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学 位 論 文 題 名

Development of a novel DNA aptamer ligand by a cell-SELEX method to discover a new drug delivery system(DDS)

（新規薬物送達システム開発のためのCell-SELEX法に基づくDNAアプタマーの探索）

学位論文内容の要旨

The goal of this research project is to obtain and characterize DNA aptamers as a novel ligand against primary cultured mouse tumor endothelial cells (mTECs), and applied it to identify novel biomarkers as well as to develop a novel aptamer-modified drug delivery system (DDS) to target tumor vasculature. Tumor growth that is dependent on angiogenesis was first reported by Folkman in 1971. Preventing or inhibiting angiogenesis, which is associated with the increased vascularity necessary for tumor progression and metastasis, is a challenging issue in combating cancer. The rationale for targeting tumor endothelial cells in this current project is based on the following assumptions: Tumor blood vessels provide nutrients and oxygen, and remove waste from tumor tissue, resulting in tumor progression. So, a single tumor endothelial cell can support many tumor cells. Tumor blood vessels have been shown to differ from their normal counterparts in that they show leakiness, the thickness of the basement membrane is uneven. This suggests that tumor endothelial cells may well express surface markers that are different from those found in normal cells. Thus, targeting tumor endothelial cells might be a much more effective strategy than targeting actual tumor cells themselves. Numerous advantages offer to choice novel DNA aptamer ligand to screen for targeting to mTECs, such as aptamers are ssDNA, ssRNA or peptide molecules, are very easy to reproduce, are generally nontoxic, have a low molecular weight (8-15 kDa) and their binding to targets is very specific and selective.

In this study first, in an attempt to develop a novel DNA aptamer ligand targeting to mTECs, a 12 round *in vitro* cell-based SELEX (Systemic Evolution of Ligands by EXponential Enrichment) method was established successfully. Negative selection was performed along with positive selection using normal endothelial cells (Skin-ECs) and renal tumor cell lines OS-RC-2 at 11 and 12 round selections. After a successful selection, followed to clone and sequence to obtain 48 novel DNA aptamers candidates. Among 48 DNA aptamer candidates, AraHH001 to specifically bind, with a high affinity, to mTECs in the nano-molar range but did not bind to normal skin endothelial cells (skin-EC) even not to bind to tumor cells. The selected DNA aptamer AraHH001 was also found to bind to cultured human tumor endothelial cells (hTEC), isolated from a clinical patient with a renal carcinoma as well as other different origin of mTECs .The newly developed aptamer AraHH001 showed significant anti-angiogenesis activity by inhibiting *in vitro* tube formation of mTECs on matrigel. Interestingly, a Confocal Laser Scanning Microscopy (CLSM) examination of *in vitro* cellular uptake showed that an AraHH001 was taken up by mTECs, and was co-localized in acidic compartments in mTECs by labeling with lysotracker Red®. Therefore, the development of a specific novel DNA aptamer AraHH001 that binds to mTECs, which is reported here for the first time, holds great promise as a targeted molecular probe that appear to play a major role in angiogenesis, and for the identification of a biomarker and for the development of a targeted new drug delivery system.

Second, since an AraHH001 selectively binds to different origin of primary cultured tumor endothelial cells, a strategy that followed Aptamer-facilitated Biomarker Discovery was applied to identify the molecular target of an AraHH001. Biotin-tagged AraHH001 was incubated with cell lysates of mTECs and aptamer-proteins were then conjugated with streptavidin magnetic beads. Finally, recovered bound proteins which were separated by SDS-PAGE with silver staining and identified molecular target of an AraHH001 named Troponin T by PMF analysis.Its presence was confirmed by a gel shift assay, measuring mRNA, protein levels, and immune staining of AraHH001 with Troponin T. Troponin T, a molecular target of an AraHH001 report here that express on mTECs, is novel findings. It is considering very promising and interesting diagnostic tool in the development of anti-angiogenic therapy by targeting tumor vasculature. Same method was applied to another potential novel aptamer candidate AraHH036 to recognize a molecular

target heat shock protein (HSP 70) from mTECs, and confirmed by expression of HSP70 on mTECs and Hela cell lines. HSP 70 is a very potential chaperon group protein that posses tumerogenic effects, and resistance during chemotherapy in different cancer types. Therefore, novel aptamer AraHH036 is promising to develop a ligand based drug delivery system by targeting HSP 70 to get anti-cancer effects.

