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論文題目	Implementation of highly sensitive small extracellular vesicle (sEV) quantification method in the identification of novel sEV production modulators and the evaluation of sEV pharmacokinetics (高感度定量法を利用した細胞外小胞の産生モジュレーターの探索と体内動態解析)		

Small extracellular vesicles (sEVs) are nano-sized, lipid membrane vesicles that are secreted from various cell types in the body. sEVs mediate intercellular communication by transferring their cargos including nucleic acids and proteins to the recipient cells, thereby modulating diverse biological and pathological processes in the body. Sensitive quantification of sEVs is crucial since sEVs have been reported to correlate with various disease progression. Small molecules that can control sEV production may become a useful tool to investigate the roles of sEV in the body. Thus, the development of an sEV quantification method that is both sensitive and rapid can facilitate the exploration of novel compounds that can directly target sEV secretion/biogenesis. Furthermore, since information regarding the specific mechanisms of sEV production during pathogenesis is still limited, the ability to quantify sEVs in *vivo* can further our understanding of the factors that influence their production and their *in vivo* fate. Therefore, I developed a rapid, highly sensitive sEV quantification method utilizing *Gaussia* luciferase (gLuc) reporter protein and screened for compounds that could modulate sEV secretion. Utilizing the gLuc protein quantification method, I then took a pharmacokinetic approach to elucidate the mechanism behind elevated sEV secretion during cancer pathogenesis and investigated the roles of surface glycans on sEV pharmacokinetics.

Chapter 1 Development of a highly sensitive sEV quantification method and the identification of novel sEV modulators via high throughput screening

The ability to regulate sEV production may become useful in sEV research. However, reports of pharmacological agents that potentially inhibit or induce sEV biogenesis/secretion is limited due to the lack of high-throughput method for sEV quantification. Therefore, I developed a rapid, highly sensitive, high-throughput sEV quantification method utilizing a fusion protein consisting of gLuc reporter protein and sEV marker CD63 (CD63-gLuc) to identify compounds that can modulate sEV production. A total of 480 compounds were screened, and two novel compounds were identified to be potent inducers or inhibitors of gLuc activity. The efficacy of the identified compounds in modulating sEV production was validated by protein quantification of the isolated sEVs. Altogether, a rapid, sensitive sEV quantification method was successfully developed, and two novel compounds were identified to modulate sEV production.

Chapter 2 Pharmacokinetic approach for the elucidation of elevated plasma sEV concentration during cancer pathogenesis

The mechanism behind elevated plasma sEV concentration during disease states is poorly understood due to the technical difficulty in tracking the *in vivo* fate of sEVs. By implementing a selective sEV labeling method utilizing gLuc reporter protein, which enabled the quantification of mouse plasma-derived sEVs (MP-sEVs) *in vivo*, I aimed to elucidate the mechanism of elevated plasma sEV

concentration during cancer pathogenesis. Considering that the steady-state concentration of MP-sEVs is maintained by a balance between the rate of secretion (k_0) and rate of elimination (k_{el}), the elevated sEV concentration is due to either increased k_0 or decreased k_{el}. Intravenous administration of MP-sEVs isolated from healthy or tumor-bearing mice into either healthy or tumor-bearing mice showed comparable pharmacokinetic parameters, suggesting no changes in k_{el} during cancer pathogenesis. To determine the k_0 of tumor-derived sEVs, CD63-gLuc stably expressing B16BL6 tumor bearing mice model was utilized. gLuc activity of MP-sEV isolated from these tumor-bearing mice was below the limit of detection; furthermore, it was approximated that tumor-derived sEVs contributed to only 0.1% of the total sEV protein isolated. Thus, it was revealed that k_{el} hardly changes, and that tumor-derived sEVs contribute very little to the overall increased MP-sEV concentration. These results indicate that cells other than tumor cells mainly contribute to the elevated blood concentration of sEV during disease states.

