博士（生命科学）　Suwarti

学位論文題名

Recognition of CpG oligodeoxynucleotides by human Toll-like receptor 9 and subsequent cytokine induction

(ヒト由来Toll様受容体9のCpGオリゴデオキシヌクレオチド認識とそれに伴うサイトカインの誘導)

学位論文内容の要旨

Toll-like receptors (TLRs) are pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs), and play a critical role in the innate immune response to invading pathogens. In human,10 kinds of TLRs, TLR1-10, have already been identified and classified into subfamily based on PAMPs type. TLR1, 2, and 6 are grouped into lipoprotein or lipopeptides recognizing-PAMPs, TLR4 and 5 are classified into flagellin and lipopolysacharide-PAMPs subfamily, while TLR3, 7, 8, and 9 are crucial for nucleic acid PAMPs recognition. TLR9 is activated by DNA from invasive bacteria and by synthetic oligodeoxynucleotides (ODNs) containing unmethylated cytosine-phosphate-guanosine (CpG) motifs. Signal transduction of this transmembrane protein induced by CpG ODN has a pivotal role as a first line of immune defense in the human body. Three-dimensional structure of TLR9 has not been reported yet, and the ligand-binding mechanism of TLR9 is still poorly understood. In this study, we constructed several human TLR9 (hTLR9) mutants, including predicted truncated mutants and single mutants in predicted CpG ODN-binding site, and analyzed the role of potential important regions of TLR9 in receptor signaling. For stimulation of hTLR9, we used using phosphorothioate (PTO)-modified CpG ODNs and also "natural" CpG ODNs consisting of only phosphodiester (PD) backbone-based ODN, because the widely used CpG ODN-PTO do not faithfully recapitulate natural DNA-mediated TLR9 activation.

In chapter 1, a general introduction of this study and previous research were addressed, including the content of functional TLR9 and related TLR family, the interaction between TLR9 and CpG ODN, the modification and medical applications of CpG ODN.

In chapter 2, we examined whether truncated hTLR9 was activated by CpG ODNs. The proteolytic cleavage of TLR9 is a prerequisite for signal transduction in mouse; however, whether the proteolytic event of TLR9 occurs in humans remains unclear. C-terminal truncated of hTLR9 extracellular domain (ECD) had been proved to be the requirements of murine TLR9 (mTLR9) activation. We designed hTLR9 recombinant and truncation mutants design similar to that of functional truncated-mTLR9 mutation to assess whether likely mechanism is also applicable hTLR9. Our mutation comprises of C-terminal (1-26 + 471-1032 amino acid (aa)), Undefined Region (440-1032 aa), and N-terminal (1-470 aa + 814-1032 aa). Then, we transfected these vectors into HEK293 cells and stimulate with both CpG ODN-PTO and PD. Our result revealed, CpG ODN-PD induce NF-κB activation at similar level of CpG ODN-PTO in transfected HEK293-hTLR9-HA (hTLR9-WT). Both of ligand also failed to induce NF-κB activity in entire truncated mutants of transfected HEK293 cells. These results showed that truncated hTLR9 alone is not sufficient enough to activate hTLR9 independently of ligand backbone modification.

In chapter 3, we identified essential residues in C-terminal and irregular Leucine Rich Repeat (LRR) in N-terminal of hTLR9 ECD for signaling receptor activity. Based on previous homology modeling study, 10 essential residues in C-terminal of hTLR9 ECD were predicted to be responsible for binding and signaling activity. We constructed vector containing mutation of each predicted essential residues and transfected into HEK293 cells. We analyzed the ligand binding and signaling properties of that CpG ODN-PTO and ODN-PD. Among those 10 essential residues, eight amino acid mutations (R481A, N483A, H505A, Q510A, H530A, K532A, Y554A, and Q557A) disrupted signaling responsiveness over CpG ODN-PTO stimulation. Mutation of H505, Q510, H530, and Y554 almost totally abrogated activity when CpG ODN-PD was used as the TLR9 ligand. H505, H530, and Y554 vicinally oriented and formed a positive charged cluster with which negative charged ODN could interact on

predicted TLR9 structure. Negative charged phosphate group or sulfate group on CpG ODN would occupy a position in close proximity to residues 505, 530, and 554 in the ligand-receptor complex. This would trigger a conformational change of TLR9, and then induce type I interferon or pro-inflammatory cytokine production. To complete the information of TLR9-signaling residues mapping, we constructed LRR mutations in N terminal of hTLR9 ECD. Recent study suggested that irregular LRR 2, 5, and 8 had a pivotal role in mTLR9 signaling activity. Hence we designed vector containing deletion of LRR 2, 5, and 8 and transfected into HEK293 cells. We noticed LRR deletion abolished the signaling response of both CpG ODN-PTO and ODN-PD stimulation. Therefore, the irregular LRRs in the N-terminal region are essential for hTLR9 function.

