

厚生労働科学研究費補助金

萌芽的先端医療技術推進研究事業

細胞内動態制御機能を有する新規細胞選択型ナノ遺伝子
キャリアの開発と遺伝子治療への応用

平成16～18年度 総合研究報告書

主任研究者 川上 茂

平成19（2007）年 4月

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研究報告書

細胞内動態制御機能を有する新規細胞選択型ナノ遺伝子キャリアの開発と遺伝子治療への応用

主任研究者 川上 茂 京都大学大学院薬学研究科

研究要旨 本研究は、生体内で細胞特異的デリバリーなど高度な機能を発揮する多機能性標的指向型ナノ・遺伝子キャリアの創製を行うため、細胞特異的認識素子の表面導入、あるいは細胞内動態制御の指針となる総合的な設計戦略を確立し、同時に実用性の高い *in vivo* レベル遺伝子発現制御技術の開発を目指す。さらに本研究を通して開発した新規多機能性標的指向型ナノ・遺伝子キャリアを遺伝子治療へと応用する。平成 16-17 年度における検討を通じて、各肝臓構成細胞が有する固有で比較的厳密な基質認識性を示す糖鎖認識機構を利用できるような分子設計した各種糖修飾カチオン性リポソームを開発した。物理化学的性質、体内動態、遺伝子導入、毒性に関する評価を通じて基本的な細胞選択的 *in vivo* 遺伝子ターゲティングシステムの確立に成功した。一方、遺伝子治療への応用を考えていく上では、*in vivo* での導入活性のみならず治療効果との相関などを議論する必要がある。平成 17 年度における検討では、樹状細胞への遺伝子ターゲティングを DNA ワクチン療法へ応用し、遺伝子を用いて導入したがん抗原特異的細胞障害性 T 細胞の誘導による新規がん遺伝子治療法開発に成功した。平成 18 年度は、本システムの核酸医薬品への遺伝子治療への応用を試みるため、炎症関連遺伝子群の発現を誘導する転写因子 NFκB に対するデコイ型 2 本鎖オリゴヌクレオチド (NFκB デコイ) を用いた新規抗炎症療法に関する評価をおこなった。その結果、細胞内動態制御機能を有するフコース修飾カチオン性リポソームにより、NFκB デコイの静脈内投与による Kupffer 細胞選択的な送達を実現し、LPS 誘発性肝疾患マウスにおける肝炎抑制効果を得ることに成功した。以上、生体内で細胞特異的デリバリーなど高度な機能を発揮する多機能性標的指向型ナノ・遺伝子キャリアの開発に成功した。これらの知見は癌や炎症性疾患に対する遺伝子治療の実現に向けて有用な基礎的知見を与える。

A. 研究目的

本研究は、生体内で細胞特異的デリバリーなど高度な機能を発揮する多機能性標的指向型ナノ・遺伝子キャリアの創製を行うため、細胞特異的認識素子の表面導入、あるいは細胞内動態制御の指針となる総合的な設計戦略を確立し、同時に実用性の高い *in vivo* レベル遺伝子発現制御技術の開発を目指す。具体的には、i) 新規細胞内動態制御型遺伝子導入キャリアの開発、ii) *in vivo* 細胞特異的遺伝子導入のための製剤設計を通じて、最終年度には iii) 齧歯類での遺伝子治療への応用を目指す。

B. 研究方法

リポソームの調製: vortex 法により調製した。物理化学的性質の測定: ゼータ電位および平均粒子径は動的光散乱法により測定した。体内動態の評価: 蛍光あるいは³²P 標識プラスミド DNA あるいは NFκB デコイを調製

した。5 週齢雌性 ICR マウス (22-25 g) の静脈内へ各種複合体を投与した。マウスを安楽死させ、肝臓、肺、心臓、脾臓、腎臓への移行を組織切片あるいは放射活性により評価した。*In vivo* 遺伝子導入の評価: 5 週齢雌性 ICR マウスへ各種複合体を投与した。マウスを安楽死させ、肝臓、肺、あるいは腹腔滲出細胞 (APCs)、大網、リンパ節を回収し、それぞれの細胞における遺伝子発現レベルを測定した。細胞障害性 T 細胞 (CTL) 誘導の評価: 腹腔内または筋肉内投与投与後、脾臓細胞を B16BL6 細胞と共存下にて培養し、B16BL6 細胞に対して CTL の評価をおこなった。抗炎症効果の評価: LPS 誘発性肝炎を誘発させ、複合体による抑制効果を肝臓中 NFκB 量、血清中サイトカイン、aspartate aminotransferase (AST)、alanine aminotransferase (ALT) の測定を指標に行った。

C.D. 結果・考察

i) 新規細胞内動態制御型遺伝子導入キャリアの開発: His を含む新規 Gal-His-C4-Chol 含有リポソームの平均粒子径・ゼータ電位は、対照のリポプレックス群とほぼ同じ値であった。³²P 標識した pDNA を用いてアジアロ糖タンパクレセプターを高発現する HepG2 細胞への取り込みを評価した結果、高い取り込みを示すことが明らかとなった。次に、HepG2 細胞への遺伝子導入能を評価した結果、Gal-His-C4-Chol 含有リポプレックスは、有意に高い遺伝子発現能を示した。また、細胞取り込みと遺伝子発現の相関より、Gal-His-C4-Chol 含有リポソーム複合体はアジアロ糖タンパクレセプターにより細胞選択的に取り込まれ、細胞内動態の改善により高い遺伝子発現に至っていることが示された。*In vivo* における遺伝子導入に関して、Man-His-C4-Chol 含有リポソーム複合体は、腹腔内投与によりマンノースレセプターを介した細胞選択的な取り込みにより効率的な抗原提示細胞への遺伝子導入が可能であることを明らかにした。以上、Gal-および Man-His-C4-Chol 含有リポソームが有用な多機能型遺伝子導入キャリアとなり得ることが示された。

