可欠な、ChxA と cRP の相互作用に関する基礎的な理解を築くものです。

研究成果の学術的意義や社会的意義
この研究の科学的意義は、ChxA とその候補受容体タンパク質（cRP）間の分子相互作用を解明したことにあります。cRP が ChxA 誘発性細胞毒性の潜在的受容体であることを確認することで、この研究は ChxA が標的細胞に結合することに関する理解を深めます。社会的観点から見ると、この研究は公衆衛生に重要な意味を持ちます。毒素とその細胞標的間の特定の相互作用を理解することで、毒素関連疾患の診断ツールや治療法が改善される可能性があります。全体として、この研究は、ChxA 誘発性コレラ菌毒性に対処し、緩和する能力を高めます。

研究成果の概要（英文）：The research confirmed that ChxA induces cytotoxicity in various cell lines, with CHO cells showing only growth inhibition which could be due to the absence of the candidate receptor protein (cRP). In-silico docking studies have shown a stable binding interface between ChxA and cRP, suggesting a potential interaction. This was experimentally supported by transformed CHO cells expressing cRP, which showed susceptibility to ChxA and subsequent cell death, unlike wild-type CHO cells. Cytotoxicity assays further confirmed that cRP-positive A431 cells were highly susceptible to ChxA, whereas cRP-negative JURKAT cells were resistant, highlighting cRP's role in ChxA-induced cytotoxicity. However, attempts to purify cRP for direct binding studies were unsuccessful due to solubility issues. These achievements lay a foundational understanding of the interaction between ChxA and cRP, essential for advancing research in toxin biology.

研究分野：Microbiology

キーワード：Vibrio cholerae　Cholix　Receptor

１．研究開始当初の背景 (Background at the beginning of the research)

In 2010, the cholix toxin (ChxA), an ADP-ribosylating toxin was discovered. It is an exotoxin produced by Vibrio cholerae, a pathogen known for causing cholera. The chxA gene is an important virulence factor present in 47% of non-O1/non-O139 Vibrio cholerae strains as well as reported in limited number of clinically important V. cholerae O1 strains. ChxA exerts its toxic effects by catalyzing the ADP-ribosylation of the eukaryotic elongation factor, a process that disrupts protein synthesis in host cells. This disruption leads to a cascade of events including mitochondrial dependent and independent caspase activation, cleavage of Rho-associated coiled-coil containing protein kinase 1, and ultimately, apoptosis in the affected cells. The cytotoxic pathway and the resulting liver injury and mouse lethality have been partially elucidated. However, much remains unknown about the toxicity of ChxA, and key receptors involved in the initial stages of toxicity expression are not known. The precise mechanisms of ChxA entry into host cells, particularly the specific cell surface receptors facilitating this entry, have not been identified. Our preliminary *in-silico* analysis has identified a potential receptor candidate, hypothesized to be critical for ChxA binding to target cells. Confirming this receptor could significantly advance our understanding of the mechanisms underlying ChxA toxicity and provide new targets for therapeutic intervention.

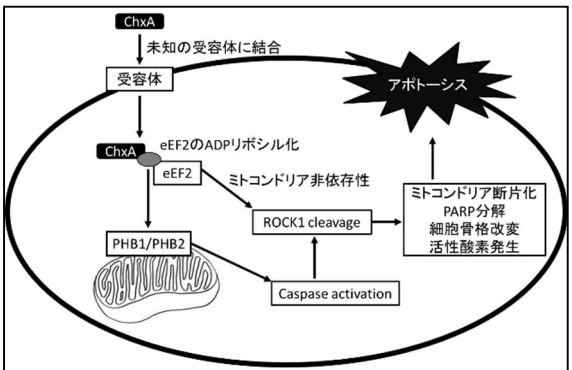

図１：ChxAの細胞に対する毒性発現機構
eEF2, eukaryotic elongation factor 2; PHB, prohibitin;
PARP, poly (ADP-ribose) polymerase
ROCK1, Rho-associated coiled coil-containing protein kinase 1

２．研究の目的 (Purpose of research)

The primary objective of this research was to identify and validate the receptor responsible for the entry of ChxA into eukaryotic cells. Specifically, the research aimed to evaluate whether the identified receptor candidate, expressed on eukaryotic cell lines, functions as the receptor for ChxA of Vibrio cholerae. The successful identification of this receptor would not only enhance our understanding of ChxA's mechanism of toxicity but also potentially inform the development of new strategies to mitigate the effects of this toxin. This research aimed to bridge the gap in knowledge about how ChxA interacts with host cells at the molecular level, which is crucial for devising effective countermeasures against its toxic effects.

