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Abstract of Doctoral Dissertation

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Exploring and Improving lung-targeting function of GALA peptide-modified lipid nanoparticle
（GALA 修飾脂質ナノ粒子の肺標的化機能の評価および改良）

A lung is a vital organ for gas exchange between the body and the environment. Pulmonary vasculature contains approximately 30% of endothelial surfaces in the body, in which becomes the major capillary network in a systemic circulation. As the pulmonary vessels receive more than 50% of the entire cardiac output, pharmacological therapeutics could benefit from the large blood circulation to reach the lung. However, a little amount of the injected dose could be distributed to the lung due to a lack of endothelial affinity. Therefore, nanocarriers are required to target drugs to lung endothelium. GALA peptide (WEAALAEALAEALAEHLAEALAEALEALAA) was previously reported for dual functions as an inducer of endosomal escape and a targeting ligand to a lung endothelium. When the liposomal nanocarrier: a multifunctional envelope-type nano device (MEND) which was encapsulated with siRNA was modified with GALA, namely GALA/MEND, silencing activity against the endothelial marker, CD31 was achieved in the lung. Furthermore, GALA/MEND greatly inhibited lung metastasis in a melanoma-bearing mouse model. As a consequence, GALA becomes the potential candidate to develop lung-targeting nanocarriers. As a Ph.D. project, GALA was applied to design the nanocarriers with more efficient delivery to the lung. In addition, targeting mechanisms were further elucidated to expand the utility of the GALA.

The lung-targeting nanocarrier was developed by combining GALA and a neutral pH-sensitive lipid, YSK. To improve intracellular kinetics, YSK becomes positively charged under the endosomal/lysosomal acidic pH. An inverted hexagonal (HII) phase of a YSK nanocarrier interacts with endosomal membrane to promote fusion and release of encapsulated compounds. GALA-modified YSK-MEND was developed by using the 1st generation lipid, YSK05, namely GALA/YSK05-MEND. The optimized GALA/YSK05-MEND improved lung silencing effect of siRNA targeting an endothelial marker, CD31, by 3.9 fold when compared to the conventional GALA/MEND which DOTMA was used as a main lipid component. In addition, GALA/YSK05-MEND increased lung accumulation by 11.3 fold, compared to the unmodified YSK05-MEND. As a result, the combination of GALA and YSK becomes an alternative approach to developing the potent lung-targeting nanocarriers.

Next, enhancement of lung-targeting activity by GALA was examined by using PEGylation approach. This strategy was reported for increasing binding affinity and cellular uptake of ligands by conjugating to PEG linkers. The GALA conjugate which was terminally conjugated with PEG_{2000} (GALA/PEG_{2000}) was compared a lung-targeting activity to the conventional conjugate which was directly conjugated to cholesterol (GALA/Chol). In HMVEC-L, GALA/PEG_{2000}-LPs had a significantly higher uptake than GALA/Chol-LPs from 2 mole% and reached the maximum uptake at 5 mole%. Lung accumulation of GALA/LPs was then observed by confocal microscopy. Few amount of unmodified PEGylated liposomes (PEG-LPs) were traced in the lung. In contrast, the accumulation increased for GALA/Chol-LPs. More interestingly, GALA/PEG_{2000}-LPs showed the highest distribution in the lung. Thus, the role of PEGylation to improve a lung-targeting function of GALA was strongly confirmed. The flexibility of PEG linkers was supposed to assist binding activity of GALA to sugar receptors on the endothelial surfaces in the multivalent manner

Next, uptake mechanism of GALA-modified nanocarriers was elucidated *in vitro* in human lung microvascular endothelial cells (HMVEC-L). Flow cytometry and confocal observation were performed to identify an endocytosis pathway of GALA-modified liposomes (GALA-LPs). An internalization of GALA/LPs was significantly inhibited by chlorpromazine which is specific for a clathrin-mediated pathway. In contrast, the inhibitory effect was not observed in the case of applying filipin III and amiloride which block a caveolae-mediated pathway and a macropinocytosis pathway, respectively. The role of the clathrin pathway was confirmed by strong colocalization between GALA/LPs and a clathrin marker, transferrin. Therefore, it was proved that GALA-modified nanocarriers enter into lung endothelial cells via clathrin-mediated endocytosis.

Finally, transendothelial activity or termed "transcytosis" of GALA was examined since extravasation of GALA-LPs was observed. It was thus hypothesized that if GALA possesses transcytosis function, GALA-modified nanocarriers would penetrate a lung endothelium and reach other lung tissues, e.g. a Type I alveolar epithelium. The accumulation of GALA-LPs was quantitated by flow cytometry in both the lung endothelium and the Type I alveolar epithelium. DiD fluorescence from all GALA-LPs were detected in more than 70% of total endothelial cells, whereas signals from the PEG-LPs were hardly detected. The fluorescent intensity was the highest for GALA/PEG_{5000}-LPs, followed by GALA/PEG_{2000}-LPs and GALA/Chol-LPs, by which confirmed an enhancing effect of GALA PEGylation on *in vivo* targeting activity to lung endothelium. More interestingly, the fluorescence from GALA-LPs could be measured in more than 30% of total Type I alveolar cells. Among GALA derivatives, the maximal uptake was observed in GALA/PEG_{2000}-LPs. These results suggested that GALA might bypass capillaries to target other tissue layers in the lung. This idea was supported by TEM images of the gold nanoparticles (AuNPs) which were encapsulated in GALA/MEND, namely GALA/MEND-AuNPs. AuNPs were taken up not only in the lung endothelium, but they also penetrated an air-blood barrier and were localized in Type I alveolar epithelium. In addition, AuNPs were accumulated in Type II alveolar epithelium and an alveolar macrophage. The concept of transendothelial targeting by GALA would open more possibilities to develop potent lung-targeting nanocarriers.

In summary, a lung-targeting activity of GALA was successfully improved by the following approaches. First, PEGylation of GALA enhanced the binding activity of GALA so that lung accumulation of GALA-modified nanocarriers would increase. Second, the combination between GALA and the pH-sensitive lipid, YSK, also improved the activity of GALA/MEND in a lung endothelium by increasing endosomal escape. In addition, the cellular mechanisms of GALA have been elucidated. GALA-modified nanocarriers are taken up into lung endothelial cells by the clathrin-mediated pathway. The uptake mechanism is similar to the entry of influenza virus which is the design model for GALA. This study has provided more insights regarding intracellular trafficking of GALA. Furthermore, GALA was found to target the tissues beyond a lung endothelium via transcytosis effect. GALA-modified nanocarriers penetrated a lung endothelium to other parts of lung tissues, such as an alveolar epithelium and alveolar macrophages.This Ph.D. work would provide an innovative concept for design and development of lung-targeting nanocarriers to treat various pulmonary diseases.