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研究課題名（和文）Developing brain-penetrating anti-inflammatory nano-medicines to treat neurodegenerative diseases

研究課題名（英文）Developing brain-penetrating anti-inflammatory nano-medicines to treat neurodegenerative diseases

研究代表者

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研究成果の概要（和文）：本研究では、治療用抗体を安全に脳内に送達するナノキャリアシステムを開発した。本システムでは、脳血管内皮細胞に多く局在するグルコーストランスポーター１(GLUT1)を標的とし、ナノキャリア表層に血液脳関門(BBB)を通過させるために、グルコースを搭載した高分子ミセル(G-NP)を構築した。血糖値を精密に制御することで効率的にBBBを通過し、脳内環境に応答してG-NP中に保持した抗体を適切に放出させることに成功した。ここでアルツハイマー病(AD)の原因物質として考えられるアミロイドβ(Aβ)を標的とする抗体を用いることで、Aβ量を劇的に減少させることを実現した。

研究成果の学術的意義や社会的意義
Treating neurodegenerative diseases is severely limited by the poor entry of therapies into the brain. We have developed two different strategies capable of delivering nanoparticles specifically to the brain, thereby paving the way to deliver a wide-range of therapies into the brain.

研究成果の概要（英文）：We created a nano-carrier system capable of delivering therapeutic payloads specifically into the brain. We designed nanoparticles decorated with glucose (G-NP) capable of encapsulating therapeutic antibodies. By modulating blood-glucose levels, we were able to induce movement of the glucose transporter Glut-1 specifically across brain endothelial cells (BEC). Hence, the G-NP bound to the Glut-1 were transported across BEC to release their therapeutic cargo specifically in the brain. We demonstrated the therapeutic potential of this strategy by delivering antibodies capable of preventing aggregation of amyloid-beta, a key pathological mechanism in Alzheimer's disease.

We also developed a new brain-targeting strategy which exploits the impermeability of the blood-brain barrer to selectively label BEC. This strategy has the benefit that it does not modify the physiology of the organism, therefore has great potential for clinical translation.

研究分野：Biomedical research/bioengineering

キーワード：Nanoparticles　Brain　Blood-brain barrier　Glucose　Alzheimer's

１．研究開始当初の背景

Delivering therapies to the brain is severely hindered by the presence of the blood-brain barrier (BBB), a cellular barrier mainly composed of the endothelial cells lining the brain vasculature which prevents passage of the vast majority of molecules from the blood into the brain. Hence, treating neurodegenerative diseases, such as Alzheimer's disease, will require strategies which can overcome the BBB to deliver drugs to their brain targets in therapeutically active amounts.

Within the Kataoka group, a new technology had been developed to selectively deliver nanoparticles across the BBB by decorating them with glucose molecules and exploiting the translocation of glucose transporter (Glut)-1 proteins across brain endothelial cells (Anraku et al, Nature Communications; 8-1001, 2017).

In addition to the lack of brain penetration, undesired accumulation of therapies/nanoparticles in peripheral organs (e.g. lung, liver, heart) limits their clinical application due to detrimental peripheral side-effects. To date, the vast majority of strategies to target nanoparticles to the brain rely on functionalizing nanoparticles with ligands expressed at the brain vasculature (e.g. transferrin receptor-1). However, these strategies have intrinsic brain-specificity limitations as the target proteins are also significantly expressed in peripheral vascular beds, leading to an increase in nanoparticle accumulation in peripheral organs. Therefore, new strategies which can improve the brain specificity of nanoparticle delivery strategies are urgently needed.

