Protein-tyrosine kinase Syk is required for pathogenengulfment in complement-mediated phagocytosis

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【 学位論文題目 】

Protein-tyrosine kinase Syk is required for pathogen engulfment in complement-mediated phagocytosis
（補体依存性の食作用におけるチロシンキナーゼＳｙｋの機能解析－食胞形成過程における必須な役割－）

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Introduction

Phagocytosis is a central event in the innate immune responses that are triggered by the association between ligands on the surface of pathogen and receptors on the membrane of phagocytes. Phagocytes then engulf and eliminate the pathogens. Among the phagocytic receptor types expressed on mammalian neutrophils and macrophages, Fc γ receptors and complement receptor 3 (CR3) have been characterized in detail. The clustering of these receptors by their ligands bound to pathogens is followed by the activation of signaling pathways that trigger dynamic rearrangements of the cytoskeleton, which lead to the formation of phagosomes. Some signaling molecules such as Rho-GTPases and PLC γ regulate actin polymerization during phagosome formation.

The most important action in complement system is to accelerate the uptake and destruction of pathogens by phagocytes. This complement-mediated phagocytosis begins with the specific recognition of bound complement components by complement receptors. In these combinations, C3bi binding to CR3 is a highly effective signal because this interaction is sufficient to stimulate phagocytosis. The protein-tyrosine kinase, Syk, plays a central role in Fcγ receptor-mediated phagocytosis in the adaptive immune system. On the other hand, the role of Syk in non-adaptive immune mechanisms is not well understood and the roles of Syk in innate immunity have received considerable focus. Besides these roles in immunoreceptor funtion, Syk is also activated upon ligation with cell surface integrin β 2 in neutrophils or monocytic cell lines. Among complement receptors, CR3 (α M β 2) and CR4 (α X β 2) are the main phagocytic receptors and both of them belong to the integrin receptor family. Considering the particular role of Syk in integrin signaling, Syk should play a critical role in innate immunity through complement-mediated phagocytosis.

The present study examined the role of Syk in complement-mediated phagocytosis using macrophage-like differentiated HL60 cells and C3bi-opsonized zymosan and indicated that Syk plays a crucial role in the process from phagosome formation to engulfment by controlling the accumulation and disassembly of polymerized actin and Rho-GTPase activation.

Materials and Methods

Cell culture and transfection. The human promyelocytic cell line HL60 was maintained in RPMI 1640 medium, supplemented with 8% heat-inactivated fetal calf serum. For stable transfection, each 10μg linealized expression construct were

transfected into 1×10^7 HL60 cells by electroporation (975μF, 350V). Stably transfected cell lines were selected by 1mg/ml hygromycin. For the rescue experiment, the Flag-rescue-Syk was introduced into Syk-siRNA/HL60 cells by electroporation and positive clones were selected with 0.2mg/ml zeocin. Cell lines were screened by the expressing level of Syk protein by the immunoblotting. The cells were induced to undergo differentiation to macrophages by seeding them on dishes in the presence of 10^{-7}M Vitamin D3 and 10ng/ml 12-o-tetradecanoylphorbol-13-acetate (TPA), and incubating for 3 days. Then macrophage-like differentiated HL60 cells were utilized for phagocytosis assay.

Phagocytosis assay. Zymosan A was incubated in 50% human serum at 37℃ for 30 minutes, and binding of C3bi to zymosan was confirmed by flow cytometry with anti-C3bi antibody. C3bi-opsonized or non-opsonized zymosan (PBS) was added to macrophage-like differentiated HL60 cells (ratio of cell : zymosan particle, 1 : 10) and incubated for indicated times at 37℃ in a 5% CO_2 humidified atmosphere. Texas Red zymosan was used similarly as described above and analyzed by flow cytometry for quantitative analysis of phagocytosis assay.

Quenching assays and actin accumulation around phagosome. To make a distinction whether zymosan particles exist inside or outside the cells, AlexaFluor 488 zymosan was pretreated as described above and added to the cells, and incubated for indicated times, then the cells were analyzed by fluorescent microscope before and after treatment of 0.2% trypan blue in PBS. For analysis of actin accumulation around phagosome, phagocytosis assay was performed and the cells were fixed and then incubated with AlexaFluor 594 labeled phalloidin and observed with a confocal laser-scanning microscope.