ii) *in vivo* 遺伝子導入のための製剤設計: 血管内へカチオン性リポソーム複合体を静脈内投与後、主に赤血球との相互作用により標的細胞へのデリバリーが阻害されていることを明らかにした。一方、中性脂質として広く用いられる pH 感受性脂質 DOPE を含有するリポソーム複合体は静脈内投与後肺からの脱出が早く、肺以外の臓器へのターゲティングに適していることが示唆された。また、腹腔内は生体成分も血管内と比べて少なく、腹腔内やリンパに存在する抗原提示細胞への長期的かつ効率的な遺伝子導入が可能であることを明らかにした。一方、腹腔内投与では DOPE を含むカチオン性リポソーム複合体の遺伝子導入能は低く、cholesterol を用いてリポソーム膜を安定化させることで遺伝子発現を改善できることが示唆された。さらに、複合体形成時の溶媒のイオン濃度が安定な複合体形成に重要であり、*in vivo* 遺伝子導入能にも影響することを明らかにした。以上、静脈内投与ならびに腹腔内投与における遺伝子導入におよぼす製剤学的な因子を明らかに

した。

iii) 遺伝子治療への応用

腹腔内投与による抗原提示細胞デリバリーに基づく DNA ワクチン療法の開発をおこなった。その結果、マンノース修飾リポソーム複合体の腹腔内投与法による遺伝子導入法では、効率的な導入抗原特異的な CTL 誘導に基づくがん免疫療法が可能であることを明らかにした。

また、オリゴヌクレオチドへの応用に関して、Fuc-C4-Chol, DOPE により細胞内動態制御機能を有するフコース修飾カチオン性リポソーム (Fuc リポソーム) を調製した。静脈内投与後、Fuc リポソーム/NFκB デコイ複合体の Kupffer 細胞選択的な送達および炎症抑制効果について検討した。静脈内投与後、Fuc リポソーム/[³²P] 標識 NFκB デコイ複合体は、対照の naked NFκB デコイおよびカチオン性リポソーム複合体に比べ、有意に高く肝臓へ集積した。また、肝臓内細胞分布に関しては、Kupffer 細胞が存在する肝非実質細胞への集積が認められた。さらに、Kupffer 細胞に障害を与える GdCl₃ を前投与したところ、Fuc リポソーム/NFκB デコイ複合体の肝臓への集積が有意に減少し、Kupffer 細胞での高い取り込みが示唆された。

LPS 誘発性肝疾患マウスに対し、Fuc リポソーム/NFκB デコイ複合体を静脈内投与したところ、対照の naked NFκB デコイ、カチオン性リポソーム複合体と比べ、血清中 TNFα量、IFNγ量の有意な抑制効果が認められた。また、本治療実験に用いたマウス肝臓核内 NFκB 量を評価したところ、有意な核内 NFκB 量の増加抑制が認められ、Fuc リポソーム/NFκB デコイ複合体による NFκB の活性化抑制が確認された。さらに、Fuc リポソーム/NFκB デコイ複合体による AST、ALT の有意な抑制も認められ、肝炎治療の可能性が強く示唆された。

E. 結論

生体内で細胞特異的デリバリーなど高度な機能を発揮する多機能性標的指向型ナノ・遺伝子キャリアの開発に成功した。また、齧歯類動物によりプラスミド DNA、オリ

ゴヌクレオチドによる癌、炎症に対する遺伝子治療を証明した。これらの知見は、今後の遺伝子治療技術の開発において有益な基礎的知見となり得る。

F. 健康危険情報

なし

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H. 財産権の出願・登録状況

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2. 実用新案登録 なし

3. その他 なし

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Optimization of tumor-selective targeting by basic fibroblast growth factor-binding peptide grafted PEGylated liposomes

Takeshi Terada, Miki Mizobata, Shigeru Kawakami, Fumiyoshi Yamashita, and Mitsuru Hashida *

Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan.

*Correspondence: M. Hashida, Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan. Tel: 81 75 753 4525. Fax: 81 75 753 4575. E-mail: hashidam@pharm.kyoto-u.ac.jp

Abstract

We have previously shown that the peptide, KRTGQYKLC (bFGF), is recognized by fibroblast growth factor (FGF) receptor (FGFR) via binding to basic FGF (bFGF), and is capable of being used for drug delivery to tumors highly expressing FGFR and bFGF. However, although the binding and uptake of the liposomes (bFGFp-liposomes) modified by the peptide increased in the presence of bFGF, the modification induced non-specific uptake. To overcome this problem, here, we prepared bFGFp-liposomes including mPEG-DSPE. The 5 and 10% mPEG₅₀₀₀/ and 10% mPEG₃₀₀₀/bFGFp-liposomes reduced most of the interaction with erythrocytes and the uptake by macrophages, suggesting the sustained blood circulation of bFGFp grafted PEGylated liposomes. Furthermore, 10% mPEG₃₀₀₀/bFGFp-liposomes produced a significant increase in uptake in NIH3T3, A549, and B16BL6 cells with the expression of FGFR following pre-incubation with bFGF, but no increase in CHO-K1 cells lacking FGFR expression. Taken together, these results lead us to believe that bFGFp grafted PEGylated liposomes possess the functions of both PEGylated stealth liposomes and the tumor-targeting liposomes. This strategy could be applied to the development of novel tumor-selective drug delivery systems.

