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論文題目	Evaluation of the pharmacokinetic and pharmaceutical characteristics of exosomes for the development of exosome-based drug delivery carrier. (エキソソームを利用したデリバリーキャリアの開発を目的とした体内動態および製剤学的特性の評価)		

Exosomes are small membrane vesicles with 100 nm in diameter and are released from various types of cells. Since exosomes play a role as intercellular communication tools by transferring their cargoes including protein and nucleic acid to the recipient cells, therapeutic application of exosomes by using exosomes as drug delivery carrier has gained much attention. To exploit exosomes as drug delivery carriers, it is important to understand the factors affecting the pharmacokinetics of exosome, such as types of exosome-producing cells, and the role of surface protein on their pharmacokinetics. In addition, preservation method is an important issue to be concerned for the development of exosome-based drug delivery carriers. However, the information about these factors is limited. Therefore, in this thesis, I investigated the pharmacokinetics of exosome from five different types of murine cell lines, and elucidated the role of exosome surface protein on their pharmacokinetics by developing method to label inner space of exosomes. In addition, I also developed a preservation method of exosomes utilizing lyophilization.

Chapter 1: Evaluation of cell type-specific and common characteristics of exosomes derived from mouse cell lines

Characteristics of exosomes that are important for the development of exosome-based delivery carriers such as yield, physicochemical properties, and pharmacokinetics, may be different among different cell types. However, there is limited information about the effect of cell type on these characteristics. Therefore, I evaluated these characteristics of exosomes derived from five different types of mouse cell lines: B16BL6 murine melanoma cells, C2C12 murine myoblast cells, NIH3T3 murine fibroblasts cells, MAEC murine aortic endothelial cells, and RAW264.7 murine macrophage-like cells. Exosomes were collected using a differential ultracentrifugation method. The exosomes collected from all the cell types were negatively charged globular vesicles with a diameter of approximately 100 nm. C2C12 and RAW264.7 cells produced more exosomes than the other types of cells. The exosomes were labeled with a fusion protein of *Gaussia* luciferase and lactadherin to evaluate their pharmacokinetics. After intravenous injection into mice, all the exosomes rapidly disappeared from the systemic circulation and mainly distributed to the liver. In summary, the exosome yield was significantly different among the cell types, and all the exosomes evaluated in this study showed comparable physicochemical and pharmacokinetic properties.

Chapter 2: Evaluation of the role of exosome surface proteins in the pharmacokinetics of exosome

Surface proteins on exosome membranes might play roles in pharmacokinetics of exosomes. One method which can be used to study the role of surface membrane of exosome is to modify the inner space of exosome. In this chapter, I constructed a plasmid DNA expressing a fusion protein of Gag protein derived from Moloney murine leukemia virus (Gag) and *Gaussia* luciferase (gLuc) (Gag-gLuc) to modify the inner space of exosome. Exosomes were collected from B16BL6 melanoma cells transfected with the plasmid. Gag-gLuc exosomes were negatively charged globular vesicles with a diameter of approximately 100 nm. gLuc labeling of the Gag-gLuc

exosomes was stable in serum. gLuc activity of Gag-gLuc exosomes was minimally decreased by proteinase K (ProK) treatment, indicating that gLuc was modified in the inner space of exosomes. Then, to evaluate the effect of the surface proteins of exosomes on their pharmacokinetics, Gag-gLuc exosomes treated with ProK were intravenously administered to mice. Volume of distribution (Vd) was significantly smaller for treated exosomes than untreated exosomes. Moreover, ProK treatment degraded various surface protein including integrin $\alpha_6\beta_1$, an integrin known to be involved in lung targeting. The ProK treatment significantly reduced the lung distribution of EVs after intravenous injection. These results indicate that the surface proteins of exosomes such as integrin $\alpha_6\beta_1$ play some roles in pharmacokinetics in terms of reducing Vd and their distribution to the lung.

Chapter 3: Development of preservation method of exosomes at room temperature by using lyophilization.

