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論文題目	Development of nucleic acid therapeutics based on the control of their intracellular distribution （細胞内動態制御を基盤とした核酸医薬品開発に関する研究）		

（論文内容の要旨）

In recent years, nucleic acid therapeutics have been developed and used in clinical practice. Native nucleic acids have negative charges, resulting in poor membrane permeability, and they are easily degraded by nucleases existing in the blood, the tissues, and inside the cells. Therefore, it is necessary to deliver them to the target site of the target cell while avoiding degradation. In this thesis, I investigated the methodologies to deliver DNA site-specifically within cells, and applied them to exert functions as nucleic acid therapeutics. First, I attempted to elucidate the mechanism underlying the cellular uptake of DNA, and found that DNA with large and complex structures are efficiently taken up by phagocytic immune cells. Based on the results, I hypothesized that long single-stranded DNA (lssDNA), obtained by rolling circle amplification, would be suitable to target those cells due to its large size. I attempted to develop functional lssDNA which can control its intracellular distribution in macrophages or dendritic cells to elicit their immunoactivities.

CHAPTER 1: Critical contribution of macrophage scavenger receptor 1 to the cellular uptake of nanostructured DNA

Macrophage scavenger receptor 1 (MSR1) is known to contribute to cellular uptake and removal of negatively-charged molecules such as oxidized low-density lipoproteins. DNA is also negatively-charged, and MSR1 has reported to be involved in DNA uptake. To investigate how much MSR1 contributes to DNA uptake, I used polypod-like nanostructured DNA (polypodna). Knockout of MSR1 in macrophages truncated cellular uptake of polypodna and the subsequent immunoreactions. In addition, binding efficiency of MSR1 and polypodna was increased as the pod number increased. These results suggested that MSR1 plays a central role in DNA uptake and that complex and large structure of DNA is efficiently taken up through efficient recognition by MSR1, leading to the use of lssDNA in the following studies.

CHAPTER 2: Development of unmethylated CpG DNA with long-lasting endosome targeting ability

Unmethylated CpG motifs are recognized by Toll-like receptor 9 (TLR9), expressed in endosomes of immune cells, followed by secretion of proinflammatory cytokines. CpG DNA is, therefore, expected to be used as vaccine adjuvants and cancer

therapeutics. However, there are many obstacles for therapeutic application, such as delivery efficiency, biostability, and short *in vivo* half-life. I constructed CpG-incorporated lssDNA with intermolecular i-motifs by quadruplex formation (i-CpG-lssDNA). This could induce fabrication of hydrogel in an acidic environment of endosomes. I selected the sequence which forms intermolecular i-motif with high efficiency, and then synthesized lssDNA containing that sequence. i-CpG-lssDNA elicited sustained proinflammatory cytokine production compared to CpG-lssDNA without i-motifs both *in vitro* and *in vivo*. These results suggested that i-CpG-lssDNA could serve as a novel type of adjuvant for the sustained induction of immune response.

CHAPTER 3: Construction of long single-stranded DNA therapeutics targeting cytosolic DNA sensor proteins

Cytosolic DNA sensors (CDS) recognize DNA in the cytosol, leading to the activation of stimulator of interferon genes (STING) and production of type 1 interferon (IFN). Type 1 IFN evokes defensive reactions from viral infections and activates immune system. Therefore, agonists for CDS are expected as antiviral agents and cancer therapeutics. Double-stranded DNA with dozens to thousands of bases are agonists of CDS, but transfection reagents are required for their delivery to cytosol because of poor cellular uptake and endosomal escape. LssDNA is efficiently taken up and is located to endosomes, and therefore, I considered facilitated intracellular transport of lssDNA from endosomes to cytosol would be a powerful strategy for the development of an effective CDS agonist. To achieve this, an endosome-disruptive GALA peptide was conjugated to oligonucleotides to obtain peptide-DNA conjugates (PDC). Compared to lssDNA, PDC/lssDNA complex composed of lssDNA hybridizing to complementary PDC was more likely to be distributed to cytosol of dendritic cells, and induced more amount of IFN-β secretion, indicating efficient stimulation of CDS. These results suggested that PDC/lssDNA complex could serve as a novel type of CDS agonists.

In conclusion, control of intracellular distribution of lssDNA can elicit its functions as novel therapeutics, which could help develop more effective nucleic acid therapeutics.

（論文審査の結果の要旨）

The applicant investigated the methodologies to deliver DNA site-specifically within cells, and applied them to exert functions as nucleic acid therapeutics. First, the applicant attempted to elucidate the mechanism underlying the cellular uptake of DNA, and found that DNA with large and complex structures are efficiently taken up by phagocytic immune cells. Based on the results, the applicant hypothesized that long single-stranded DNA (lssDNA), obtained by rolling circle amplification, would be suitable to target those cells due to its large size. The applicant attempted to develop functional lssDNA which can control its intracellular distribution in macrophages or dendritic cells to elicit their immunoactivities.

CHAPTER 1: Critical contribution of macrophage scavenger receptor 1 to the cellular uptake of nanostructured DNA

Macrophage scavenger receptor 1 (MSR1) is known to contribute to cellular uptake and removal of negatively-charged molecules such as oxidized low-density lipoproteins. The applicant has demonstrated that MSR1 plays a central role in DNA uptake and that complex and large structure of DNA is efficiently taken up through efficient recognition by MSR1, leading to the use of lssDNA in the following studies.

CHAPTER 2: Development of unmethylated CpG DNA with long-lasting endosome targeting ability

Unmethylated CpG motifs are recognized by Toll-like receptor 9 (TLR9), expressed in endosomes of immune cells, followed by secretion of proinflammatory cytokines. The applicant constructed CpG-incorporated lssDNA with intermolecular i-motifs by quadruplex formation (i-CpG-lssDNA). This could induce fabrication of hydrogel in an acidic environment of endosomes. I selected the sequence which forms intermolecular i-motif with high efficiency, and then synthesized lssDNA containing that sequence. i-CpG-lssDNA elicited sustained proinflammatory cytokine production compared to CpG-lssDNA without i-motifs both *in vitro* and *in vivo*. These results suggested that i-CpG-lssDNA could serve as a novel type of adjuvant for the sustained induction of immune response.

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In conclusion, control of intracellular distribution of lssDNA can elicit its functions as novel therapeutics, which could help develop more effective nucleic acid therapeutics.

以上、本論文は博士（薬学）の学位論文として価値あるものと認める。また、令和５年２月 14 日、論文内容とそれに関連した事項について試問を行った結果、合格と認めた。

要旨公表可能日：　　年　　月　　日以降