Keywords: Drug delivery system; Liposomes; Targeting; bFGFp-liposomes, Cancer

1. Introduction

Tumor cells, such as melanoma, breast cancer, and prostate cancer, are reported to exhibit overexpression of basic fibroblast growth factor (bFGF) and FGF receptors (FGFR) (1-3). Furthermore, such tumors have high concentrations of bFGF (4), a multi-functional protein, which has mitogenic, chemotactic, and angiogenic activities and plays an autocrine role in tumor angiogenesis and progression (5-9).

It has been reported that residues 117-126 of bFGF might interact with bFGF itself (10). Recently, we have shown that the peptide KRTGQYKLC (bFGFp) containing cysteine at the carboxyl terminal of residues 119-126 of bFGF is able to bind to bFGF and is recognized by FGFR via this binding (11). The binding of bFGFp to bFGF was also higher than that to albumin which is the major serum protein, and the dissociation constant was very low. Based on these findings, we have demonstrated that bFGFp grafted liposomes (bFGFp-liposomes) are specifically taken up by tumor cells highly expressing FGFR via binding to bFGF, suggesting that it could be used as a novel drug delivery carrier to target tumors. However, it was also shown that the non-specific binding of bFGFp-liposomes by bFGFp is high. Therefore, further optimization is needed to reduce the non-specific binding for the *in vivo* use of bFGFp-liposomes for tumor-specific targeting.

It has been reported that polyethyleneglycol (PEG)-grafted liposomes are not highly recognized by the reticulo-endothelial system (RES) and they remain longer in the blood circulation than conventional liposomes (12-15). Accordingly, in the design of bFGFp-liposomes, one approach is to conjugate bFGFp to the liposomal PEG terminals. This strategy has been reported to be an effective targeting approach to reduce non-specific binding (16). However, Savva et al. showed that PEG-grafted liposomes bearing genetically modified recombinant tumor necrosis factor- α on the PEG terminals exhibited rapid plasma elimination and high accumulation in liver and spleen (17). Furthermore, they suggested that not all biological molecules are suitable as targeting ligands when exposed to the grafted PEG extremities of liposomes circulating for long periods. Since bFGFp contains many basic amino residues, the bFGFp-liposomes possess a positive charge on the liposomal surface even if bFGFp is conjugated to the liposomal PEG terminals. The positive charge may induce rapid uptake by RES and non-specific binding with unexpected cells, such as erythrocytes, after intravenous administration.

In the present study, to overcome this problem, we prepared bFGFp-liposomes containing mPEG-DSPE (bFGFp grafted PEGylated liposomes). This mPEG is expected to limit the interactions with RES and other non-target cells. Therefore, we evaluated the inhibitory effects on the uptake by macrophages, used as model cells of the RES, and the interaction with erythrocytes *in vitro*. Furthermore, the binding of bFGFp grafted PEGylated liposomes to bFGF can induce the specific uptake by the tumor cells expressing FGFRs due to the protrusion of the binding bFGF from the PEG layer.

2. Materials and Methods

2.1. Materials

Recombinant human bFGF was obtained from PeproTech EC. (London, UK). Recombinant human FGFR1 α (IIIc)/Fc chimera (FGFR1) was purchased from Techno Co. (Minneapolis, MN, USA). Distearoyl phosphatidylcholine (DSPC) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Cholesterol (Chol) and Clear-Sol I were obtained from Nacalai Tesque Inc. (Kyoto, Japan). [3 H] CHE was purchased from NEN Life Science Products Inc. (Boston, MA, USA). The peptide KRTGQYKLC (bFGFp) was custom-made by Toray Research Center Inc. (Tokyo, Japan). mPEG₃₀₀₀-DSPE was purchased from Avanti Polar Lipids Inc. (Alabama, AL, USA). mPEG₃₀₀₀-DSPE and Maleimide-PEG₂₀₀₀-DSPE was purchased from NOF Co. Inc. (Tokyo, Japan). All other chemicals were reagent grade products obtained commercially.

2.2. Cell lines

NIH3T3 mouse fibroblasts, A549 human lung cancer cells, and B16BL6 mouse melanoma cells were routinely grown in DMEM medium supplement with 10% FBS, 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine (all from Invitrogen Co., Carlsbad, CA, USA) in 5% CO₂, humidified air at 37 °C. Chinese hamster ovary (CHO)-K1 cells were cultured in Ham's F12 medium supplemented with 10% FBS.

2.3. Synthesis of bFGFp-PEG-DSPE

Synthesis of bFGFp-PEG-DSPE was carried out by the method described previously (11). Briefly, 28 mg bFGFp (26 μ mol) was dissolved in 2 mL 0.1 M HEPES buffer (pH 7.0) then 50 mg maleimide-PEG₂₀₀₀-DSPE (17 μ mol) and 0.2 mL methanol were added to the bFGFp solution at 4 °C while stirring. The reaction was continued at 4 °C for 2 days. To remove the unreacted bFGFp, the solution was dialyzed against distilled water using a suitable dialysis membrane (3.5 kDa cut-off), and lyophilized. TLC analysis and ninhydrin assay showed the disappearance of free maleimide-PEG₂₀₀₀-DSPE and the appearance of bFGFp-PEG₂₀₀₀-DSPE, indicating that the reaction had gone to completion. The purity of the synthesized bFGFp-PEG-DSPE was calculated by fluorescamine assay (18) and found to be over 90% on the basis of the bFGFp amino acids. Furthermore, 10 mM L-cysteine solution and the product were mixed at room temperature for 1 h to consume any free maleimide-PEG₂₀₀₀-DSPE, followed by dialysis and lyophilization.