Application of exosomes as drug delivery vehicles can be expanded by the development of the preservation method of exosomes. Although exosomes are generally stored at -80ºC, it is not suitable for handling or transportation exosomes and other storage method that is easier to handle is desirable. Lyophilization is a promising storage method that can be used to preserve variable substances at room temperature. In this study, I tried to develop a preservation method of exosomes at room temperature using lyophilization, and compared the properties of the lyophilized exosomes with ones stored at -80ºC. Lyophilization without cryoprotectant resulted in aggregation of B16BL6 melanoma-derived exosomes while the addition of trehalose, a cryoprotectant, prevented aggregation during lyophilization. PAGE analysis revealed that lyophilization in the presence of trehalose could protect proteins and RNA of exosomes that were stored at 25ºC. Lyophilization hardly affected the pharmacokinetics of gLuc-labeled exosomes after intravenous injection into mice. Moreover, it was found that lyophilized exosomes retained the activity of loaded gLuc and immunostimulatory CpG DNA for about 4 weeks even when stored at 25ºC. In summary, lyophilization with trehalose is an effective method for the storage of exosomes for various applications.

In conclusion, I found that types of cells have negligible effect on pharmacokinetics of exosomes. Moreover, I found that surface proteins of exosomes play role on their pharmacokinetics. In addition, I have succeeded in the development of preservation method of exosomes at room temperature utilizing lyophilization, without altering physical properties, pharmacokinetics and the function of molecules loaded to exosomes. The findings in this thesis provide useful information for the development of drug delivery carriers using exosomes.

（論文審査の結果の要旨）

種々の細胞から分泌される粒子径100nm程度の膜小胞エキソソームは、内因性の細胞間物質輸送機構であることから、ドラッグデリバリーキャリアとしての利用が期待される。エキソソームを利用したキャリアの開発には、エキソソームの産生細胞種の違いがエキソソームの特性に及ぼす影響の解明や、エキソソームの体内動態特性の解析が必要である。また、エキソソームの保存方法も必要となる。しかしながら、これらの情報については乏しかったことから、本学位論文では以下の3章にわたりこれらの要因についての検討を行った。

第I章　Evaluation of cell type-specific and common characteristics of exosomes derived from mouse cell lines

（マウス細胞株を用いた産生細胞種特異的・共通のエキソソーム特性の評価）

産生細胞種の違いがエキソソームの産生量・物理化学的性質・体内動態特性等に及ぼす影響について検討した。マウス由来の5種類の株化細胞を用いて検討した結果、検討した細胞種すべてからほぼ同等の物理化学的性質を有するエキソソームの産生を確認した。産生細胞種により産生量は異なっていた。静脈内投与後は、産生細胞種を問わず速やかに血中より消失し主に肝臓へ移行した。以上の結果より、産生細胞種の違いはエキソソーム産生量に影響を与えるものの、体内動態特性にはほとんど影響を与えないことを明らかとした。

第 II 章　Evaluation of the role of exosome surface proteins in the pharmacokinetics of exosome

（エキソソーム表面タンパク質のエキソソーム体内動態に及ぼす影響の評価）

エキソソームの表面タンパク質が体内動態特性に及ぼす影響を評価するために、レポータータンパク質*Gaussia* luciferase (gLuc)を利用したエキソソームの内部標識法を開発した。開発した方法を用いて調製したgLuc内部標識エキソソームはプロテアーゼで処理してもgLuc活性を保持可能であったことから、内部標識法開発の成功を確認した。プロテアーゼで処理して表面タンパク質を除去したgLuc内部標識エキソソームを静脈内投与後の体内動態を評価したところ、分布容積が上昇し、エキソソームの肺への移行の低下を見出した。以上の結果から、エキソソームの表面タンパク質が肺への移行に関与することを明らかとした。

第III章　Development of preservation method of exosomes at room temperature by using lyophilization

（凍結乾燥を利用した室温でのエキソソーム保存方法の開発）

エキソソームの室温での保存を可能とする方法の開発として、凍結乾燥を利用したエキソソームの保存方法を開発した。凍結乾燥保護剤としてトレハロースを用いることで、エキソソームの凍結乾燥を可能とした。エキソソームを凍結乾燥することで、エキソソームに内包されたタンパク質・RNAは室温保存後も保持されており、搭載した機能性核酸・タンパク質の活性も保持されていた。また、体内動態特性についてもほとんど変化しなかった。以上、室温での安定な保存を可能とするエキソソームの凍結乾燥保存法の開発に成功した。

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、平成30年８月27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