In chapter 4, we analyzed the impact of CpG ODN-PTO and ODN-PD stimulation to natively-hTLR9 expressing cells, human peripheral blood mono nuclear cells (PBMCs). ODN-PTO is extensively used as a TLR9 ligand instead of ODN-PD because of its high stability and stimulating effect; however, CpG ODN-PTO is assumed to induce unspecific reaction leading to antibody production and safety concerns. We stimulate human PBMC with both CpG ODN-PTO and ODN-PD, and detect the IL-6 secretion level. Interestingly, we found CpG ODN-PTO induces IL-6 secretion level almost three times higher of CpG ODN-PD in PBMC. Furthermore, PBMC fractionation using anti $CD20^-$ B cells showed, CpG ODN-PTO induce higher level of IL-6 in $CD20^+$ B-cells compare to CpG ODN-PD. We assumed this differences is thought to be PTO unspecific binding since GpC ODN-PTO also induced IL-6 secretion instead of GpC ODN-PD, whereas GpC sequence is non-immunostimulatory ODNs. Our assumption was also supported by the fact that inhibitory sequence of TLR9 pathway, IRS 869 inhibit the IL-6 secretion when simultaneously added with CpG or GpC ODN-PTO in human PBMC, resembling the IL-6 signal is secreted via TLR9 pathway. These data collectively suggest CpG ODN-PD induce specific immune response in a CpG-sequence-dependent manner in PBMC and B-cells.

In conclusion, we have successfully analyzed the role of potential important regions of TLR9 in receptor signaling. Our recent data suggest hTLR9 truncation could not be actively alone in HEK293 cells. Using site-directed mutagenesis, we were able identify both 4 residues in the C-terminus and 3 LRR in N-terminus of ECD of hTLR9 that are required for hTLR9 signaling. Further characterization of the function and three-dimensional structure of TLR9 will allow for the development of potential therapeutic agents. Additionally, our CpG ODN2006x3-PD displays specific recognition in TLR9. By using CpG ODN-PD on behalf of CpG ODN-PTO, we were able to resolve the ligand recognition of hTLR9 and hTLR9-mediated pro-inflammatory cytokine induction mechanism to accurately mimic the immune reaction in mammalian cells.

学位論文審査の要旨

主　査　客員教授　花方信孝（連携分野）
副　査　教　授　幸田敏明
副　査　教　授　出村　誠
副　査　客員准教授　山崎智彦（連携分野）

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博士学位論文審査等の結果について（報告）

近年、自然免疫獲得において外部から体内に侵入した細菌等の核酸、特にゲノムDNAを感知する受容体としてToll様受容体9（TLR9）に関する研究が盛んに行われている。TLR9はリガンド認識により、炎症性サイトカインを誘導し炎症反応を形成する一方で、I型インターフェロン(IFN)を誘導する。I型IFNは，樹状細胞の成熟，CD8+T細胞の活性化を誘導することにより免疫活性化剤として機能する。この機能により、TLR9のリガンド分子は感染症，癌，アレルギー治療などでの臨床応用が期待されている。TLR9に関しては、立体構造解析が未だ報告されておらず、リガンド認識部位ならびにリガンド結合による構造変化、それに伴うシグナル伝達に関しては未解明な部分が多い。Suwarti氏はヒトTLR9のDNA認識に関与している部位を予測し、蛋白質工学的手法を用いて変異体を作成し、細胞内でのTLR9の活性化を指標にTLR9のDNA認識ならびにシグナル伝達に必須な部位を明らかとした。

現在までヒトTLR9についてはDNA認識機構についての報告がほとんどない。マウスTLR9とヒトTLR9はアミノ酸配列の相同性が80%以上と非常に高いが、TLR9が認識する配列が異なることから分子機能は同一ではないと考えられる。マウスTLR9については、リガンド結合領域であるロイシンリッチリピート（LRR）ドメインのプロテアーゼによる切断が起き、切断されたTLR9がエンド/ライソソームでDNA認識ならびにシグナル伝達機能を示すと報告されている。Suwarti氏はヒトTLR9の変異体を多数作成し、非メチル化CpGモチーフを有する合成オリゴデオキシリボヌクレオチド（ODN）を用いて機能評価を行った。その結果、ヒトTLR9はヒト胎児腎細胞株HEK293細胞においては、プロテアーゼによる切断は起こらず、マウスTLR9とは異なって未切断TLR9が機能を有し炎症性サイトカインの誘導に関与していることが示された。また、同じTLRファミリーですでに構造が明らかとされているTLR3のリガンド結合部位との構造比較によりTLR9のリガンド結合部位を予測し、変異導入を行ったところH505, Q510, H530, Y554をアラニンに置換したTLR9変異体は機能を失った。H505, H530, Y554はLRRドメインにおいてプラスにチャージしたクラスターを形成しており、この部位にマイナスにチャージしたDNAが結合することが予測された。また、TLR9のリガンドとして安定性を向上させるために一般に用いられているホスホロチオエート（PT）修飾CpG ODN-PTと天然DNAと同じ構造を持ち、かつ安定性の向上したCpG ODN-PDの比較を行ったところ、ODN-PTは配列非依存的に免疫細胞を活性化させるのに対して、ODN-PD配列依存的かつTLR9を介してのみ活性化させたことから、TLR9のヒト細胞内での機能解析を行う上でCpG ODN-PDのリガンドとしての有用性が示された。

これを要するに、Suwarti氏はヒトTLR9のDNA認識ならびにシグナル伝達に必須な部位について新知見を得たものであり、ヒトTLR9の機能解明に貢献するところ大なるものがある。

よって著者は，北海道大学博士（生命科学）の学位を授与される資格あるものと認める。