2.4. Preparation of liposomes

Liposomes were prepared by our previously reported method (19-21). Each lipid mixture (DSPC, Chol, mPEG₃₀₀₀-DSPE, and mPEG₅₀₀₀-DSPE) was dissolved in chloroform and evaporated to dryness. bFGFp-PEG-DSPE was solved in a mixed organic solvent (chloroform:methanol = 1:1) beforehand. [³H] CHE or PE-fluorescein (1 mol %) was added to the liposomes for the uptake study and confocal microscopy, respectively. The dried lipid films were hydrated in PBS, sonicated at 65 °C for 3 min, and then extruded through 0.2 μm pore size polycarbonate filters. Mean particle diameters were determined by laser light scattering using a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK). All liposomes were similar in size (an average diameter of approximately 100 nm) as shown in Table 1.

2.5. Quartz crystal microbalance (QCM) biosensor system

Binding analysis between bFGF and bFGFp-liposomes was performed with a 27 MHz QCM instrument (AffinixQ, Initium Inc, Tokyo, Japan). bFGF was immobilized on a ceramic sensor chip using an amine coupling reaction. A drop of 5 mM DTDP was applied to a sensor chip to immobilize the DTDP directly on the gold electrode surface of the chip by an Au-thiol interaction. After 24 h, the sensor chip was then activated by adding a coupling solution including 50 mg/mL EDC and 50 mg/mL NHS. Immobilization of bFGF onto the QCM was achieved by coating the surface with 50 μg/mL bFGF solutions for 2 h at room temperature, followed by rinsing with water. To block the activated surface and nonspecific binding sites, the surface was exposed to 50 mM Tris buffer (pH 8.0) and the purchased blocking solution (Initium Inc., Tokyo, Japan) for 30 min and then washed with water. After immersing the prepared sensor chip in 8 mL 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, with gentle stirring at 37 °C, each sample was added to the buffer and the interaction was investigated by monitoring the alterations in frequency (ΔF) resulting from changes in mass at the electrode surface.

Binding analysis between erythrocytes and each mPEG/bFGFp-liposome preparation was performed with a 27 MHz QCM instrument. Fresh blood from male ICR mice was collected in a heparinized syringe. The mice were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). Erythrocytes were washed three times on ice in order to remove serum proteins and resuspended in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl. Erythrocytes were immobilized on a ceramic sensor chip by an amine coupling reaction. Briefly, 10% erythrocyte suspension (erythrocyte volume/erythrocyte suspension volume %) was added to the activated surface of the sensor chip by the method described above. After 1 h at 4 °C, the surface was rinsed with PBS. To block the activated surface and nonspecific binding sites, the surface was exposed to 50 mM Tris buffer (pH 8.0) containing 150 mM NaCl and the purchased blocking solution (initium Inc., Tokyo, Japan) for 30 min at 4 °C and then washed with PBS. After immersing the prepared sensor chip in 8 mL 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, with gentle stirring at 37 °C, each liposome preparation was added to the buffer in the following order: 10 and 5% mPEG₅₀₀₀/bFGFp-liposomes, 10% mPEG₃₀₀₀-bFGFp-liposomes, and bFGFp-liposomes. The interaction was investigated by monitoring alterations in frequency (ΔF) resulting from changes in mass at the electrode surface.

2.6. Surface plasmon resonance (SPR) spectroscopy assay

SPR measurement was performed using a BIAcore X instrument (BIAcore, Uppsala, Sweden) by our previously reported method (11, 22). FGFR1 was immobilized on the surface of a CM5 sensor chip using the standard amine coupling procedure of the manufacturer. Briefly, the surface of the chip consisting of flow cells 1 and 2 was activated by exposing them to a mixture of 0.05 M *N*-hydroxysuccinimide (NHS) and 0.2 M *N*-ethyl-*N'*-dimethylaminopropyl carbodiimide (EDC) for 7 min. Flow cell 1 was immobilized with FGFR1 in acetate buffer (pH 4.0). The amount of the immobilization was approximately 5000 resonance units (RU). Flow cell 2 was immobilized with the same amount as flow cell 1 by BSA to be used as a blank sensorgram for subtraction of the bulk refractive index background. Finally, the unreacted sites of both immobilized flow cells were blocked with 0.1 M ethanolamine (pH 8.5). All reagents were injected at a flow rate of 5 μL/min.

In the binding measurements, each sample was adjusted to an appropriate concentration by using a buffer (10 mM HEPES, 150 mM NaCl, pH 7.4). Each sample solution was allowed to flow at 20 μL/min for 3 min at 25 °C and dissociated for 3 min. The regeneration of the sensor chip was carried out by injection of 50 mM HCl.

2.7. Cellular uptake study in macrophages

A cellular uptake study in macrophages was performed using our previously reported method (23). Briefly, five-week-old male ICR mice were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). Resident macrophages were collected from the peritoneal cavity of unstimulated mice with serum free RPMI 1640 medium. Washed cells were suspended in RPMI 1640 medium supplemented with serum and plated on 12-well culture plates at a density of 1×10^6 cells / well. After incubation for 2 h at 37 °C in 5% CO₂ - 95% air, adherent macrophages were washed three times with RPMI 1640 medium to remove non-adherent cells and then cultured under the same conditions. After 48 h incubation, the cells were washed 3-times and incubated with HBSS containing 100 μM (total lipids) of each [³H] liposome form at 37 °C for 2 h. The radioactivity was measured as described above.

