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≪若手研究者紹介≫

Development of Cell-Selective Delivery Systems and Research Life in Japan

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1. Introduction

Study abroad was one of my goals after graduated and it was strongly suggested from my colleagues when I worked in Chulalongkorn University. In 2003, I received a Monbusho scholarship from the government of Japan. Based on my interests, I came to Prof. Mitsuru Hashida's laboratory, Kyoto University, to study pharmaceutics and pharmacokinetics. Of course, studying in Japanese is very difficult for foreign students, including me. [Do my best] I told myself and keep doing until now. Based on the specialty of Prof. Hashida's laboratory in the development and evaluation of drug delivery system (DDS), my main research during master and doctoral program was the development of macrophage-selective delivery using mannosylated lipid nanoparticles for anti-inflammatory therapy. After graduated, I extended my study to the development of amino acid dendrimers. It was quite different from previous study by requiring more skills in chemical synthesis and analysis. Although there are sometime difficult, time-consuming, and many problems for planning and understanding an unfamiliar research, I am really enjoy challenging in this research.

In this forum, my previous and current researches are summarized in section 2 and 3. In addition, my research life, experience, and opinion are discussed in section 4.

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2. Mannosylated Lipid Nanoparticles for Macrophage Targeting

Macrophages play a key role in the inflammatory response against noxious stimuli and infection to release a variety of inflammatory mediators. They function as instigators and orchestrators in inflammatory response both acute and chronic inflammation. To increase therapeutic efficacy and decrease systemic side effects of drug/drug candidate, macrophage-selective delivery systems using mannosylated lipid nanoparticles were developed based on the recognition of mannose moiety by mannose lectin receptors. As for the systemic therapy, Kupffer cells (liver resident macrophages) are the main effector cells for inflammatory response. The intravenously injected mannosylated emulsions (110–130 nm in diameter) showed several advantages including high incorporation of water-insoluble anti-inflammatory drugs and high accumulation in the liver, especially in the non-parenchymal cells (NPC), consisting of Kupffer and endothelial cells, via mannose receptor-mediated mechanism. As for local inflammatory therapy such as in the lung which is the major organ exposing to external stimuli for lung inflammation, the intravenously injected mannosylated emulsions showed no advantages due to very low lung accumulation.

Activation of alveolar macrophages, resident macrophages in the lung, is a hallmark of lung inflammation which is a fundamental pathologic process in pulmonary diseases. Pulmonary surfactants functioning in lung homeostasis and structure are cleared by alveolar macrophages. Therefore, to develop targeting carriers compatible with lung microenvironment, liposomes were modified with varied mannose density and their

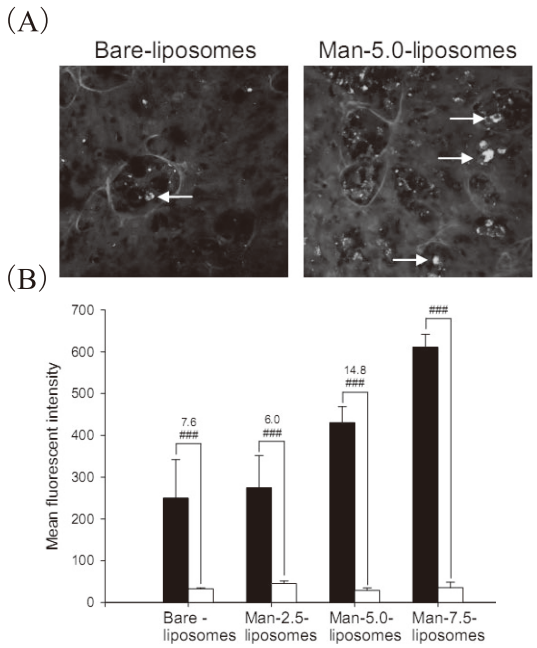

Fig. 1. Intrapulmonary distribution by confocal (A) and FACS analysis (B) of FITC-labeled liposomes (green) after intratracheal instillation in rats. Alveolar macrophages (arrow or black bar), Alveolar epithelial cells (white bar). The accumulation ratio in alveolar macrophages to alveolar epithelial cells is indicated in B. ###; $p<0.001$

