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論　文　名	「Molecular mechanism of the toxic action of Clostridium septicum alpha-toxin on mammalian cells」 （クロストリジウム・セプチカムα毒素の動物細胞に対する毒性発現機構に関する研究）

論文要旨

Introduction

Clostridium septicum is a ubiquitous, spore-forming, gram-positive anaerobic bacterium that causes traumatic（wound derived）and non-traumatic（endogenous）myonecrosis, which are rapidly fatal diseases in man and animals. Although the pathogen secretes several extracellular factors such asa alpha-toxin, deoxyribonuclease, hyaluronidase and neuraminidase, alpha-toxin has been implicated as a major virulent factor such that it possesses hemolytic, lethal and necrotizing properties. However, the exact role of the toxin in disease remains unclear. *C. septicum* alpha-toxin belongs to a family of toxin termed pore-forming toxins. These toxins disrupt cellular membrane permeability by forming unregulated channels. Previous studies have shown that the toxin is secreted as a water-soluble protoxin which binds to cell surfaces via glycosylphosphatidylinositol (GPI) -anchored proteins. Once the toxin binds to the cell, it is activated by proteolytic cleavage at the carboxyl-terminal propeptide with cell surface proteases. Activation lets the activated monomers facilitate oligomerization on the membrane followed by formation of functional transmembrane pores. The oligomerization on cell surface is considered to be a critical step in the channel formation process. However, the precise mechanism on oligomerization of the toxin on cells has not been fully understood. The present study has been made to investigate the mechanism of toxic action on mammalian cells, in particular focusing upon toxin binding and subsequent oligomerization on cell membranes. During the present investigation, a new toxin-binding protein

was discovered and characterizad.

Chapter 1: Hemolytic activity and binding of *C. septicum* alpha-toxin on erythrocyte membranes of various animals

In the preliminary experiments, the activity of *C. septicum* alpha-toxin was determined in erythrocytes of various animals. The sensitivity of erythrocytes to the toxin was observed in the order of mouse, rat, canine, equine, rabbit, chicken, bovine, swine and ovine. Temperature and protease treatment affected the sensitivity of erythocytes to the toxin. Proteinase K tretment decreased the sensitivity of murine, canine, equine and bovine erythocytes, while ovine erythrocytes did not change the sensitivity to the toxin. To the contrary, the activity of the toxin on swine erythrocytes increased after treatment with proteinase K, trypsin, chymotrypsin or lysyl endopeptidase. In toxin overlay assay, the toxin was shown to bind to erythrocyte membrane proteins with a molecular mass of 30 to 45-kDa in mouse, equine, bovine, swine and chicken, while the toxin reacted with a 100-kDa protein in rat erythrocyte membranes. The treatment of murine and swine erythrocyte membranes with phosphatidylinositol-specific phospholipase C resulted in liberation of the toxin-binding protein from individual membranes in a native state. These results show that toxin associates with specific erythocyte membrane proteins in any animal species, and are subsets of GPI-anchored proteins in various animal species.

Chapter 2: Cytotoxicity and oligomerization of *C. septicum* alpha-toxin on mammalian cells

The cytotoxic activities were also examined with various mammalian cells. In contrast with the activity to erythrocytes, mammalian cells were more sensitive to protoxin than trypsinized toxin interacted with various molecular sizes of cellular proteins. The size and SDS-PAGE pattern of the proteins were different among cell lines, but they were liberated from the cells by the treatment with phosphatidylinositol-specific phospholipase C. The toxin appeared to target and utilize detergent resistant membranes (DRMs) for binding and subsequent oligomerization. In discontinuous sucrose density gradient followed by immunoblotting, the toxin bound to DRMs contained in L929 (fibroblast) cells and

caused oligomer formation. Furthermore, cholesterol depletion with cholesterol interacting agents reduced toxin oligomerization and lowered cytotoxicity of the toxin toward cells. These results suggest that the toxin preferentially exploits DRMs for oligomerization.

Chapter 3: Interaction of *C. septicum* alpha-toxin with alpha-actin

3.1. Identification of alpha-actin as a *C. septicum* alpha-toxin binding protein

Alpha-toxin was found to interact with a 43-kDa protein in L929 cells, which was not associated with DRMs. In order to characterize the toxin-binding protein, the protein was digested with lysyl endopeptidase and the liberated peptides were separated by high performance liquid chromatography. When the peptides obtained were applied to amino acid sequencing, the toxin-binding protein was identified as a mouse cardiac alpha-actin. The cardiac alpha-actin gene was then cloned, and the fusion protein was expressed as a histidine-tagged protein *in Escherichia coli*. After purification by affinity chromatography, the toxin binding activity of the recombinant protein was examined. The recombinant alpha-actin retained the toxin-binding ability, whereas no toxin binding was observed with bera-actin. The recombinant alpha-actin also appeared to accelerate oligomer formation of the toxin *in vitro*.

3.2. Analysis of alpha-actin interacting with alpha-toxin in murine cells and tissues

A rat polyclonal anti-alpha-actin IgG was prepared and used to screen the presence of alpha-actin in various murine cell lines and tissues. Alpha-actin was present in C_2C_{12} (myoblast) cells, L929 cells, heart and skeletal muscles as a monomer and polymer. After alpha-actin containing tissue lysates was entrapped with beads coupled with anti-alpha-actin antibody, the immunoprecitates were subjected to SDS-PAGE followed by toxin overlay assay. The results indicated that the toxin seemed to bind preferentially to alpha-actin polymer rather than monomer. To confirm further the interaction of the toxin with alpha-actin, toxin-treated cell lysates were applied to immunoprecipitation with beads coupled with anti-alpha-toxin. It was found that alpha-actin was entrapped together with the toxin, suggesting that the toxin really associates with alpha-actin in cell

membranes.

