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Abstract of Doctoral Dissertation

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Title of Doctoral Dissertation

A study on the effect of guanine-quadruplex topology on immunostimulatory properties of CpG oligodeoxynucleotides
(グアニン四重鎖構造のトポロジーが CpG オリゴ核酸の免疫活性化能に与える影響)

Synthetic cytosine-phosphate-guanine oligodeoxynucleotides (CpG ODNs) are promising candidates for vaccine adjuvants, as they activate immune responses through the Toll-like receptor 9 (TLR9) pathway. However, unmodified CpG ODNs are quickly degraded by serum nucleases, and their negative charge hinders cellular uptake, limiting their clinical application. The phosphodiester bond of CpG ODNs have been modified with sulfur to a phosphorothioate bond in order to stabilize CpG ODNs. The phosphorothioate modification has reported to have toxicity towards humans causing renal damages and non-specific protein binding. To overcome these complications, we use natural CpG ODNs with guanine-quadruplex (G-quadruplex or G4) as a scaffold to aid delivery into the cells and its nuclease resistance in serum. G4 structure has three topologies depending on their orientation and bond angles of guanine present in the G4 sequence. G4 topologies have different spatial specifications, their properties when used as a scaffold for CpG ODNs have been studied in this work. Also, a study has also been conducted to further improve the adjuvant potential of naked G4 CpG ODNs by conjugating G4 ligands that bind to its G-tetrad and stabilize the G4 structure.

In **chapter one**, we provide a background about general understanding of topics related to this study. We explain G-quadruplex, its structure and properties in detail, CpG ODNs and its uses also G4 ligands as well.

In **chapter two**, immunomodulatory properties of different topologies of G-quadruplex have been investigated. Clinical applications of CpG ODNs are limited because of their poor stability in serum due to rapid degradation by serum nuclease. Non-canonical G4 structures have been used to stabilize CpG ODNs by using them in the loop of G4s. G4 structures can form in parallel, anti-parallel, or hybrid topologies, depending on the orientation of G-tracts resulting from different bond angles in guanine residues, but the effects of these topologies on CpG ODNs have not yet been explored. In this chapter, our objective is to firstly compare the immunostimulatory effects posed by three different topologies of G4 CpG ODNs and then find their difference in cellular uptake in mouse macrophage RAW264 cells. For these, we designed three distinct G4 topologies as scaffolds for CpG ODNs with comparable length. They were characterized in circular dichroism (CD) to determine the topology and polyacrylamide gel electrophoresis (PAGE) to confirm their monomeric G4 structure. The parallel G4 CpG ODN demonstrated the highest serum stability in fetal bovine serum (FBS) and cellular uptake in RAW264 macrophage cells, compared to other topologies, resulting in the strongest immune response from the cells, as quantified by ELISA. Additionally, we investigated the binding affinity of different G4 topologies to macrophage scavenger receptor-1 (MSR-1), which facilitates the entry of G4 CpG ODNs into the cells with a preference towards parallel topology. Upon internalization to the cells all topologies of G4 CpG ODNs bind to TLR9 with no topological preference, leading to activation of immune system. In short, the parallel G4 CpG ODN induced highest immune response from RAW264 cells due to higher stability in serum and higher cellular uptake in RAW264 in comparison to anti-parallel and hybrid G4 CpG ODNs using MSR-1 receptors on the cell membrane.

In **chapter three**, we have tried to enhance the immune response induced by G4 CpG ODNs. In the previous chapter we have systematically demonstrated how the parallel G4 CpG ODN exhibited highest immunostimulatory effects *in vitro*, due to higher serum stability and cellular uptake. Even though, immune response posed by naked G4 CpG ODNs is not sufficient for them to be used as vaccine adjuvants like phosphorothioate modified CpG ODNs. In this chapter, our primary objective is to enhance stability of G4 CpG ODNs and use it for *in vitro* studies in mice to study its adjuvant effects. Firstly, we have designed a parallel G4 CpG ODN, which has shown to be the best performing topology in terms of activating immune system, for enhancing its potent adjuvant effects. A parallel G4 CpG ODN was characterized, and its molecularity was determined. The synthesized majorly monomeric G4 CpG ODN showed higher immune response than some of the already reported G4 CpG ODNs. The enhancement in immunostimulatory effect was achieved by conjugating the naked G4 CpG ODNs with G4 chemical ligands which stabilized G4 structures, as demonstrated by thermal stability studies using CD. During *in vivo* studies, mice vaccinated with G4 CpG ODN/ligand/OVA's complex exhibited a higher concentration of OVA specific immunoglobulin G2c (IgG2c) concentration in blood in comparison with naked G4, indicating a strong Th1 mediated immunity. In short, in this chapter we have designed a parallel G4 CpG ODN and conjugated it with G4 ligands which enhanced its thermal stability, resulting to induction of more immune response from RAW264 macrophage cells and higher adjuvant effects in mice.

In **chapter four**, the key findings of this study have been summarized. For the first time, a meticulous study was conducted where the best performing topology was found in terms of immunomodulatory properties and its properties has been further enhanced to develop a molecule with potency of a vaccine adjuvant. This study as a whole provides information about cytokine profiles of different topologies of G4 CpG ODNs and insights about using G4 ligands in *in vitro* manner. Knowledge obtained from this study can be further optimized and used in development on natural CpG based vaccine adjuvants in future.