2.8. Cellular association experiments

A cellular association study was performed using our previously reported method (11, 23). Briefly, NIH3T3, A549 and CHO-K1 cells were plated on 12-well culture plates at a density of 1×10^5 cells/well. After 24 h incubation, cells were washed three times with Hanks' balanced salt solution (HBSS) and incubated with 100 μM (total lipids) of each [³H] liposome form, with or without

pretreatment of 1 $\mu\text{g/mL}$ bFGF, for the indicated time in HBSS at 37 °C. After 5 h, the solution was removed and cells were washed with ice-cold HBSS. Cells were then solubilized with 0.3 N NaOH with 0.1 % Triton-X-100 and the radioactivity in the cell lysate was measured using a well-type NaI-scintillation counter (ARC-500, Aloka, Tokyo, Japan). The amount of cellular protein in each cell lysate was estimated using a protein quantification kit (Dojindo Laboratory).

2.9. Confocal microscopic study

The confocal microscopic study was performed using a standard method (24, 25). Briefly, NIH3T3, A549, and B16BL6 cells were seeded on glass coverslips in 12-well plates at a density of 1×10^5 cells/well. After complete adhesion, the cells were washed 3 times with HBSS and incubated at 4 or 37 °C for 5 h with the fluorescent 10% mPEG₃₀₀₀/bFGFp-liposomes, with or without the pretreatment of 1 $\mu\text{g/mL}$ bFGF, for 24 h in HBSS. After incubation, the liposome solution was removed, and the cells were washed 3 times with HBSS, followed by fixing with 4% paraformaldehyde in PBS, and incubating at room temperature for more than 20 min. After washing twice with PBS, the cells were mounted in glycerol:PBS (1:1) containing 2.5% 1,4-diazobicyclo (2,2,2) octane. Images were obtained by confocal laser scanning microscopy (MRC-1024, Bio-Rad, Hercules, CA, USA).

2.10. Statistical analysis

Statistical analysis was performed using Student's paired *t*-test for two groups. Multiple comparisons with the control group were performed by ANOVA with Dunnett's multiple-comparison test. $P < 0.05$ was considered to be indicative of statistical significance.

3. Results

3.1. Interaction analysis of bFGFp-liposomes with bFGF

The interaction of bFGFp-liposomes with bFGF was investigated using the QCM biosensor system (Fig. 1). The 27-MHz QCM biosensor system used in this study has a sensitivity of 0.6 ng cm^{-2} of mass change per 1 Hz of frequency decrease (26). bFGF was immobilized on a sensor chip coated with DTDPA by amine coupling. Fig. 1 shows the typical frequency changes for the bFGF-immobilized QCM sensor chip, responding to additions of mPEG-liposomes or bFGFp-liposomes in solution. The addition of mPEG-liposomes produced no change in the frequency (mass). On the other hand, in the case of bFGFp-liposomes, the frequency decreased (mass increased), and the decrease was inhibited by the presence of excess bFGF. Therefore, the frequency decrease indicated by the addition of bFGFp-liposomes is consistent with the mass increase due to the binding of bFGFp-liposomes to bFGF via the bFGFp present in the QCM biosensor system.

3.2. The binding of bFGFp-liposomes to FGFR1 via bFGF

Recently, we used SPR spectroscopy to show that BSA modified by bFGFp (bFGFp-BSA) interacts with FGFR1 via binding to bFGF (11). Here, we confirmed the interaction of bFGFp-liposomes with FGFR1 via binding to bFGF using the same method as in the previous report (11) (Fig. 2). FGFR1 was immobilized on the surface of a CM5 sensor chip by amine coupling. Non-specific binding of an analyte and any change in bulk refractive index were eliminated by immobilizing BSA as a control. Although bFGF showed a significantly high response signal (Fig. 2), no response signal was observed with bFGFp-liposomes. In addition, the response signal of bFGFp-liposomes pre-incubated with bFGF was much higher than that of bFGF itself, suggesting that bFGFp-liposomes pre-incubated with bFGF are capable of binding to FGFR1 via bFGF. These results were in good agreement with our previous report using bFGFp-BSA (11).

3.3. The effect of mPEG-DSPE on the uptake of bFGFp-liposomes by macrophages

Our previous study showed that bFGFp-liposomes exhibit a high degree of unspecific uptake in cells (11). As shown in Fig. 3, we also found a high uptake by macrophages used as model cells of mononuclear phagocytes in the RES. The high uptake by macrophages would lead to a reduced targeting efficiency to the tumor cells because of the high trapping by RES after intravenous administration. Therefore, we prepared mPEG/bFGFp-liposomes, without or with 1, 2.5, 5, and 10% of mPEG₅₀₀₀-DSPE and mPEG₃₀₀₀-DSPE, to reduce the non-specific binding and/or uptake. The cellular association in macrophages decreased with an increase in the amount of mPEG-DSPE. In particular, mPEG/bFGFp-liposomes containing 5 or 10% mPEG₅₀₀₀-DSPE (Fig. 3 (A)) and 10% mPEG₃₀₀₀-DSPE (Fig. 3 (B)) showed a significantly reduced uptake, suggesting prolonged circulation in the body after intravenous administration.