Immunoprecipitation and immunoblotting. C3bi-opsonized zymosan was added to macrophage-like differentiated cells and incubated for indicated times. For the immunoprecipitation studies, cells were solubilized in 0.05% SDS lysis buffer. Then the immunoprecipitated proteins and total cell lysates were separated by SDS-PAGE and electronically transferred to PVDF membrane and visualized by the chemiluminescence reagent.

GST pull-down assay for activated RhoA. Rho Binding Domain (RBD) cDNA was produced by polymerase chain reaction (PCR) from cDNA corresponding to the residues 7-89 amino acids of mouse rhotekin. The construct was introduced into E.coli DH5α and the proteins were purified with GST-Sepharose beads. C3bi-opsonized zymosan was added to macrophage-like differentiated cells and

incubated for indicated times. The cells were lysed in pull-down lysis buffer. Then the lysates were incubated with GST-rhotekin RBD immobilized on glutathione-Sepharose 4B beads. The proteins on the beads were separated by SDS-PAGE and GTP-bound RhoA was detected by immunoblotting with anti-RhoA antibody.

Results

Syk is tyrosine-phosphorylated during complement-mediated phagocytosis in macrophage-like differentiated HL60 cells. We investigated the role of Syk in innate immunity, especially in complement-mediated phagocytosis using macrophage-like differentiated HL60 cells incubated with Vitamin D3 and TPA for three days. After three days, the cells became morphologically macrophage-like and the cell surface expression of complement receptor 3, CR3 gradually increased, while the amount of Syk remained unchanged. Microscopy showed that C3bi-opsonized, but not non-opsonized zymosan particles promptly attached to and were ingested by the cells. In order to determine whether Syk is involved in complement-mediated phagocytosis, macrophage-like differentiated HL60 cells were incubated with zymosan pretreated with PBS, human serum, IgG-removed serum, serum in the presence of compstatin (a C3-convertase inhibitor). Syk was tyrosine-phosphorylated after incubation with zymosan pretreated with both serum and IgG-removed serum (C3bi-zymosan), but not with zymosan pretreated with either PBS or with serum in the presence of compstatin. Furthermore, the phagocytosis was clearly suppressed when the cells were incubated with zymosan pretreated with PBS or compstatin.

Dominant-negative Syk and Syk-siRNA inhibit the C3bi-CR3-mediated phagocytosis. To investigate the role of Syk in complement-mediated phagocytosis, stable cell lines expressing DN-Syk or Syk-siRNA were generated. Further, to support the effects of Syk-siRNA, Flag-rescue-Syk was transfected into Syk-siRNA/HL60 cells and stable cell line was isolated. The expression of CR3 in the surface of these cell lines was similar to that of parental HL60 cells. Tyrosine-phosphorylation of Syk induced by C3bi-zymosan was suppressed in DN-Syk/HL cells. Using C3bi-opsonized zymosan, we compared the effects of phagocytosis among these cell lines. The phagocytic activity was suppressed in DN-Syk/HL and Syk-siRNA/HL cells, but transfer of Flag-rescue-Syk restored the phagocytosis. These results indicate that Syk plays a significant role in the process of C3bi-CR3-mediated phagocytosis.

Syk is essential for pathogen engulfment rather than for attachment via

complement receptor. After the phagocytosis assay with C3bi-opsonized fluorescent zymosan, the cells were analyzed before and after trypan blue staining by fluorescent microscopy and fluorescent zymosan particles were counted. Most of the C3bi-zymosan attached to the parental HL60 cells and the Flag-rescue-Syk expressing cells was rapidly engulfed and that the number of C3bi-zymosan outside the cells was similar among all of these cell lines. The defective complement-mediated phagocytosis of DN-Syk/HL cells and Syk-siRNA/HL cells was not due to decreased C3bi-binding to CR3 but to loss of internalization of the CR3-bound zymosan.

