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論文題目	Study on formulation to improve pharmacokinetics of extracellular vesicles based on their physicochemical properties （細胞外小胞の物性に基づく体内動態改善を目的とした製剤化に関する研究）		

（論文内容の要旨）

Extracellular vesicles (EVs) are cell-derived membranous vesicles and are expected to be applied as a carrier for novel drug delivery systems. The improvement of EV productivity and pharmacokinetics is an important issue for EV-based formulation. Furthermore, EVs are heterogenous vesicles and shows different pharmacokinetic properties depend on their physicochemical and biological properties. I focused on EV's charge or size in physicochemical properties of EVs as the factors affecting on pharmacokinetic properties, because these factors are directly relating with EV productivity. In the formulation process, it is necessary to improve the productivity of the target EV subpopulation. The purpose of this study is a basic investigation for the development of EV formulations, focusing on the effects of size and surface charge on the pharmacokinetics of EVs.

Chapter 1: Improvement of productivity and development of detection methods for PS low expression small extracellular vesicles

Small extracellular vesicles (sEVs), EVs with diameters of approximately 100 nm, is the most popular EV population. It has been reported that sEVs are rapidly eliminated after intravenous injection. On the other hand, EVs are heterogenous vesicles with different physicochemical and biological properties. Minor subpopulation of sEVs, phosphatidylserine (PS)-deficient sEVs ($PS^{(-)}$ sEVs), which can be isolated from bulk sEVs, show long circulation. Therefore, $PS^{(-)}$ sEVs are attractive candidate for drug delivery systems. However, limited productivity of $PS^{(-)}$ sEVs and lack in a method to perform qualitative analysis of $PS^{(-)}$ sEVs makes it difficult for the development of $PS^{(-)}$ sEVs-based formulation for delivery systems. In chapter 1, I attempted to increase productivity of $PS^{(-)}$ sEVs subpopulations and to develop a labeling method for sEVs to check the quality of sEVs.

Section 1: Preparation of small extracellular vesicles (sEVs) with low PS expression by enzymatic treatment to improve productivity of sEVs with high blood retention

Bulk sEVs collected via ultracentrifugation are rapidly eliminated by macrophages in the liver after intravenous injection because of negative charge derived from PS. On the other hand, $PS^{(-)}$ sEVs subpopulations with less PS exposed on their outer membrane show long circulation. Therefore, $PS^{(-)}$ sEVs subpopulations are considered to be a novel drug delivery carrier. However, these populations are suffered from limited production, which makes the sEV subpopulation difficult for therapeutic application. To improve the productivity, I developed the enzymatic reaction using phosphatidylserine decarboxylase (PSD) to deplete PS of the surface on the bulk sEV collected via ultracentrifugation, in order to increase the amount of $PS^{(-)}$ sEVs. By the treatment of liposomes or bulk sEVs with PSD, the PS levels on liposomes surface lower than untreated liposomes, and increased the yield of $PS^{(-)}$ sEVs. Moreover, the serum concentration and pharmacokinetic parameters of $PS^{(-)}$ sEVs derived from PSD-treated bulk sEVs showed long blood-circulation half-life compared with bulk sEVs. These results indicate that the treatment with PSD can be used to increase effectively the amounts of $PS^{(-)}$ sEVs.

Section 2: Development of a detection method for a subpopulation focusing on PS status in small

extracellular vesicles

Although bulk **s**EV-labeling methods have developed and used, a method to label a particular population of EVs have hardly been developed. In this section, I developed a labeling method for qualitatively analyzing $PS^{(-)}$ sEV subpopulation using two types of fusion protein containing fluorescent protein. To achieve this, a fusion protein of lactadherin (LA; PS-binding protein) and EGFP (EGFP-LA) to detect PS-positive sEVs, and the other is a fusion protein of Vn96 peptide (sEV marker, HSP70 affinity peptide) and mCherry (mCherry-Vn96) to detect bulk sEVs. I attempted to develop a simple method for detecting PS-positive sEV with PS on the surface using these two fusion proteins. Bulk sEVs derived from cells transfected with plasmid DNA encoding the fusion proteins were collected and were detected by fluorescence microscopy. Various fluorescence patterns from bulk sEVs were detected and suggested subpopulation with PS status are present in bulk sEVs. Moreover, I isolated $PS^{(-)}$ sEVs from bulk sEVs using TIM4-conjugated beads and observed their fluorescence. The fluorescence ratio derived from EGFP-LA was decreased but not mCherry-Vn96. These results indicate that I successfully developed a method for labeling sEVs using two fusion proteins. The method developed in this study can be a useful method for detecting the PS status of collected sEVs.