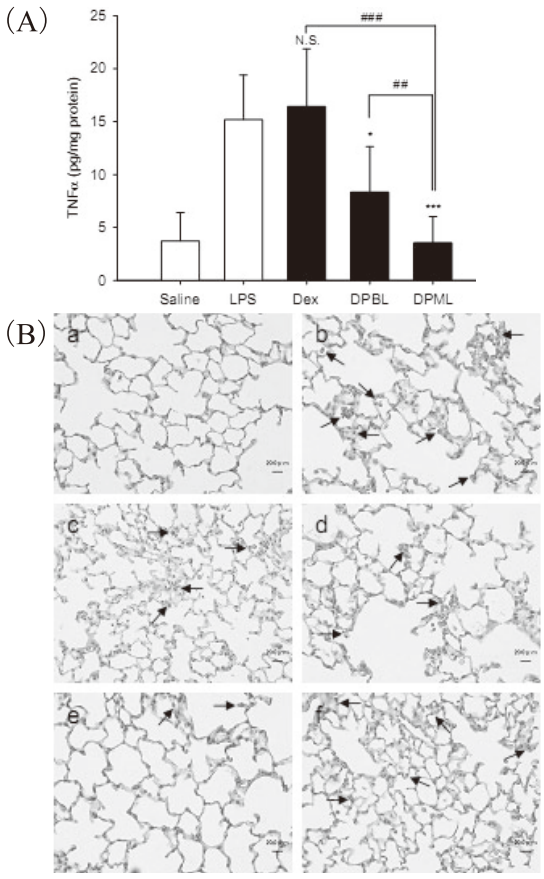

Fig. 2. TNFα release (A) and neutrophil infiltration (B, arrow) in the lung after treatment with intratracheally instilled dexamethasone (Dex, c), dexamethasone palmitate-incorporated in bare liposomes (DPBL, d) or mannosylated liposomes (DPML, e) in the presence of mannan (f) in LPS-induced lung inflamed rats (b) compare to naïve rats (a). *$p<0.05$, ***$p<0.001$ vs LPS, ## $p<0.01$, ### $p<0.001$.

targeting effectiveness was examined following direct pulmonary delivery via intratracheal in rats. Corresponding to *in vitro* study, the selective uptake of mannosylated liposomes with at least 5% of mannose content was significantly demonstrated in alveolar macrophages through mannose-receptor mediated manner (Fig. 1). Although pulmonary surfactant is considered to alter the uptake of lipid particles, there was no effect in the uptake and stability of these mannosylated liposomes. These results suggest the achievement in alveolar macrophage delivery using mannosylated liposomes after intratracheal administration.

Inhalation of free or incorporated glucocorticoids such as dexamethasone (Dex) is a first-line treatment of lung inflammation; however, there is some drawbacks including short half-life, potential toxicity and release from formulations. Meanwhile, prodrug of Dex which is more lipophilic than free form was stably incorporated in liposome formulation. For the treatment of lipopolysaccharide (LPS)-induced lung inflammation, Dex palmitate was incorporated in mannosylated liposomes (DPML) and intratracheally administered at 0.5 mg/kg in rat model. Three hours after administration, DPML significantly inhibited pro-inflammatory cytokines (TNFα, IL-1β) and chemokines (cytokine-induced neutrophil chemoattractant-1, CINC-1), suppressed neutrophil recruitment, and down-regulated NFκB and p38 mitogen-activated protein kinase activation compared to bare-liposomes (DPBL) and free Dex solution (Fig. 2). Furthermore, there was no systemic side effect in elevation of blood glucose after administration of liposomal Dex palmitate compared to free Dex as a result of their negligible systemic distribution. The findings prove the value of inhaled mannosylated liposomes as powerful targeting systems to alveolar macrophages to improve drug efficacy and safety.

Recently, potent anti-inflammatory agents such as steroids have been of limited for the treatment of several chronic inflammatory lung diseases. Then, a new therapeutic regimen is required to be explored. As known that NFκB is critically responsible for the expression of inflammatory mediators, therefore, inactivation of this transcription factors using NFκB decoy oligonucletides could successfully attenuate these diseases. Cationic liposomes have been widely used as vector to deliver and protect degradation of nucleic acid drugs. To characterize the fate of cationic liposomes, N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium

chloride (DOTMA)/cholesterol and 1,2-dioleoyl-3-trimethylammoniopropane (DOTAP)/cholesterol liposomes were intravenously injected in mice. After administration, both cationic liposomes were rapidly eliminated from the blood circulation and greatly accumulated not only in the lung but also in the liver. This observations support the non-specific disposition of cationic liposomes after intravenous administration.