３．研究の方法 (Research method)

The *V. cholerae chxA* gene was cloned into the pET-28a(+) vector and transformed

into *E. coli* BL21(DE3) for expression and purification. The recombinant ChxA (rChxA) was purified to homogeneity using affinity and gel filtration chromatography. The cytotoxicity induced by rChxA was evaluated in various eukaryotic cell lines (HeLa, Y-1, NIH-3T3, Int407, Hep-2, Vero, CHO) to determine cell tropism. An *in-silico* binding assay was performed using the open-source programs Z-Dock and PyMOL. The extracellular domain of the candidate receptor protein (cRP) was purchased, and used for the binding assay. Additionally, a plasmid (pTJNeo) containing the cDNA of the full-length cRP was obtained. CHO cells were transformed with the pTJNeo-cRP plasmid. Both wild-type and transformed CHO cells were used in the cytotoxicity assay with ChxA. Furthermore, A431 and Jurkat cells were cultured in their respective media and also used in the cytotoxicity assay against ChxA. The cRP was cloned for expression and purification in pET-28a(+) for use in *E. coli* or in pFASTBac™ for use in the insect cell baculovirus expression system. The purification of cRP was attempted from cell lysates or inclusion bodies to be used in binding assay.

4. 研究成果 (Research result)

ChxA cytotoxicity assay and cell tropism: The previously reported cytotoxicity assay and cell tropism results were confirmed using the newly purified recombinant ChxA (rChxA). Among the seven tested cell lines, ChxA induced cytotoxicity and cell death in six. In contrast, only cell growth inhibition was observed in CHO cells. A literature review revealed that CHO cells lack a specific receptor. This receptor is hypothesized to be the candidate receptor protein (cRP) for ChxA.

Cell Line	Origin	Source	Cytotoxicity	rChxA I (CD_{50}) µg/mL
HeLa	Human	Cervical cancer		1.25
Y-1	Mouse	Adenocortical tumor		1.25
NIH-3T3	Mouse	Embryonic fibroblast		1.25
Int407	Human	Embryonic intestine		1.25
HEp2	Human	Epidermoid cancer		1.25
Vero	Monkey	Kidney epithelium		2.5
CHO	Hamster	Ovary		2.5*

*only cell growth inhibition was observed.
CD50: concentration of ChxA killing cells or inhibiting cell growth by 50%.

In-silico binding assay between ChxA and candidate receptor protein: The full length ChxA (2Q5T) and cRP structures were downloaded from protein data bank (PDB). The structure based in-silico binding was carried out with Z-dock and Pymol programs. The top-ranking docking pose was further analyzed and visualized using PyMOL. The visual representation confirmed a stable and plausible binding interface between the two proteins. The in-silico analysis suggested that ChxA and cRP can indeed interact with each other. The predicted binding interface and the nature of interactions indicated a potential functional relevance, which warrants further experimental validation. These findings provided a foundational step towards understanding the molecular mechanism underlying the interaction between these two molecules.

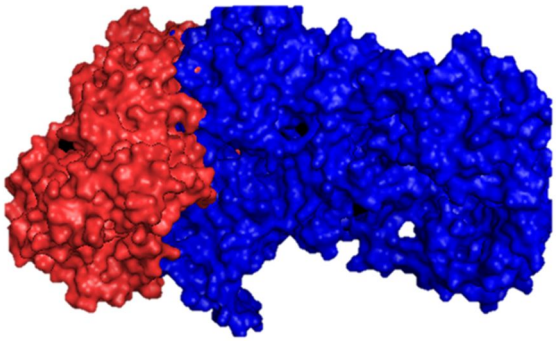

Fig. In-silico binding model for ChxA (red) and candidate receptor protein (blue)

ChxA cytotoxicity assay with CHO cells transformed with plasmid carrying cRP-cDNA and expressing cRP: The cytotoxicity assay was evaluated using wild-type and transformed (expressing cRP) CHO cells. HeLa cells were used as control. The wild-type CHO cells which did not express cRP did not show any cytotoxicity when treated with rChxA up to 20 µg/mL, although growth inhibition was observed at this concentration. On the other hand, transformed cells expressing cRP were susceptible to rChxA and demonstrated cytotoxicity with CD50 of 5 µg/mL rChxA. The results further supported the idea that the cRP could be the target of ChxA.

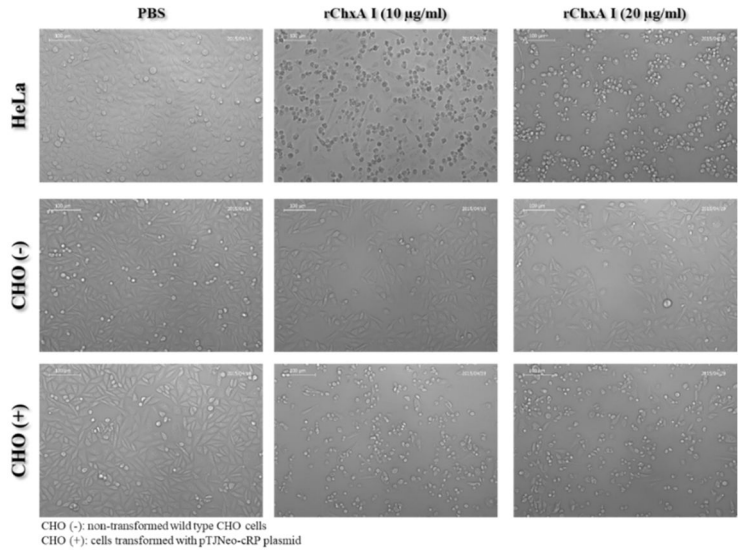

Fig. Cytotoxicity assay of rChxA with HeLa, CHO (-) and CHO (+pTJNeo~cRP)