Syk affects actin dynamics around the C3bi-mediated phagosomes. After synchronized phagocytosis, actin fibers and Syk accumulated in the region of the forming phagosome that surrounded the C3bi-zymosan particles at 5 minutes in the parental HL60 cells and the Flag-rescue-Syk expressing cells. At 30 minutes, actin began to disassemble, but Syk was sustained around phagosomes. In both DN-Syk/HL cells and Syk-siRNA/HL cells, the number of phagosomes surrounded by actin were clearly decreased at 5 minutes, but the accumulation of actin was sustained even at 30 minutes. At the beginning of the C3bi-CR3-mediated phagocytosis, PLCγ2 was co-precipitated with Syk promptly, but attenuated at 30 minutes.

Syk activates the RhoA-pathway in C3bi-CR3-signaling. Using GST-rhotekin-RBD pull-down assays, we investigated whether Syk regulates RhoA-signaling in complement-mediated phagocytosis. Incubation with C3bi-opsonized zymosan led to the prompt activation of RhoA in the parental HL60 cells and the Flag-rescue-Syk expressing cells, but transfer of DN-Syk or Syk-siRNA suppressed RhoA activation. Furthermore, Vav, one of guanine nucleotide exchange factors (GEFs), was tyrosine-phosphorylated in the process of phagocytosis in the parental HL60 cells and the Flag-rescue-Syk expressing cells, but not in DN-Syk or Syk-siRNA expressing cells. These results indicated that Syk acts as an activator of the RhoA-pathway and Vav acts as a GEF downsteam of Syk in C3bi-CR3-signaling.

Disscusion

It han been demonstrated that transfer of DN-Syk or Syk-siRNA suppressed the C3bi-opsonized-zymosan-induced phagocytosis in macrophage-like differentiated HL60 cells. Among the signaling molecules, transfer of DN-Syk or Syk-siRNA dramatically down-regulates activation of RhoA and tyrosine phosphorylation of Vav in complement-mediated phagocytosis. Therefore, Syk is a crucial upstream

regulator of Vav-RhoA signaling that generates contractile force. Quenching assays revealed that Syk is essential for pathogen engulfment rather than for attachment via complement. Furthermore, at the early stage of the C3bi-opsonized-zymosan-induced phagocytosis, Syk accumulated around the phagosomes and actin also accumulated around the phagosomes, but decreased late while Syk was sustained. It is reported that PI(4,5)P_2 contributes to the termination of actin assembly during the late stage of phagosome-completion. Immunoblotting assays revealed that PLC γ 2 was co-precipitated with Syk promptly, then attenuated at 30 minutes in this process. These results suggest that Syk has a function to activate PLC γ 2, then regulates actin accumulation. Taken together, both the PLC γ 2- and RhoA-pathways are under the control of Syk in complement-mediated phagocytosis.

Therefore , we propose a mechanism of complement-mediated phagocytosis as follows: the ligation of pathogen-bound C3bi to the receptor CR3 directly aggregates CR3; this ligation induces (1) actin polymerization at the forming phagosome that might be controlled by GTPases of the Rho family Rac1 and Cdc42 (not RhoA); (2) after the accumulation of actin, disappearance of PI(4,5)P_2 after hydrolysis by PLC γ 2 has a critical role in the termination of actin assembly and in disassembly from the phagocytic cup that liberate elements of the cytoskeletal machinery for assembly elsewhere; (3) contractile force created by the downstream of Vav-RhoA signaling might engulf the phagosome after the completion of phagosome.

In conclusion, Syk plays an indispensable role in complement-mediated phagocytosis by regulating both actin dynamics and Vav-RhoA-activation pathway and that these functions of Syk lead to phagosome formation and pathogen engulfment.