Chapter 2: Evaluation of the effect of extracellular vesicles particle size on the permeability of extracellular vesicles in the gastrointestinal tract

Biopharmaceuticals available for oral administration has been desired for noninvasive and simple method. Although various studies on oral administration of EVs have been reported, PK of EVs in GI tract remains unclear. In this chapter, I evaluated absorption efficiency and distribution of EVs after intraduodenal administration using different size of EVs. I considered the smaller EVs would improve their permeability in GI tract. To investigate this, I collected three different sizes of EVs, approximately 30 nm, 100 nm and 500 nm. I used fusion protein (Gag-gLuc) composed of EV tropic protein (Gag) and *Gaussia* luciferase (gLuc) to label EVs. After intraduodenal (i.d.) administration of each EV, gLuc activity in serum, small intestine and liver was measured to evaluate in vivo behavior of EVs. To visualize recipient cell types, EV samples were labeled with fluorescent dye. Cryosections of the small intestine were prepared and observed by fluorescence microscopy after immunostaining. After i.d. administration, little gLuc activity was detected in blood and liver for each EV. Immunohistochemical observation showed that all types of EVs localized in the intestinal lamina propria, and the degree of localization decreased with EV size. In addition, all types of EVs co-localized with a macrophage marker, but not an endothelial cell marker. These results indicate EVs are hardly translocated into the systemic circulation after i.d. administration and are mainly taken up by immune cells such as macrophages in intestinal lamina propria. In addition, the size of EVs can be one of the important factors affecting their behavior in the GI tract.

In conclusion, I have succeeded in developing methods on $PS^{(-)}$ sEV, which could help more effective productivity and analysis for them. Furthermore, I revealed that EV size could affect their PK in GI tract. These findings in this thesis could be useful the utilization of EV-based formulation.

（続紙 ２ ）

（論文審査の結果の要旨）

細胞外小胞（EV）は、ドラッグデリバリーシステムのキャリアとして期待されているが、製剤開発のためには、EV の生産性を向上させ、その薬物動態を理解して制御することが必要である。

ホスファチジルセリン（PS）を欠く EV 画分は、ほかの画分の EV と比べて表面電荷が中性よりで、血中滞留性が高いことが知られているが、全 EV における PS を欠く画分の数は極めて少ない。そこで、申請者は、生産性の向上を目的に、ホスファチジルセリン脱炭酸酵素（PSD）による酵素処理で、PS を欠く EV 画分の収量を増加させることに成功した。この手法で得られた EV 画分は、粒子径や表面電荷などの性質、および、血中滞留性において、天然に得られる PS を欠く EV 画分と同様の性質を有していた。

全 EV 画分における PS を欠く EV 画分の存在割合を簡便に評価できる方法は、EV の実用化において有用であると考えられる。申請者は、EV 表面に発現するマーカータンパク質に着目し、２種類の色の異なる蛍光タンパク質に、全 EV のマーカーに結合するペプチド、あるいは、PS に結合するペプチドをそれぞれ融合させたペプチド融合蛍光タンパク質を設計した。これらのペプチド融合蛍光タンパク質を EV と混合し蛍光顕微鏡で観察することで、全 EV 画分における PS を欠く EV 画分の割合を評価できるようになった。

最後に、EV の消化管吸収は、経口投与製剤による治療において重要な因子となる。遠心分離法で得られた粒子径の異なる各 EV 画分を得て、十二指腸内投与後の EV の吸収効率と分布を評価したところ、いずれの粒子径の EV も腸管固有層に局在し、その局在の程度は EV の大きさとともに減少した。さらに、どの粒子径の EV もマクロファージに取り込まれていた。EV は静脈内投与後ほとんど全身循環に移行せず、主に腸管前膜のマクロファージなどの免疫細胞に取り込まれることが示された。EV のサイズは、消化管での挙動に影響を与える重要な因子のひとつである可能性を示した。

以上、EV の表面電荷や粒子径は、体内動態に影響を及ぼす因子のひとつであり、これらの画分の生産性や検出法を開発し、治療に適した薬物動態を有する EV を効率よく回収することが EV の製剤開発に貢献すると考えられる。

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。2024 年 2 月 16 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。なお、本論文は、京都大学学位規程第１４条第２項に該当するものと判断し、公表に際しては、（2026 年 3 月 24 日までの間）当該論文の全文に代えてその内容を要約したものとすることを認める。

要旨公表可能日：　　年　　月　　日以降