２．研究の目的

The research supported by the Kakenhi grant encompassed two purposes. The first purpose was to exploit the glucose-decorated nanoparticle platform developed by the Kataoka group to generate BBB-penetrating nanoparticles capable of delivering therapeutic cargos to specific brain cells. We pursued two nanoparticle designs for therapy delivery. In the first instance, we aimed to generate surface-decorated nanomicelles capable of carrying both a therapeutic protein (namely, the potent anti-inflammatory protein CD200) and a brain-cell targeting protein (namely, ligands against receptors on microglial cells) on their surface. With this, we aimed to generate nanoparticles capable of penetrating the BBB, be directed towards inflammatory microglial cells and deliver the anti-inflammatory protein to dampen the overactive (inflamed) microglial cells responsible for the chronic neurodegeneration seen in Alzheimer's disease. In the second strategy, we aimed to develop nanomicelles capable of carrying the therapeutic cargo within the micelle (as opposed to on the surface as in the first strategy) and be able to release it within the brain following crossing of the BBB. To this end, we generated nanomicelles capable of incorporating an antibody fragment (Fab') within their

core in a pH- and redox-dependent manner. To apply this system to fight Alzheimer's disease we incorporated a Fab' against amyloid-β aggregation (derived from the 3D6 antibody).

The second purpose supported by the Kakenhi grant concerned the development of a new strategy to deliver nanoparticles to the brain which could overcome the limitations of brain specificity. To this end, we aimed to exploit the physiology of the BBB to generate artificial brain targets for nanoparticle delivery. A crucial characteristic for the high impermeability of the BBB is a reduced level of endocytosis in brain endothelial cells compared to peripheral endothelial cells. This characteristic was exploited to selectively retain molecular targets on the surface of brain endothelial cells following binding of the molecular label on the surface of all endothelial cells throughout the body (i.e., both peripheral and brain endothelia). Nanoparticles functionalized with ligands specific for the molecular targets would then specifically interact with the surface of brain endothelial cells only, thereby achieve specific brain targeting.

３．研究の方法

For the first nanoparticle design we created polymeric nanomicelles composed of oppositely charged PEGylated polymers. The surface of these micelles could be decorated with different molecules through functionalization of the polymers. As such, we employed polymers functionalized with glucose to allow BBB-penetration, as well as polymers functionalized with azide (N_3) reactive groups to allow attachment of DBCO-conjugated proteins onto the surface of the nano micelles. The proteins employed were the potent anti-inflammatory protein CD200, which binds to its cognate receptor CD200R expressed on macrophages, and an antibody fragment (Fab') recognizing the macrophage integrin protein CD11b. BBB-penetration was examined in *in vitro* BBB models composed of primary rat brain endothelial cells plated on collagen/fibronectin-coated transwell porous membranes. Anti-inflammatory potential was examined by treating amyloid-β/LPS-inflammed mouse brain macrophages (microglia) (BV2 cell line) with CD200-functionalized nanomicelles and measuring production of nitric oxide, reactive oxygen species and pro-inflammatory cytokines (TNFα, IL-1β).

For the second nanoparticle design, we employed glucose-functionalized PEGylated cationic polymers capable of complexating with negatively charged Fab' proteins to generate therapy-loaded nanomicelles. The polymers were capable of forming disulfide bonds following nanomicelle assembly, which could be broken under reductive conditions (as those found within the brain parenchyma), thereby conferring redox sensitivity to the nanomicelle. pH sensitivity was achieved by converting the positive charge of the Fab' proteins into negative charge with citraconic anhydride (thereby allowing complexation with cationic polymers). This charge conversion could be reversed at low pH, thereby promoting nanomicelle disintegration in endosomes following cellular uptake. Nanomicelle delivery was examined in vitro (primary brain endothelial cells) as well as in vivo. The ability to deliver therapeutically active Fab' proteins was examined by treating Alzheimer's Disease (AD) mouse models (APP/PS1) and monitoring the development of soluble and insoluble amyloid-

β_{1-42}.