3.4. The effect of mPEG-DSPE on the interaction of mPEG/bFGFp-liposomes with erythrocytes

Furthermore, the effect of mPEG-DSPE on the interaction of mPEG-bFGFp-liposomes with erythrocytes was evaluated using a QCM biosensor system. Fig. 4 shows the typical frequency changes for the isolated erythrocyte-immobilized QCM sensor chip, responding to additions of each form of liposomes in the following order: 10 and 5% mPEG₅₀₀₀/bFGFp-liposomes, 10% mPEG₃₀₀₀/bFGFp-liposomes, and bFGFp-liposomes. The three kinds of mPEG/bFGFp-liposomes indicating the controlled uptake in macrophages exhibited no change in frequency (mass) in the QCM biosensor system, suggesting no interaction with erythrocytes. On the other hand, the frequency decreased (mass increased) following the addition of bFGFp-liposomes. The three

mPEG/bFGFp-liposomes are capable of controlling the interaction with erythrocytes via the bFGFp present on the surface of the liposomes.

3.5. The effect of bFGF on the uptake of mPEG/bFGFp-liposomes by various cells

The cellular uptake via bFGF of the three mPEG/bFGFp-liposomes was examined (Fig. 5). NIH3T3 and A549 cells express FGFR, and CHO-K1 cells are known to lack these receptors (27). In these cells with or without FGFRs, the uptake of mPEG/bFGFp-liposomes was almost the same as that in macrophages, suggesting that the each incorporation of 5 or 10% mPEG5000-DSPE and 10% mPEG3000-DSPE is enough to inhibit the non-specific cellular uptake of bFGFp-liposomes. Furthermore, pre-incubation of mPEG₃₀₀₀/bFGFp-liposomes with bFGF produced a significant increase in the uptake in NIH3T3 and A549 cells. However, CHO-K1 cells exhibited no such increase while the two PEG₅₀₀₀/bFGFp-liposomes exhibited no uptake in NIH3T3 and A549 cells, even if pre-incubated with bFGF.

3.6. The binding or internalization of mPEG₃₀₀₀/bFGFp-liposomes to cells

To further demonstrate the binding of the labeled mPEG₃₀₀₀/bFGFp-liposomes with PE-fluorescein via bFGF to cells using confocal microscopy, the liposomes were incubated with NIH3T3 cells at 4 °C. Fluorescent signals of the liposomes pre-incubated with bFGF were observed on the surface of the cells, and these were much stronger than those of liposomes without bFGF (Fig. 6). In addition, the cellular internalization of the liposomes by confocal microscopy was examined in NIH3T3, A549, and B16BL6 cells, all of which are known to express FGFR (Fig. 7). In all the cells used, the images showed that the liposomes pre-incubated with bFGF were internalized, and the fluorescent signal was stronger than that of the liposomes without bFGF. These results were in good agreement with our previous study of cellular internalization using bFGFp-BSA.

3.7. The effect of the pre-incubation time of mPEG₃₀₀₀/bFGFp-liposomes with bFGF on the uptake in NIH3T3 cells

mPEG₃₀₀₀/bFGFp-liposomes were incubated with bFGF for 1 and 24 h, and the uptakes in NIH3T3 cells were compared with those of liposomes without bFGF (Fig. 8). Although the incubation for 1 h significantly increased the uptake, incubation of mPEG₃₀₀₀/bFGFp-liposomes for 24 h produced a higher increase in uptake. In spite of high association of bFGFp with bFGF, it took long time to equilibrate the interaction of mPEG₃₀₀₀/bFGFp-liposomes with bFGF. This suggests that the incorporation of mPEG-DSPE in bFGFp-liposomes hinders the interaction of bFGFp with bFGF, and the successful targeting of the tumor takes some time.

4. Discussion

PEGylation provides drug carriers with steric stabilization, subsequently leading to reduce uptake by the RES and prolonged circulation in the body (15, 28, 29). Pharmacokinetic analysis and therapeutic studies with tumor-bearing mice have shown that PEG-liposomes have considerable potential as drug carriers for tumor therapy. These liposomes can exploit the “enhanced permeability and retention (EPR)” effect (30-32) for preferential extravasation from tumor vessels, which results in increased liposome accumulation in tumor tissues (33, 34). However, to produce more effective targeting of tumor cells, requires the development of novel liposomes exhibiting both a long circulation by PEGylation and the active targeting by the ligand.

In a previous report, we showed that the peptide KRTGQYKLC (bFGFp) can interact with FGFR1 via binding to bFGF, and is useful as the targeting ligand for tumor cells (11). The binding characteristics allow the development of novel PEG-liposomes for tumor therapy. First of all, we confirmed the binding of bFGFp-liposomes to bFGF and FGFR1 by using a QCM biosensor system and SPR spectroscopy, which has been widely used as an instrument to study a variety of biomolecular interactions, including liposome-protein (35, 36), DNA-protein (26) and protein-protein (37, 38). All liposomes were prepared with an average diameter of 100-200 nm (Table 1), which has been reported to result in a prolonged circulation time and high tumor accumulation (39, 40). bFGFp-liposomes interacted with bFGF via bFGFp (Fig. 1) and with FGFR1 via binding to bFGF (Fig. 2). These findings agree with our previous report using bFGFp-BSA (11).

To achieve prolonged blood circulation of bFGFp-liposomes in the body, we prepared mPEG/bFGFp-liposomes by adding mPEG-DSPE to bFGFp-liposomes. The steric hindrance of the added mPEG on the surface of bFGFp-liposomes results in a reduction in the non-specific binding caused by the bFGFp present. In this study, we selected two kinds of mPEG-DSPE containing PEG with an MW of 5000 or 3000. Since the MW of the bFGFp-PEG of the synthesized bFGFp-PEG-DSPE is approximately 3000, it appears that the two mPEG-DSPEs have a sufficient PEG to cover the bFGFp present. The high uptake by macrophages and strong interaction with erythrocytes in bFGFp-liposomes, which leads to early elimination from the blood and a low targeting efficiency, were significantly reduced by the addition of 5 and 10% mPEG₅₀₀₀/bFGFp- and 10% mPEG₃₀₀₀/bFGFp-liposomes (Figs. 3 and 4). These results lead us to believe that these bFGFp grafted PEGylated liposomes would exhibit a prolonged circulation in the body.