Conclusions

1. Alpha-toxin binds to specific GPI-anchored proteins in various mammalian cells.
2. The protoxin binds to GPI-anchored proteins in non-DRMs for activation by cell surface proteases before moving to DRMs and oligomerize leading to pore-formation.3 . Cholesterol plays a significant role in alpha-toxin oligomerization and cytotoxicity.4 . Alpha-actin was identified as a new toxin-binding protein, which provides some information to elucidate peculiarity in terms of symptoms such as malignant edema involving *C. septicum* infection.

審査結果の要旨

ヒトのガス壊疽や動物の悪性水腫の原因菌である *Clostridium septicum* は皮膚や腸管の創傷部位から侵入し、産生される数種類の毒素･酵素の相乗作用により壊死や毒血症を引き起こす。*C. septicum* の主要な病原因子であるα毒素は細胞膜上に pore を形成することにより細胞を傷害する細胞溶解毒素（溶血毒）である。α毒素は活性のない分子量 46kDa の前駆体(protoxin)として産生された後、蛋白分解酵素により限定分解を受け、41kDa の活性化毒素となる。α毒素は GPI アンカー蛋白を受容体として細胞膜上に結合後、6～7 量体の高分子複合体(oligomer)として孔を形成して膜を破壊する代表的な細胞溶解毒素である。

本研究では、α毒素の細胞障害機構における受容体への結合から孔形成に至るまでの細胞膜上でのα毒素の動態について分子レベルで詳細に解析することにより、次のような成果を得た。

１．種々の動物由来赤血球を用いて *C. septicum* α毒素の溶血活性と受容体との関係を比較検討した。各赤血球を proteinase K で処理したとき、マウス、イヌ、ウマ、ウシ赤血球の感受性が低下した。ヒツジ赤血球では変化がみられず、ブタ赤血球では proteinase K、トリプシン、キモトリプシン、リシルエンドペプチダーゼのいずれの蛋白分解酵素処理によってもα毒素の活性は増加した。Toxin overlay assay でα毒素はマウス、ブタ、ウシ、ウマ、ニワトリ赤血球膜の 35～45ｋDa 蛋白と結合し、ラットでは 100ｋDa の膜蛋白と反応することが分かった。マウスおよびブタ赤血球膜を phosphatidylinositol-specific phospholipase C(PI-PLC)処理すると、α毒素結合蛋白は膜から遊離することを明らかにした。これらの結果は、α毒素が動物種によって異なる赤血球膜蛋白と結合し、これらの蛋白はいずれも GPI-アンカー蛋白であることを示しており、このことがα毒素の動物種による溶血活性の違いに影響を与えていると考えられた。

２．動物由来株化細胞に対するα毒素の細胞障害および受容体結合活性、オリゴマー形成に関係する細胞側分子について解析を行った。α毒素は様々な分子量の異なる蛋白と結合したが、これらの結合蛋白は細胞株間で異なっていた。また、PI-PLC で処理するとα毒素結合蛋白は細胞から遊離することから、赤血球同様、株化細胞においてもα毒素は種々の GPI-アンカー蛋白と結合することにより活性を発現する明らかにした。α毒素で処理した細胞を界面活性剤で可溶化後、不連続ショ糖密度勾配遠心により分画し抗α毒素抗体を用いた immunoblotting により局在を調べた。α毒素は細胞膜の detergent 不溶画分（DRM)、いわゆるラフトでオリゴマーを形成することが分かった。さらにコレステロール阻害剤で処理するとα毒素のオリゴマー形成が減少し、細胞障害活性も低下した。これらの結果か

ら、α毒素は受容体に結合した後、ラフトに集積しオリゴマーを形成すること、さらにラフト集積およびオリゴマー形成には膜に存在するコレステロールが重要な役割を担っていることを明らかにした。

３．α毒素に結合する細胞の新たな蛋白としてαアクチンを同定した。L929 細胞を界面活性剤で処理後、ショ糖密度勾配遠心により分画し、toxin overlay assay を行った。α毒素は不溶画分の GPI-アンカー蛋白以外に、可溶画分に存在する分子とも結合した。この分子の部分アミノ酸配列からマウス心臓αアクチンであることが分かった。種々の臓器、組織の可溶分画を抗αアクチン抗体で免疫沈降し、その沈降物を用いて toxin overlay assay を行った。その結果、α毒素は心臓および骨格筋では単量体より重合体αアクチンと結合することが分かった。α毒素を結合させた筋肉由来株化細胞を可溶化した後、抗α毒素抗体で免疫沈降した結果、沈降物にはαアクチンが含まれていた。これらの結果は、α毒素が細胞に結合し孔を形成した後、αアクチンと特異的に結合すること示し、さらにαアクチンはα毒素の細胞障害活性を発揮するために重要な役割を担っていることを初めて明らかにした。

この成果は、細胞溶解毒素の持つ病原性発現機構における新たな見地を与え、感染制御学の分野に貢献するところが大きく、最終試験の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。