To develop the new brain targeting strategy, molecular labels were generated by labelling the surface of endothelial cells with biotin. Molecular label-recognition by nanoparticles was achieved by decorating the surface of polymeric nanomicelles with the protein avidin. The biotin-avidin interaction was chosen for its extremely high binding strength (ensuring for efficient molecular target recognition), as well as for the fact that avidin binds biotin in a multimeric fashion (each avidin protein binding four biotins), promoting endocytosis through protein clustering.

Retention of the biotin label on brain endothelial cells was firstly examined in vitro by binding biotin-NHS to primary amines of cell-surface proteins on primary brain endothelial cells vs. peripheral endothelial cells (HUVECs). Specific retention of molecular labels on brain endothelial cells was examined by treating brain and peripheral endothelial cells with avidin-functionalized nanomicelles following increasing incubation times to allow for internalization of the biotin labels.

To corroborate molecular target retention on brain endothelial cells in vivo we employed intravenous injection (in mice) of biotin-conjugated antibodies against the endothelial protein PECAM-1. Avidin-functionalized nanomicelles were then injected at increasing time-periods following biotin-anti-PECAM-1 injection. Nanomicelle accumulation was quantified through ex vivo IVIS imaging, fluorescence quantification in homogenized organs, and immunohistochemistry of fixed brain tissue.

４．研究成果

Employing in vitro models of the BBB, we demonstrated that decorating the surface of glucose-functionalized nanomicelles with proteins did not negatively affect BBB penetration, indicating surface-bound proteins could be carried into the brain with our nanoparticle platform. Furthermore, by employing in vitro models of brain inflammation we demonstrated functionalization of nanomicelles with the anti-inflammatory protein could indeed prevent microglia inflammation, as measured by a decrease in production of nitric oxide and reactive oxygen species. Similarly, by decorating nanomicelles with antibody fragments against different cell-type receptors, we could direct their binding towards different cell types. Namely, by decorating with anti-CD11b, we could detect higher binding of nanomicelles towards microglial cells vs. control breast cancer cells. Conversely, decorating them with antibodies against proteins predominantly expressed by astrocytes (Excitatory Amino Acid Transporter-1), we could shift the nanomicelle binding from microglia to astrocytes. Hence, we were able to generate nanoparticles capable of binding proteins onto their surface to confer anti-inflammatory properties or be directed to a specific cell type without significantly affecting brain penetration. However, these three characteristics were tested separately. A single particle with all three properties was not examined. Furthermore, we did not corroborate our results in vivo.

For the second nanoparticle design, we were able to demonstrate in vitro that the glucose-

nanomicelles entered brain endothelial cells in a Glut-1 dependent manner. We also demonstrated Fab' release in pH and redox conditions mimicking those in the endothelial endosome and brain parenchyma, indicating Fab' release would occur in vivo during the transcytosis process and release into the brain tissue. Furthermore, we demonstrated the released Fab' could recover its biological activity, as measured by its ability to prevent amyloid-beta aggregation to a similar extent as native Fab'. We were able to corroborate brain penetration in vivo in control mice as well as AD mouse models, both by nanomicelle quantification in organ homogenates and through intravital confocal laser scanning microscopy. Importantly, we demonstrated the brain entry of nanomicelle-Fab translated to a therapeutic effect in AD mice, as demonstrated by a decrease in the content of soluble and insoluble amyloid-β in the brain of AD mice treated with nanomicelle-Fab vs. Fab alone.

