博 士（生命科学） 孟 文 君

学 位 論 文 題 名

Nuclease-resistant Phosphodiester CpG Oligodeoxynucleotide as Toll-like Receptor 9 Agonist and its Intracellular Delivery

(Toll-like receptor 9アゴニストとしての核酸分解酵素耐性天然型 CpG オリゴデオキシヌクレオチドとその細胞内デリバリー)

学位論文内容の要旨

Unmethylated cytosine-phosphate-guanine oligodeoxynucleotides (CpG ODN) can be recognized by Toll-like receptor 9 (TLR9), a member of the Toll-like receptors family which plays a critical role in the innate immune response to invading pathogens. The stimulation of TLR9 by CpG ODN triggers an intracellular signaling, leading to the activation of dendritic cells and B cells, and production of proinflammatory cytokines and type I interferons (IFNs). Consequently, CpG ODN can be used either as a standalone molecule or as a vaccine adjuvant for the immunotherapeutic treatment of cancer, allergy and infectious diseases. However, application of the unmodified natural CpG ODN with phosphodiester (PD) backbone to immunotherapy has been greatly limited by their extreme susceptibility to nuclease degradation which renders them inactive in the free form. Modifications in the CpG ODN backbone, and various delivery methods including mixing and cross-linking of ODN to other carrier materials such as liposomes, biodegradable microparticles and inorganic nanoparticles have been shown to significantly enhance the biological activity of ODN. But the backbone modification by substituting the oxygen with sulphur to create a phosphorothioate (PTO) ODN is associated with several inherent disadvantages including unspecific binding to various proteins and renal damage. Therefore, it is still required to develop some nuclease-resistant CpG ODN with entirely natural PD backbone to avoid the side effects of the chemical modification. Furthermore, many of the reported delivery systems are still involved in modified CpG ODN; so it is still challenging to develop suitable vehicles for natural CpG ODN delivery. Hence, this dissertation focuses on the development of a novel nuclease-resistant TLR9 agonist without any backbone modification as well as an intracellular delivery system for the natural CpG ODN.

In chapter 1, a general introduction of this study and previous research was addressed, including the content of immune system, the ligands and signal pathways of the identified 10 human and 12 mouse TLRs, the interaction between TLR9 and CpG ODN, the modification and medical applications of CpG ODN, and drug delivery systems for CpG ODN.

In chapter 2, a safe and effective CpG ODN consisting of a PD backbone for human TLR9 (hTLR9) activation was developed. Firstly, we systematically investigated the structure features of the modified PTO-ODN2006 (5'-tcgtcgttttgtcgttttgtcgtt-3') for NF-κB activation via a TLR9-dependent pathway. The activity increased 50 times when PTO CpG ODN was used at a final concentration of 0.1 μM comparing with natural PD-ODN2006. The stimulation experiment showed that TLR9 can be activated in a CpG-dose-dependent and sequence-dependent manner. Therefore, PD-ODN2006 was selected as a basic structure for the development of suitable natural CpG ODNs. Then we modified 3'-end of PD-ODN2006 with organic molecules such as biotin, FITC and amino group, which increased the NF-κB activity due to the increased resistances to nucleolytic degradation by the modification. Furthermore, enhancement of

hTLR9 activity was found to be dependent on the number of CpG core motifs (GTCGTT) in the PD ODN containing the 3'-end oligonucleotides. In particular, ODN sequences consisting of 2 or 3 linked ODN2006 sequences with a PD backbone (e.g., PD-ODN2006-2006 and PD-ODN2006-2006-2006) acted as effective agonists of hTLR9 even at lower concentrations. The cytokine production *in vitro* was also examined, suggesting that these novel natural CpG ODNs have similar properties as class B CpG ODN which consisting of a PTO backbone.

In chapter 3, a potential PD-ODN2006 delivery system, which combines amino-modified mesoporous silica SBA-15 (SBA-NH_2) particles with polycation poly (allylaminehydrochloride) (PAH) to form SBA-ODN-PAH complexes, was presented. Stability, cell uptake, in vitro cytotoxicity, and NF-κB activity of the SBA-ODN-PAH complexes were evaluated. Gel electrophoresis indicated that the SBA-ODN-PAH complexes exhibited enhanced serum stability due to the dual protection effects of the SBA-NH2 particles and the PAH coating. The SBA-ODN-PAH complexes were taken up by 293XL-hTLR9 cells and little cytotoxicity was expressed in a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Most importantly, the SBA-ODN-PAH complexes significantly enhanced NF-κB activity, stimulated by interaction between the internalized CpG ODN and hTLR9. Thus, positive-charged silica nanoparticles provide a promising strategy for increasing the nuclease-resistance of natural CpG ODN and enhancing ODN the delivery efficiency.

