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Title of Doctoral Dissertation

Immunostimulatory properties of CpG oligodeoxynucleotides
forming monomeric guanine-quadruplex structure
(単量体グアニン四重鎖構造を形成する
CpG オリゴデオキシヌクレオチドの免疫活性化特性)

Toll-like receptor 9 activation by oligodeoxynucleotides (ODNs) containing unmethylated cytosine-phosphate-guanine (CpG) motifs triggers the innate immune system and establishes an adaptive immune response, and therefore these ODNs have been studied as vaccine adjuvants against infectious diseases. The phosphodiester backbone of ODNs is prone to degradation by nucleases; therefore, in clinical trials, phosphorothioate-modified CpG ODNs are used to increase DNase stability. However, phosphorothioate ODNs have been reported to cause thrombocytopenia, anemia, and neutropenia. These data suggest that CpG ODNs with a phosphodiester backbone are more suitable for clinical applications. Recently, our group was the first to report that the guanine-quadruplex (G4) structure enables higher nuclease stability, cellular uptake, and inflammatory cytokine induction compared to the linear phosphodiester CpG ODN. As such, the use of G4 ODNs in immunotherapy is still in its infancy, and little knowledge about their immunomodulatory activities is known. Understanding the preclinical characteristics will make progress in studies of G4 CpG ODNs as vaccine adjuvants. Therefore, this dissertation focuses on further investigation of the immunostimulatory properties of CpG ODNs forming G4 structures, particularly monomeric G4.

In **chapter one**, we present a general background for this study with information about innate and adaptive immunity, CpG ODNs, evaluation of CpG ODNs as vaccine adjuvants, CpG ODNs forming high order structures, as well as G-quadruplex structures.

In **chapter two**, the new monomeric G-quadruplex-based CpG ODNs (G4 CpG ODNs) named GD1, GD2, and GD3, with different lengths and numbers of CpG sequences, are designed with CpG motifs in the central loop region of the G4 structure. Success in controlling the G4 molecularity into a monomer in this chapter facilitates further investigation of intrinsic factors governing the efficiency of G4 CpG ODNs, which is presented in Chapter 3. We also confirm that the immunostimulatory properties of monomeric G4 CpG ODNs are mediated by TLR9 activation. In addition, we reveal the advantages of monomeric G4 in increasing nuclease resistance, intracellular uptake, and cytokine induction *in vitro* and *in vivo* of a phosphodiester CpG ODNs.

In **chapter three**, the effects of intrinsic factors, including the position of the loop containing the CpG motif, the position of the CpG motif within a loop, spaces between a CpG motif and G-tracts, and the number of CpG ODNs on immunostimulatory activity, are examined. Adding CpG motifs into the second loop rather than the first or third loop satisfies the requirement of a stable and potent monomeric G4 CpG ODNs. An increase in the length of the loop containing the CpG motifs is necessary but not adequate for effective TLR9

stimulation. The number of CpG motifs and the spaces between CpG motifs and G-tracts are critical factors governing immunostimulatory efficiency of G4 CpG ODNs, probably because of improved flexibility in the loop region. The findings we report in this chapter provide further understanding of the mechanisms underlying the immunostimulatory activities of G4 CpG ODNs and provide hints for the rational design of G4 CpG ODNs for vaccine adjuvant applications.

In **chapter four**, we evaluate the influences of G4 ligands on the immunostimulatory properties of G4 CpG ODNs. G4 ligands are small molecules developed to stabilize G4 through binding to G4 ODNs with high affinity. Some G4 ligands have been developed to induce a specific G4 topology, such as parallel or anti-parallel. With the ability to stabilize G4 ODNs, we expect that G4 ligands may also stabilize G4 CpG ODNs, which may further increase their immunostimulatory activity. We examine the effects of two type of G4 ligands named L2H2-6OTD and L2G2-2M2EG-6OTD on a G4 CpG ODN, GD3. We find that both ligands increase the thermodynamic stability and nuclease resistance of GD3. Furthermore, the ability to enhance cytokine induction depends on the type of G4 ligand. Indeed, L2G2-2M2EG-6OTD, a parallel-inducing G4 ligand, may not be suitable for increasing the immunostimulatory properties of G4 CpG ODNs. Meanwhile, L2H2-6OTD is a potential G4 ligand that promotes an immune response to G4 CpG ODNs. These findings may represent a step forward towards development of a more effective vaccine adjuvant based on G4 CpG ODNs.

In **chapter five**, the effects of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) on the immunostimulatory properties of G4 CpG ODNs are demonstrated. Cationic lipids such as DOTAP have been widely studied as carriers for antigen and CpG ODNs in vaccine adjuvant applications. Therefore, understanding the immunostimulatory properties of the lipoplex of G4 CpG ODNs and DOTAP is an important step toward the application of G4 CpG ODNs in the field of vaccine adjuvant. Association with DOTAP enhances cytokine induction of GD2 and GD3 in mouse macrophage cell lines, as well as human B cell, and plasmacytoid dendritic cell lines. GD2 and GD3 function as TLR9 agonists when they are combined with DOTAP. Combination with DOTAP enhances G4 CpG ODN-induced interleukin (IL)-6 production in human PBMCs. Notably, the GD3-DOTAP lipoplex is able to induce IFN- α production, which is an important cytokine for anti-viral immune response, while GD3 alone does not. Surprisingly, GD2 in a complex with DOTAP does not induce IFN- α production. These results suggest that G4 CpG ODN-DOTAP is a potential Th1-inducing adjuvant system for human vaccines against infectious diseases. In addition, the provoked adaptive immune response by G4 CpG ODN DOTAP lipoplexes can be tailored by controlling lipoplex preparation.

Chapter six summarizes the concluding remarks of this study. In conclusion, we successfully developed monomeric G4 CpG ODNs, and for the first time, we investigated the parameters affecting their immunostimulatory activities. The monomeric G4 CpG ODNs developed in this study are not only effective, safe, and nuclease resistant, but also do not require a complicated conformation process. Therefore, G4 CpG ODNs are promising potent vaccine adjuvants that can be produced on a large scale without high costs. In future, the work improving the efficiency of G4 CpG ODNs based on hints given by this study and clarifying the mechanism by which G4 CpG ODN-DOTAP lipoplexes regulate IFN- α production will be conducted.