In the bFGFp grafted PEGylated liposomes, the targeting to tumor cells via binding to bFGF indicates the success of novel PEG-liposomes exhibiting both prolonged blood circulation and active tumor targeting. In practical terms, we showed that the pre-incubated mPEG₃₀₀₀/bFGFp-liposomes with bFGF exhibit specific binding to the cells expressing FGFR (Fig. 5). The addition of 10% mPEG₃₀₀₀-DSPE to bFGFp grafted liposomes abolished the binding by the bFGFp present on the liposomal surface, and the binding of bFGF to bFGFp could allow the targeting of tumor cells via FGFR by protrusion of the binding bFGF from the PEG layer. Furthermore, the receptor-mediated internalization demonstrated by confocal microscopy (Fig. 7) would allow the 10% mPEG₃₀₀₀/bFGFp-liposomes to avoid the action of multidrug resistant (MDR) transporters like P-glycoprotein and enhance the pharmacological effects of the antitumor drug as reported recently (41). We also found that pre-incubation with 10% mPEG₃₀₀₀/bFGFp-liposomes enhances the binding on the surface of NIH3T3 cells as shown by an *in vitro* study at 4 °C (Fig. 6),

suggesting that the targeting potential obtained by pre-incubation is not due to the physiological effects of bFGF itself. The difference in uptake between pre-incubation with 10% mPEG₃₀₀₀-bFGFp-liposomes for 1 and 24 h also suggests that the targeting is not due to physiological effects (Fig. 8). Furthermore, this result leads us to surmise that targeting via binding to bFGF would take a comparatively long time. Some tumor cells are reported to exhibit overexpression of bFGF and FGFRs (1-3), and such tissues contain high concentrations of bFGF (4). Taking these findings into consideration, liposomes with a prolonged blood circulation may accumulate in tumor tissues containing high concentrations of bFGF by the EPR effect, and subsequently lead to active targeting via binding to bFGF.

mPEG₅₀₀₀/bFGFp-liposomes exhibited no targeting effect on cells even if pre-incubated with bFGF. One possible reason for this is that the long PEG-chains could completely inhibit the interaction with bFGF, or else the bFGFp present could bind to bFGF, and the interaction of the bound bFGF with FGFR was blocked by the long PEG-chains on the liposomal surface. This finding agrees with a previous report showing that the immuno-specific antibody-antigen binding by immunoliposomes conjugated with a monoclonal IgG antibody could be reduced by the addition of PEG with an MW of 2000 (42). In the case of complicated binding, such as antibody-antigen and bFGF-FGFR, the addition of short PEG-chains could suppress the targeting effects of ligand-modified liposomes due to steric hindrance.

In the targeting system using ligands such as peptides, species differences are a problem as far as successful clinical application is concerned. It has been reported that the homology between murine and human FGF-2 is high (95% homologous) (43). Moreover, the peptide KRTGQYKL used in the present investigation is completely homologous with both murine and human FGF-2. Therefore, further investigation of this strategy will provide us with information about the potential application to tumor therapy in humans.

In conclusion, we have succeeded in the development of novel PEG-liposomes for tumor targeting. mPEG/bFGFp-liposomes were prepared as novel PEG-liposomes by adding mPEG-DSPE to bFGFp-liposomes capable of targeting tumor cells expressing FGFR via binding to bFGF. The addition of 5 and 10% mPEG₅₀₀₀-DSPE and 10% mPEG₃₀₀₀-DSPE reduced the uptake by macrophages and interaction with erythrocytes. Furthermore, 10% mPEG₃₀₀₀/bFGFp-liposomes exhibited specific targeting of tumor cells via binding to bFGF. Further investigations involving *in vivo* studies, including the interaction with serum proteins and the targeting efficiency of the local bFGF concentrations in tumor tissue, are needed. However, our results suggest that the bFGFp grafted PEGylated liposomes exhibit both prolonged blood circulation by limiting the transfer to normal tissues and active targeting to cells in the tumor tissues. This strategy could be applied to the development of novel tumor-selective drug delivery systems.

Acknowledgments

This work was supported in part by Grant-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by Health and Labour Sciences Research Grants for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare of Japan, and by the 21st Century COE Program "Knowledge Information Infrastructure for Genome Science".

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Figure legends

Fig. 1. QCM assay of binding to the immobilized bFGF for mPEG-liposomes and bFGFp (2.5%)-liposomes, with or without 50 $\mu\text{g}/\text{mL}$ bFGFp. Two μM (total lipids) liposomes were injected over immobilized bFGF. Each response signal was overlaid and zeroed on the y-axis to the average baseline. The start injection time for each form of liposomes was set to zero on the x-axis.

Fig. 2. SPR sensorgrams of binding with the immobilized FGFR1 for 5 $\mu\text{g}/\text{mL}$ bFGF, and 100 μM (total lipids) bFGFp (2.5%)-liposomes, with or without 5 $\mu\text{g}/\text{mL}$ bFGF. Each sensorgram was overlaid and zeroed on the y-axis to the average baseline. The start injection time for each form of liposomes was set to zero on the x-axis.

