Summary of Doctoral Dissertation

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Title of Doctoral Dissertation

The impact of complex formation and size of guanine quadruplex structure-based CpG oligodeoxynucleotides on immunomodulatory responses

グアニン四重鎖構造形成 CpG オリゴデオキシヌクレオチドの
免疫活性化機能に対する複合体の構成とサイズの影響に関する研究

Oligodeoxynucleotides (ODNs) containing unmethylated cytosine-phosphate-guanine (CpG) motifs serve as potent vaccine adjuvants by stimulating humoral and cellular immune responses through the activation of toll-like receptor 9. However, their clinical use is limited owing to the rapid nuclease-mediated degradation. Phosphorothioate-modified CpG ODNs (PT-CpG ODNs) exhibit an increased resistance to nucleases; however, these ODNs are significantly associated with side effects. We have reported that CpG ODNs, with fully phosphodiester backbones, form guanine quadruplexes (G4) and demonstrate enhanced resistance to nuclease and immunostimulatory properties compared to linear CpG ODNs. However, the insufficient cellular uptake and nuclease stability of these G4-CpG ODNs lead to reduced immunostimulatory activity compared to that of PT-CpG ODNs. To overcome these limitations, we used cationic carriers, such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) cationic liposome, to deliver G4-CpG ODN. Our findings reveal that the G4-CpG ODNs-DOTAP liposome complex-mediated cytokine response, specifically the production of interleukin (IL)-6 and interferon (IFN)-α, is influenced by the complex's composition and the ODN sequence. Nonetheless, comprehensive knowledge of the mechanism underlying the effects of these factors on the immunomodulatory characteristics of G4-CpG ODNs is lacking. We hypothesize that variations in the ODN structure and charge ratio of the complex potentially influence their size, and regulate cellular uptake and cytokine induction. In this study, we aim to investigate the effects of the key regulatory parameters of nanoparticle-mediated G4-CpG ODN delivery on cytokine profiles.

In the **first chapter,** we provide general background information related to this study through discussions on CpG ODNs, G4-structure, structure-dependent immunostimulatory effects of CpG ODNs, and their various delivery systems.

In **chapter two**, the influence of the charge ratio of the G4-CpG ODN-DOTAP liposome complex on its physicochemical properties and cytokine profiles has been elucidated. The efficiency of CpG ODN delivery largely depends on the cationic carrier/DNA charge ratio, defined as the ratio of the number of positive charges in the cationic carrier to the number of negative charges in the DNA backbone. G4-CpG ODN-DOTAP liposome complexes were formulated at different charge ratios by adjusting the relative quantity of DOTAP in the complex. The altered charge ratio of the complex significantly affected its properties, including topology, particle size, surface charge density, and stability. Linear structure-based CpG ODN-DOTAP liposome complexes resulted in micrometer-sized aggregates at all charge ratios, while CpG ODNs with the G4 structure

formed nanosized complexes. G4-CpG ODN-DOTAP formed small (<350 nm) particles at a lower charge ratio (0.5–1.5), and larger microparticles (1200–2200 nm) only at a higher charge ratio (>2). The results reflect the greater effectiveness of the G4 structure in forming stable ODNs-cationic liposome complexes compared to that of linear CpG ODNs. Moreover, G4 formation enhances the immunostimulatory activity of the CpG ODN-DOTAP complex. Our results indicate that G4-CpG ODN-DOTAP complexes, associated with smaller and more stable particle sizes, induced significantly higher cytokine production than that induced by linear CpG ODN-DOTAPs forming larger aggregates. The cytokine induction depends on the formation of CpG ODN and the charge ratio of the complex. Complexes with low charge ratios (0.5 and 1.5) simultaneously induced significantly higher levels of IL-6 and IFN-α than those with high charge ratios (2 and 2.5) and larger microparticle size. Confocal microscopy revealed that large microparticles (with size in the micrometer range) were not taken up into the cell. These analyses confirmed a direct correlation between cellular uptake of these complexes and cytokine production, highlighting the significance of complex formation and particle size in modulating immune responses.

In **chapter three**, we demonstrated the effect of the small particle size (<100 nm) on the immunostimulatory properties of G4-CpG ODNs using gold nanoparticles (AuNPs: 5, 20, 100 nm) as carriers. Although AuNPs alone triggered pro-inflammatory cytokines, they did not induce IFN-α production. In contrast, G4-CpG ODN-loaded AuNP significantly induced IFN-α production in a size-dependent manner via the TLR9 pathway. G4-CpG ODN-loaded 20 nm and 100 nm AuNPs significantly stimulated IFN-α responses; however, G4-CpG ODN-loaded 5 nm AuNP was ineffective in promoting IFN-α production. Further analyses validated that particle size influences the intracellular trafficking of the complexes, thereby affecting the TLR9-mediated cytokine production pathway. Fluorescence dye-labeled G4-CpG ODN-loaded 20 and 100 nm AuNP were localized in early endosomes, activating TLR9 and the MyD88–IRF7 signaling pathway, crucial for type-I interferon production. Contrastingly, fluorescence dye-labeled G4-CpG ODN-loaded 5 nm AuNP rapidly transitioned to late endosomes, and led to reduced IFN-α response. These results suggest that the G4-CpG ODNs-induced immune response can be fine-tuned by controlling the size of the G4-CpG ODN-carrier complex.

Chapter four summarizes the outcomes of this study and conveys the concluding remarks. We, for the first time, have demonstrated the parameters that influence the immunostimulatory activities of G4-CpG ODNs delivered via nanoparticles, including cationic liposomes and gold nanoparticles. Overall, the charge ratio and particle size of G4-CpG ODN/carrier complexes emerged as critical factors that regulate the physicochemical properties, which potentially modulate cytokine profiles. This study elucidates the significance of optimizing structural parameters of G4-CpG ODN/carrier complexes to enhance the immunostimulatory effects.