論文審査の結果の要旨			
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審査委員 Examiner	主　査 Chief Examiner　中村俊一 印 副　査 Vice-examiner　堀田博 印 副　査 Vice-examiner　南康博 印		
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（要旨は１，０００字～２，０００字程度）

マクロファージなどの食細胞によるファゴサイトーシス（食作用）は感染微生物からの生体防御に必須の役割を果たします。その主な分子メカニズムとして、Fcg 受容体を介した機構、および補体受容体 CR3 を介した機構があります。とりわけ補体系路は自然免疫における機能が注目を集めていますが、抗体のシステムに比べ研究が遅れていました。申請者らはヒト血清の補体活性化機構およびヒト白血病細胞株を利用することにより、補体を介したファゴサイトーシスをインビトロで再現する系を確立し、補体依存性のファゴサイトーシスにおいて、チロシンキナーゼ Syk がファゴゾームの形成と細胞内輸送に必須の役割を果たしている事を明らかにしました。

まず申請者らは食細胞として、マクロファージ様に分化したヒト白血病細胞株 HL60（ビタミン D3 と TPA により 3 日間処理）を、貪食される微生物として主に酒酵母死菌、Zymosan を用いました。ヒト血清における補体活性化システムを利用して、Zymosan を C3bi によりオプソナイズしました（C3bi-Zymosan）。これらを用いて貪食過程を動画にて解析することにより、C3bi-Zymosan は分化した HL60 に速やかに貪食されますが Non-opsonized-Zymosan の場合はほとんど捕捉されず補体依存性貪食の良い解析系である事が確認できました。

次に申請者らは補体を介した貪食における Syk の役割を明確にするため、優勢抑制型変異株および Syk-siRNA の安定的発現株を作製しました。また、貪食のプロセスをより定量的、定性的に解析するため、蛍光標識した Zymosan を利用しました。

貪食評価法として、1. フローサイトメトリーによる蛍光 Zymosan の接着または取り込みの計測をおこないました。C3bi-Zymosan を用いた場合には約 1/3 の細胞が陽性でしたが Non-opsonized-Zymosan の場合には陽性細胞はほとんど認められませんでした。この方法で比較しますと Syk-優勢抑制型変異株および Syk-siRNA の安定的発現株ではそれぞれ、約 15％～20％の細胞が陽性であり野生型 HL60 に対して抑制効果が認められ、Syk が補体依存性のファゴサイトーシスに重要であることがわかりました。しかしこの方法では Zymosan が細胞の内側にあるか外側にあるかを区別することができません。申請者らは Syk の関わるプロセスをより明確にするため、クエンチ剤により細胞外の Zymosan を消光して解析しました。野生型 HL60 細胞では補体受容体、CR3 に結合した C3bi-Zymosan はすみやかに取り込まれますが Syk-優勢抑制型変異株および Syk-siRNA の安定的発現株では、受容体に結合するだけで取り込まれないことがわかりました。この結果よりは Syk が補体を介した貪食において、その細胞内への取り込み、おそらく食胞の形成機構に関わると判断しました。

申請者らはさらに、Syk の下流でどのようなシグナル伝達がこのプロセスに関わるのかを解析しました。貪食細胞が食胞を形成して微生物を細胞内へ取り込むには細胞骨格系の再構築が必要です。そこで細胞運動、とくに細胞の収縮に関わるスモール G タンパク質として Rho ファミリーGTPase に着目しました。野生型 HL60 細胞では補体を介した貪食によりはすみやかに RhoA の活性化が認められたが Syk-優勢抑制型変異株および Syk-siRNA の安定的発現株では抑制されていました。また RhoA の GEF である Vav について、その活性化に必要なチロシンリン酸化を解析しました。RhoA 同様に野生型 HL60 細胞ではリン酸化の亢進が認められましたが Syk-優勢抑制型変異株および Syk-siRNA の安定的発現株では抑制されていました。このように補体依存性のファゴサイトーシスの一連の過程においてチロシンキナーゼ Syk が Vav-RhoA シグナリングを介して細胞内への取り込みに必須の役割を果たす事を明らかにしました。

本研究はファゴサイトーシスの分子機構を解析したものであり、これまで明らかにされていなかった補体を介するファゴサイトーシスの系に於いて、チロシンキナーゼ Syk が重要な役割を演じていることを実験的に初めて証明した価値ある集積である。よって、本研究者は博士（医学）の学位を得る資格があると認める。