According to effective targeting as described previously, NFκB decoy was delivered to alveolar macrophages using cationic (DOTMA)/mannosylated cationic liposomes via intratracheal administration in LPS-induced lung inflammation model. The complex of mannosylated cationic liposome/NFκB decoy was stable during spraying measured in term of change of particle size, zeta potential and release of oligonucleotides. In the lung inflammation model, pre-treatment with mannosylated cationic liposome/50 μg NFκB decoy complex via intratracheal administration significantly ameliorated cytokine (TNFα, IL-1β)and chemokine (CINC-1) release, neutrophil infiltration and NFκB activation (Fig. 3). The more potent anti-inflammatory effects of mannosylated cationic liposome/NFκB decoy were strongly supported by the more uptakes in alveolar macrophages confirmed by confocal microscopy and cell sorting analysis (FACS). These results indicate the sufficient targeting of nucleic acid drugs by cationic mannosylated liposomes after intratracheal instillation.

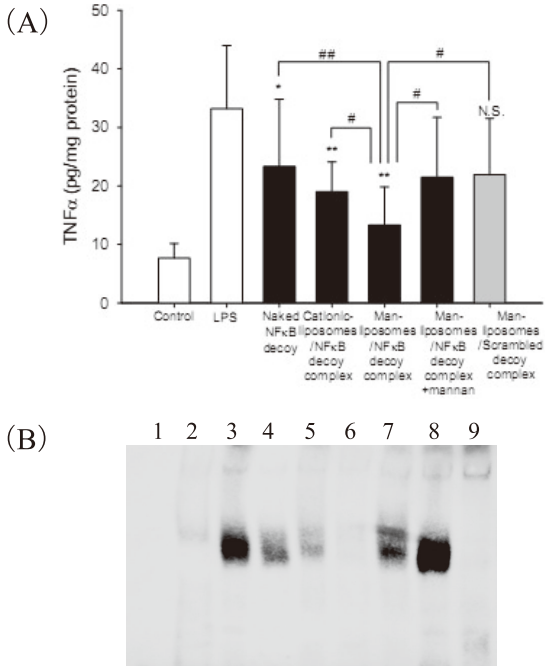

Fig. 3. TNFα release (A) and NFκB activation (B) in the lung after treatment with intratracheally instilled NFκB decoy in naked (lane 4), complexed with cationic liposomes (lane 5), complexed with mannosylated liposomes (lane 6) in the presence of mannan (lane 7) or scramble decoy (lane 8) in LPS-induced lung inflamed rats (lane 3) compare to naïve rats (lane 2). Lane 1 and 9 are the label control and competitive control groups, respectively. *$p<0.05$, **$p<0.01$ vs LPS, # $p<0.05$, ## $p<0.01$.

In brief, mannose modified lipid nanoparticles including emulsions and liposomes is a promising carriers for selective targeting systems to macrophages. Furthermore, the targeting to tissue resident macrophages is crucially depended on the route of administration. Application of mannosylated liposomes for such as steroid prodrug and NFκB decoy successfully attenuates lung inflammation by targeting to alveolar macrophages. These findings in this thesis provide valuable information for the rational drug design of mannosylated carrier systems for efficient macrophage-targeting by systemic and local administration in inflammatory disorders.

3. Amino Acid Dendrimer as Drug Carrier for Cancer Targeting

Dendrimer, a new class of polymer, is highly branched macromolecules possessing a unique dendritic structure with attractive features, including a nano-size range, monodispersity, rigid globular structure with high physical stability, and a large number of peripheral functionality for versatile chemical modification. Nevertheless, conventional dendrimers, such as cationic polyamidoamine (PAMAM) and poly-L-lysine dendrimers, tend to exhibit non-specific dose-dependent interactions and cytotoxicity due to the positive charge of the peripheral amino groups. Although acetylation of PAMAM can reduce these problems, acetylated modification possibly alters i) the physicochemical properties of the dendrimers including their water solubility, ii) structural homogeneity and iii) the number of peripheral amino groups available for antibody/drug conjugation.