Chapter 3 Determination of the effect of surface glycans in sEV pharmacokinetics

Tumor cell-derived sEVs have been reported to exhibit altered glycosylation patterns and contribute significantly to its progression and metastasis. Since glycans are located on the outermost region of sEV surface, it can be expected that surface glycans participate in various sEV functions, which ultimately influence their *in vivo* pharmacokinetics. To determine the role of surface glycans in sEV pharmacokinetics, gLuc fusion protein consisting of Gag protein and gLuc (Gag-gLuc) was utilized to label the inner spaces of the sEVs. The inner labeling method preserves the chemiluminescence derived from the sEVs after the enzymatic deglycosylation of N- and O-glycans from the sEV surface, thus enabling the pharmacokinetic analysis of the deglycosylated sEVs. Deglycosylation had minimal impact on the physicochemical properties of the sEVs, however showed enhanced uptake by the peritoneal macrophages for N-glycan removed sEVs. Nonetheless, the increased uptake efficiency observed *in vitro* did not alter the sEV clearance of the glycosidase-treated sEVs *in vivo*, suggesting that the rate-determining process in sEV clearance at the whole-body level is blood-flow dependent, and not the uptake efficiency of macrophages. Thus, it was determined that surface glycans have minimal impact on sEV clearance rate.

In conclusion, I successfully developed a simple yet powerful quantification tool for sEVs and found two novel compounds that could induce or inhibit sEV production. Furthermore, I revealed that tumor-derived sEVs had minimal contribution to the elevated sEV concentration observed during cancer pathogenesis, and that surface glycans do not significantly influence sEV pharmacokinetics. The findings in this thesis contribute to our understanding on the biological and pathological roles of sEV and to the development of sEV-based therapies.

（続紙 ２ ）

（論文審査の結果の要旨）

Small extracellular vesicle (sEV)は粒子径100nm程度の細胞由来の膜小胞であり、内因性の細胞間輸送機構として機能する。sEV量と病態との相関が報告されていることから、sEVの生理機能の解明のためには、高感度なsEV定量法が必要である。高感度なsEV定量法によりsEVの産生を制御可能な化合物の同定が期待でき、さらにはそのような定量法を利用することにより生体内におけるsEVの機能解明にも大きく寄与する。そこで本学位論文では、以下の3章にわたり、sEVの定量的解析法を開発し、それを利用したsEV産生制御可能な化合物の探索と生体内におけるsEVの体内動態について定量的に解析した。

第一章 Development of a highly sensitive sEV quantification method and the identification of novel sEV modulators via high throughput screening

（高感度sEV定量法の開発と新規sEV産生制御化合物のハイスループットスクリーニング）

CD63とGaussia luciferase（gLuc）の融合タンパク質を利用した、高感度なsEV定量法を開発し、sEVの産生を制御可能な化合物をスクリーニングした。計480化合物についてgLuc活性を指標とした検討を行い、sEVの産生を抑制あるいは促進可能な2種類の新規化合物を同定した。同定した化合物が真にsEV産生を制御可能であるかについて、化合物を添加した細胞より産生されるsEVを回収することで評価したところ、実際に回収されるsEVの回収量 を制御可能であることを明らかとした。以上、sEVの定量法の開発とそれを利用したsEV産生制御可能な化合物のスクリーニングに成功した。

第2章 Pharmacokinetic approach for the elucidation of elevated plasma sEV concentration during cancer pathogenesis

（がん病態時における血中sEV濃度上昇機構の薬物速度論的解析）

がん病態においてsEVの血中濃度の上昇が、どのような要因によりもたらされるかについて検討した。担がん、あるいは正常なマウスの血液より回収したsEVをgLucにて標識後、担がんあるいは正常なマウスに静脈内投与後の血中濃度推移を評価したところ、担がん状態はsEVの消失にほとんど影響を与えなかった。従って、担がん状態における血中sEV濃度の上昇をsEV産生速度の上昇によることが示された。そこで、担がん時の血中総sEVに占めるがん細胞由来のsEVの割合について、sEVを標識可能なCD63とgLuvの融合タンパク質を安定に発現するがん細胞を移植した担がんマウスを用いて評価したところ、がん細胞由来sEVは血中sEVの0.1%程度を占める子おtが明らかとなった。以上の結果から、担がん時における血中sEVの濃度上昇は、がん細胞以外のsEVの産生速度の上昇によることを明らかとした。

第3章 Determination of the effect of surface glycans in sEV pharmacokinetics

（sEVの体内動態にsEV表面の糖鎖が及ぼす影響の決定）

sEVの体内動態における糖鎖の役割について、GagとgLucの融合タンパク質を利用して内側をgLuc標識したsEVを用いて検討した。N型糖鎖あるいはO型糖鎖、あるいはその両者を除去した内側標識sEVを調製し、その体内動態を評価した。その結果いずれの糖鎖を除去してもその血中からの消失速度はほとんど変化しないことを明らかとした。

よって、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３年８月２０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、（令和６年９月２３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