Third, to establish an aptamer-modified new drug delivery system, Thiol modified AraHH001 aptamer-distearoylphosphoethanolamine-polyethylene-glycol-2000 conjugate (Apt-PEG-DSPE) was synthesized successfully, and applied it to prepare aptamer-modified PEG liposomes (Apt-PEG-LP) by a lipid hydration method, where aptamer was decorated outer surface of PEG-spacer. Apt-PEG-LP was significantly up-taken by mTECs and the cellular up-take was observed quantitatively and qualitatively by spectrofluorometer and CLSM respectively. Since aptamers can be chemically modified to enhance their stability in biological fluids, because of their small size; they can easily and rapidly diffuse into tissues and organs and thus permit faster targeting in drug delivery.

In conclusion, Cell-based SELEX method with improvised form has been established to develop a panel of novel DNA aptamer candidates. AraHH001, a novel DNA aptamer candidate, selectively binds to mTEC that has been recognizes a novel molecular target Troponin T. The technique for picking up molecular target of aptamer was established in very simple and improvised form and can be applicable for other cell types. This AraHH001 aptamer modified new drug delivery system has been established. In this study another novel DNA aptamer AraHH036 identified that recognizes the promising binding partner named heat shock protein (HSP 70).

学位論文審査の要旨

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博士学位論文審査等の結果について（報告）

近年、ガン細胞を標的とするDDSの分野ではアクティブターゲッティングに関する研究が盛んに行われている。しかし、現在のところ真に成功した例はなく、高性能なリガンドの開発が望まれていた。

本論文は、このような現況にあるDDSのリガンド開発において、腫瘍血管内皮細胞を標的とするDNAアプタマーを探索し、DDSの標的リガンドとして用い、ガン治療を達成することを目的としたものである。

著者は腫瘍血管内皮細胞を標的としてCell-SELEXを行い、12サイクルのセレクションの末、腫瘍血管内皮細胞に結合するDNAライブラリーを得た。FACSにより、ライブラリーの濃縮が行われていることが観察された。そのライブラリーをシークエンシングし、48個のDNA配列を得、これらの配列を網羅的に解析し、腫瘍血管内皮細胞に強く結合するDNAアプタマーAraHH001を同定した。このアプタマーの解離定数は43 nMであり、また非標的細胞である、腫瘍実質細胞OS-RC-II, 正常血管内皮細胞Skin-ECに対しては結合しないことを確認した。さらにこのアプタマーは標的分子に結合したのち、細胞内へ取り込まれることを共焦点レーザー走査型顕微鏡観察により示した。これらの調査からAraHH001はリガンドとして有効に機能する可能性が示された。また、このアプタマーを腫瘍血管内皮細胞に作用させると脈管形成を阻害することが明らかになった。このことはアプタマー単体でも新生血管阻害療法に使用できる可能性があることを示すものである。

続いて、著者はAraHH001アプタマーの標的分子を探索した。ビオチン標識アプタマーを用いる腫瘍血管内皮細胞溶解物のアフィニティー精製に続くSDS-PAGEによりアプタマーに結合するタンパク質を得、そのゲル断片をペプチドマスフィンガープリンティング法により解析して、標的タンパク質はトロポニンTと同定された。また、単離トロポニンTタンパク質とAraHH001アプタマーの結合をEMSAにより調べ、AraHH001アプタマーとトロポニンTが結合することを示した。さらに、腫瘍血管内皮細胞においてトロポニンTが発現しているかどうかを、mRNAの発現量およびFACSにより調べ、腫瘍血管内皮細胞では2倍程度mRNA量が増加していること、トロポニンT抗体が腫瘍血管内皮細胞に結合することを示した。さらにヒトの組織切片においても、トロポニンT抗体が血管に集積している様子が観測された。このようにmRNAおよびタンパク質レベルで、細胞、組織共にトロポニンTの発現が亢進していることを明らかにし、トロポニンTが新しいマーカー分子として使用できることを示した。

さらにAraHH001アプタマーを脂質と共有結合させ、リポソームを作製し、腫瘍血管内皮細胞へトランスフェクションさせたところ、アプタマー未修飾リポソームと比較して有意に取り込み量が向上することを明らかにした。

これを要するに，著者は，腫瘍血管内皮細胞を標的するDNAアプタマーの発見とDDSリガンドとしての評価を行った。さらに、アプタマー標識リポソームを調製し、取り込み量の向上を発見し、DDSのリガ

ンドとして有用であることを示した。さらにその標的分子がこれまで腫瘍血管内皮細胞に存在するとは考えられていなかったトロポニン T タンパク質であるという新知見を得たものであり，DDS に使用可能なリガンドの開発だけでなく、ガン生物学の見地からも興味深い発見である。

よって著者は，北海道大学博士（生命科学）の学位を授与される資格あるものと認める。