In chapter 4, based on the conclusions from chapter 2 and chapter3, another ODN delivery system combing the novel PD-ODN2006-2006-2006 and positively-charged silica nanoparticles was prepared. The negatively-charged silica nanoparticles were modified with polyethylenimine (PEI), a cationic polymer, to achieve a positive surface charge; and made the negatively-charged ODN prone to bind on the nanoparticles. Surprisingly, when stimulating the peripheral blood mononuclear cell (PBMC), the production of IFN-α by the complex was significantly increased as well as IL-6, compared with free PD-ODN2006-2006-2006. These results demonstrate that it is possible to endow our novel nuclease-resistant CpG ODN, which is similar to class B CpG ODN in its free form, with additional immune properties of type A CpG ODN, by loading it onto the nanoparticles. This might be explained by the formation of complicated structures similar to type A CpG ODN when applying our novel CpG ODN to nanocarriers.

In conclusion, 3'-modified CpG ODNs with oligonucleotides consisting of a PD backbone are effective at resisting nucleolytic degradation and increasing their biological activity in hTLR9 stimulation. In addition, mesoporous silica nanoparticles significantly enhance the delivery efficiency of natural CpG ODN. Finally we applied the PEI-modified silica nanoparticles to our novel PD-ODN2006-2006-2006, endowing the novel PD-ODN with some immune properties of class A CpG ODN, indicated by the significantly increase of IFN-α production compared with the free PD-ODN2006-2006-2006. It is expected that combination of the natural novel CpG ODN and the novel silica drug delivery system can be applied to some promising immunotherapeutic applications to varieties of diseases.

学位論文審査の要旨

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Nuclease-resistant Phosphodiester CpG Oligodeoxynucleotide as Toll-like Receptor 9 Agonist and its Intracellular Delivery

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近年，自然免疫獲得において異物の侵入を感知する受容体であるトール様受容体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 のリガンド分子として、非メチル化 CpG モチーフを有する合成オリゴヌクレオチド(ODN)が盛んに研究されており、体内でのヌクレアーゼでの分解を防ぐためにヌクレオチド間のあるリン酸基をホスホロチオエート化した ODN（S化 ODN）が広く研究に用いられている。しかしながら、S化 ODN は非特異的に蛋白質に吸着し、副作用・生体毒性を細胞内で示すことが報告されている。本論文では、上記の問題を解決すべく、天然の DNA の骨格を持ちながらもヌクレアーゼ耐性を持ち、かつ細胞内で TLR9 のリガンドとして機能する核酸分解酵素耐性天然型 CpG ODN である PD-ODN2006-2006-2006 を開発し、既存リガンド分子との比較を行い、優位性を示した。PD-ODN2006-2006-2006 は既存のリガンド分子である S化 ODN と比較して、低濃度で TLR9 を活性化することができ、またヒトの初代細胞においても、有効的に IL-6 の産出を誘導することを示した。特に樹状細胞において IL-6 を高く誘導する結果が得られ、今まで報告された CpG リガンドでは達成できなかった特性を示した。これらのことから、本論文で報告した PD-ODN2006-2006-2006 の有用性、新規性が高いと評価された。また、核酸分解酵素耐性天然型 CpG ODN を標的細胞に輸送するためのナノ粒子の開発を報告した。細胞毒性が低く、ポリカチオン処理を行ったアミノ基修飾シリカナノ粒子を開発し、CpG ODN を効果的に細胞内に取り込ませ、IL-6 の誘導を示すことを見出した。さらには、カチオンナノ粒子に PD-ODN2006-2006-2006 を固定することにより、CpG 単独では誘導できない IFNα を誘導することができることを見出した。すなわち、炎症性サイトカインとI型 IFN の誘導を自在にコントロールすることができるという結果を導き、誘導のスイッチングのメカニズムを考察し、新たな知見を得た。

これを要するに、筆者は TLR9 のアゴニストについて、天然 DNA 型の CpG オリゴデオキシヌクレオチドを新規に開発し、またカチオンナノ粒子と組み合わせることにより細胞への輸送性を向上させ、またサイトカイン誘導のスイッチングについて新知見を得たものであり、治療薬としての Toll-like receptor 9 のリガンド開発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。