Amino acid dendrimers are stepwise synthetic macromolecules of amino acid branch units which typically are lysine, obtained by peptide or amide bond formation. The designed structure of the amino acid dendrimers can be controlled by organization of the repeated amino acid branches at the internal or periph-

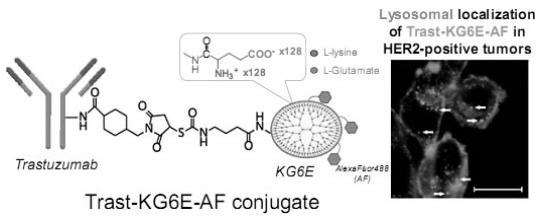

Fig. 4. Schemic structure of AlexaFluor488-labeled trastuzumab-KG6E conjugate and its localization in the lysosomes after internalization in HER2-positive human breast cancer cells (SKBR3).

eral layers called "generations". Controlling of peripheral generations by conjugation of lysine dendrimer generation 6 (KG6) with anionic amino acids, glutamic acid, called "KG6E", offers a neutral to negatively charged surface potentials due to an equal number of positive amine and negative carboxyl groups. This new approach can maintain chemical functionality, water soluble property of dendrimer while compromise cytotoxicity compared with cationic PAMAM G5 dendrimers. Due to a well-controlled particle size around 5–6 nm with low polydispersibility and chemical modification platform for antibody/drug, KG6E could be a candidate carrier for tumor targeting.

Trastuzumab, a humanized monoclonal antibody against human epidermal growth factor receptor 2 (HER2), offers a promising strategy of anti-cancer drug targeting to HER2-expressing cancer cells including breast cancer. KG6E was conjugated with trastuzumab and then labeled with AlexaFluor488, called Trast-KG6E-AF (Fig. 4). Trast-KG6E-AF conjugate was specifically bound to HER2-positive human breast cancer cells (SKBR3) in a dose-dependent manner with low binding affinity to HER2-negative cells (MCF7). As far as intracellular delivery of anticancer drugs in tumor cells is concerned, dendrimer-drug conjugates should be ideally cleaved only upon internalization in the lysosomal compartment to release free anticancer drugs in the tumor cells. As expected, the conjugate was significantly internalized in SKBR3 cells and then trafficked to lysosomes. These results indicate the potential of trastusumab-KG6E conjugates as HER2-targeting carriers for therapeutic and diagnostic approaches to cancer therapy.

To verify the potential of KG6E for tumor targeting and therapy, KG6E was conjugated with anticancer drugs and delivered to target cancer cells. This research is under investigation. The targeted KG6E-anticancer drug conjugates are expected to show the more efficient cytotoxicity specific to target cancer cells.

4. Research Life in Japan

Although studying in Japan provides different Ph.D. course, such as a variety of lectures and seminar, from that in the United States, I found that there are many opportunities which can support or fulfill my expectations. The outstanding point is the opportunity for doing research without restriction because of a number of funding support. There are many facilities in Kyoto University such as high-technology machines, scientific network and collaboration with the world class institute, iCeMS, and many supports for foreign students. In addition, there is an opportunity to attend a lot of Japanese and international symposium/conferences which motivates and inspires me for a new ideas/researches and provides a scientific/friend network. Finally, the research atmosphere is the most reasons for studying or doing research in Japan.

5. Summary and Acknowledgments

The DDS is an important research field in new drug design/formulation for clinical application although only few can be actually used in patient at this moment. Many researchers put much afford to make its real use in the clinic, not only stop at the laboratory bench. Although the DDS research I did for eight years in Japan is the bench-scale, I really wish to do more impact research towards this purpose.

Finally, I wish to express my utmost gratitude to Prof. Mitsuru Hashida, Associate Prof. Fumiyoshi Yamashita, and Assistant Prof. Shigeru Kawakami, Kyoto University for their encouragements, guidances, and supports during my stay in Japan. I am greatly indebted to the government of Japan and the Japan Society for the Promotion of Science (JSPS) for financial support.

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