For development of the new brain targeting strategy, we were able to demonstrate *in vitro* that biotin labels are selectively retained on the surface of brain endothelial cells vs. peripheral endothelial cells, as demonstrated by a steady decrease in avidin-nanomicelle binding at increasing time-intervals following biotin-target generation on peripheral endothelial cells, while the binding of avidin-nanomicelles on primary brain endothelial cells was maintained virtually constant throughout all the examined time-points following biotin-target generation. This was corroborated by visualizing nanomicelle binding through confocal microscopy. We also demonstrated the successful internalization of the avidin-nanomicelles into brain endothelial cells following biotin-target engagement, and demonstrated this process to be energy dependent, indicating a proper endocytic process. These results were echoed in vivo, where injection of avidin-nanomicelles a short time-interval (15 mins) after biotin-anti-PECAM1 injection led to an increase in avidin-nanomicelle accumulation in the lung, brain, heart and pancreas (in that order of magnitude). However, as the time-interval increased, the accumulation into all peripheral organs steadily decreased, while the accumulation into brain was kept constant. This resulted in the brain being the only organ with a significant increase in nanoparticle accumulation following an 8hr time-period, demonstrating specific brain target generation for nanoparticle delivery. While we were able to specifically increase nanoparticle accumulation in the brain, nanoparticle accumulation was observed only in the vasculature, indicating nanoparticles were being sequestered within endothelial cells without reaching the parenchyma. While brain-vasculature targeting itself would have therapeutic benefits, future studies will optimize the targeting strategy to increase nanoparticle release into the parenchyma.

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　0件）

１．著者名 Jinbing Xie, Daniel Gonzalez-Carter, Theofilus A. Tockary, Noriko Nakamura, Yonger Xue, Makoto Nakakido, Hiroki Akiba, Anjaneyulu Dirisala, Xueying Liu, Kazuko Toh, Tao Yang, Zengtao Wang, Shigeto Fukushima, Junjie Li, Sabina Quader, Kouhei Tsumoto, Takanori Yokota, Yasutaka Anraku*, and Kazunori Kataoka	４．巻 -
２．論文標題 Dual-Sensitive Nanomicelles Enhancing Systemic Delivery of Therapeutically Active Antibodies Specifically into the Brain	５．発行年 2020年
３．雑誌名 ACS Nano	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/acsnano.9b09991	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Daniel Gonzalez-Carter, Xueying Liu, Theofilus A. Tockary, Anjaneyulu Dirisala, Kazuko Toh, Yasutaka Anraku, and Kazunori Kataoka	４．巻 -
２．論文標題 Targeting nanoparticles to the brain by exploiting the blood-brain barrier impermeability to selectively label the brain endothelium	５．発行年 2020年
３．雑誌名 Proceedings of the National Academy of Science of the United States of America	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.1073/pnas.2002016117	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

〔学会発表〕　計3件（うち招待講演　3件／うち国際学会　0件）

１．発表者名 Gonzalez-Carter, Daniel
２．発表標題 Targeting nanoparticles to the brain by exploiting the blood-brain barrier impermeability to selectively label the brain endothelium
３．学会等名 Nanobio2020 (post-poned due to Coronavirus)（招待講演）
４．発表年 2020年

１．発表者名 Gonzalez-Carter, Daniel
２．発表標題 Delivering anti-inflammatory therapies into the brain through brain-targeted polymeric nano-vehicles
３．学会等名 12th International Symposium on Nanobiotechnology（招待講演）
４．発表年 2019年

１．発表者名 Gonzalez-Carter, Daniel
２．発表標題 Developing Cell-targeted, Brain-penetrating Polymeric Nano-micelles to Treat Neurodegenerative diseases
３．学会等名 11th European and Global Summit for Clinical Nanomedicine (CLINAM)（招待講演）
４．発表年 2018年

〔図書〕　計0件

〔出願〕　計2件

産業財産権の名称 A METHOD OF ADMIINISTERING A NANOPARTICLE	発明者 片岡　一則、ゴンザレス　カーター　ダニエル	権利者 公益財団法人川崎市産業振興財団
産業財産権の種類、番号 特許、特願2020-055478	出願年 2020年	国内・外国の別 国内

産業財産権の名称 A METHOD OF ADMIINISTERING A NANOPARTICLE	発明者 片岡　一則、ゴンザレス　カーター　ダニエル	権利者 公益財団法人川崎市産業振興財団
産業財産権の種類、番号 特許、PCT/JP2021/011290	出願年 2021年	国内・外国の別 外国

〔取得〕　計0件

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関