ChxA cytotoxicity assay with cRP positive A431 and cRP negative JURKAT cells: The cytotoxicity assay was evaluated using A431 (cRP+/+) and JURKAT (cRP-/-) cells. The A431 cells expressing cRP were susceptible to rChxA and demonstrated cytotoxicity with CD50 of 1.25 μg/mL rChxA. On the other hand, JURKAT cells which did not have cRP did not show any cytotoxicity when treated with rChxA up to 80 μg/mL.

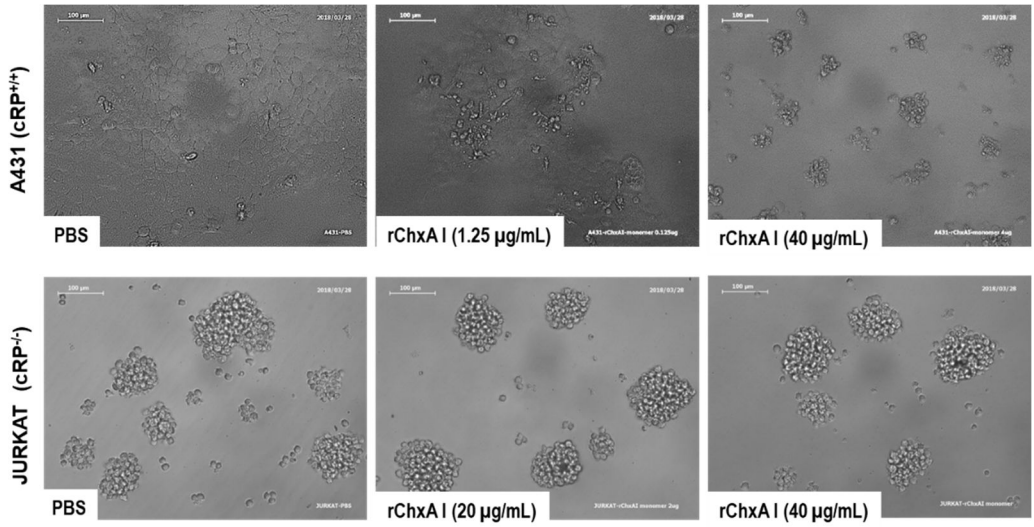

Fig. Cytotoxicity assay of rChxA with A431 (cRP+/+), and JURKAT (cRP-/-) cells

Expression and purification of cRP using baculovirus expression system and binding assay: The commercially available extracellular domain of the cRP failed to bind to the rChxA during protein overlay assay. This led us to express and purify the cRP. The cRP DNA was amplified using pTJNeo~cRP plasmid as template. This amplified cDNA was used for the cloning and expression in baculovirus expression system. The cRP could be expressed in Sf9 cells. Unfortunately, the cRP was insoluble and could not be purified. The cRP was attempted to be purified from inclusion bodies using 8M urea. Extensive efforts to purify cRP using solubilization with 8M urea followed by nickel affinity chromatography were unsuccessful. The large size of the protein or other unknown factors may have contributed to these challenges. The inability to purify cRP prevented us from doing the direct binding studies between ChxA and cRP. Previous binding attempts also failed, suggesting potential barriers to establishing this interaction. Future studies should explore alternative purification methods, different solubilization conditions, and new binding assays. Further investigation into factors influencing ChxA and cRP interactions is essential for advancing toxin biology and protein-protein interaction research.

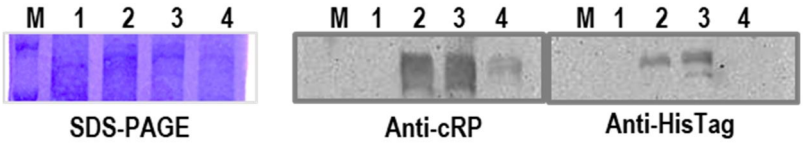

Fig. SDS-PAGE and Western blotting results to detect the cRP in transformed SF9 cells, and to see the solubility of the cRP. M. Marker, 1. Sf9_non-transformed, 2. Sf9_His-cRP-Whole, 3. Sf9_His-cRP-Sonic pellet, 4. Sf9_His-cRP-Sonic supernatant

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　0件）

１．著者名 Awasthi Sharda Prasad、Chowdhury Nityananda、Hatanaka Noritoshi、Hinenoya Atsushi、Ramamurthy Thandavarayan、Asakura Masahiro、Yamasaki Shinji	４．巻 70
２．論文標題 Quantification of Vibrio cholerae cholix exotoxin by sandwich bead-ELISA	５．発行年 2021年
３．雑誌名 Journal of Medical Microbiology	６．最初と最後の頁 NA
掲載論文のDOI（デジタルオブジェクト識別子） 10.1099/jmm.0.001311	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関