Fig. 3. Effect of the content and length of mPEG-DSPE on the uptake of [^3H] mPEG/bFGFp-liposomes (\square) by mouse peritoneal macrophages. Peritoneal macrophages were incubated with mPEG₅₀₀₀/bFGFp-liposomes (A) and mPEG₃₀₀₀/bFGFp-liposomes (B) containing different amounts of mPEG-DSPE at 37 $^\circ\text{C}$ for 2 h. The uptake of [^3H] bFGFp-liposomes (\blacksquare) was used as a comparison. Each result represents the mean \pm S.D. values ($n = 3$). Statistical significance was analyzed by Dunnett's test versus bFGFp-liposomes (**, $P < 0.01$; N.S., not significant).

Fig. 4. QCM assay of binding to the immobilized erythrocytes for mPEG/bFGFp-liposomes and bFGFp-liposomes. Two μM liposomes were injected over immobilized erythrocytes in the following order: 10% mPEG₅₀₀₀/bFGFp-liposomes (A), 5% mPEG₅₀₀₀/bFGFp-liposomes (B), 10% mPEG₃₀₀₀/bFGFp-liposomes (C), and bFGFp-liposomes (D).

Fig. 5. Effect of bFGF on the cellular association of [^3H] mPEG/bFGFp-liposomes by different cells. 10% mPEG₅₀₀₀/bFGFp-liposomes,

5% mPEG₅₀₀₀/bFGFp-liposome, and 10% mPEG₃₀₀₀/bFGFp-liposomes were pre-incubated with bFGF at 37 °C for 24 h. NIH3T3 (A), A549 (B), and CHO-k1 (C) cells were incubated with the mPEG/bFGFp-liposomes without (■) or with (□) the pre-incubation of bFGF at 37 °C for 5 h. Each result represents the mean ± S.D. values (n = 3). Statistical significance was analyzed by Student's t-test versus each group without the pre-incubation of bFGF (**, *P* < 0.01; ***, *P* < 0.001; N.S., not significant).

Fig. 6. Confocal microscopy images of the binding of 10% mPEG₃₀₀₀/bFGFp-liposomes by NIH3T3 cells. NIH3T3 cells were incubated with 10% mPEG₃₀₀₀/bFGFp-liposomes containing 1% PE-fluorescein, without or with the pre-incubation of bFGF at 4 °C for 5 h.

Fig. 7. Confocal microscopy images of the binding and internalization of 10 % mPEG₃₀₀₀/bFGFp-liposomes by various cells. NIH3T3 (A, D), A549 (B, E), and B16BL6 cells (C, F) were incubated with 10 % mPEG₃₀₀₀/bFGFp-liposomes containing 1% PE-fluorescein, without (A, B, C) or with (D, E, F) the pre-incubation of bFGF at 37 °C for 5 h.

Fig. 8. Effect of the preincubation time with bFGF on the cellular association of [³H] mPEG₃₀₀₀/bFGFp-liposomes by NIH3T3 cells. 10% mPEG₃₀₀₀/bFGFp-liposomes pre-incubated with bFGF for the indicated times were used for the uptake study. Each result represents the mean ± S.D. values (n = 3).

Table 1. Lipid composition and mean particle size of liposomes investigated

Lipid composition (molar ratio)	Particle size (nm) ^a
mPEG-liposomes (DSPC/Chol/mPEG-DSPE =60:37.5:2.5)	101 ± 1.71
bFGFp-liposomes (DSPC/Chol/bFGFp-PEG-DSPE =60:37.5:2.5)	100 ± 1.76
1% mPEG ₃₀₀₀ /bFGFp-liposomes (DSPC/Chol/bFGFp-PEG-DSPE/mPEG ₃₀₀₀ -DSPE=60:36.5:2.5:1)	115 ± 1.99
2.5% mPEG ₃₀₀₀ /bFGFp-liposomes (DSPC/Chol/bFGFp-PEG-DSPE/mPEG ₃₀₀₀ -DSPE=60:35:2.5:2.5)	113 ± 7.06
5% mPEG ₃₀₀₀ /bFGFp-liposomes (DSPC/Chol/bFGFp-PEG-DSPE/mPEG ₃₀₀₀ -DSPE=60:32.5:2.5:5)	114 ± 0.651
10 mol% mPEG ₃₀₀₀ /bFGFp-liposomes (DSPC/Chol/bFGFp-PEG-DSPE/mPEG-DSPE ₃₀₀₀ =60:27.5:2.5:10)	101 ± 1.36
1% mPEG ₅₀₀₀ /bFGFp-liposomes (DSPC/Chol/bFGFp-PEG-DSPE/mPEG ₅₀₀₀ -DSPE=60:36.5:2.5:1)	116 ± 0.961
2.5 % mPEG ₅₀₀₀ /bFGFp-liposomes (DSPC/Chol/bFGFp-PEG-DSPE/mPEG ₅₀₀₀ -DSPE=60:35:2.5:2.5)	113 ± 1.21
5% mPEG ₅₀₀₀ /bFGFp-liposomes (DSPC/Chol/bFGFp-PEG-DSPE/mPEG ₅₀₀₀ -DSPE=60:32.5:2.5:5)	106 ± 1.26
10% mPEG ₅₀₀₀ /bFGFp-liposomes (DSPC/Chol/bFGFp-PEG-DSPE/mPEG ₅₀₀₀ -DSPE=60:27.5:2.5:10)	101 ± 5.02

^a The mean particle sizes of the liposomes were measured using a laser light scattering particle size analyzer. Results are expressed as the mean ± S.D. of three experiments.

Fig. 1

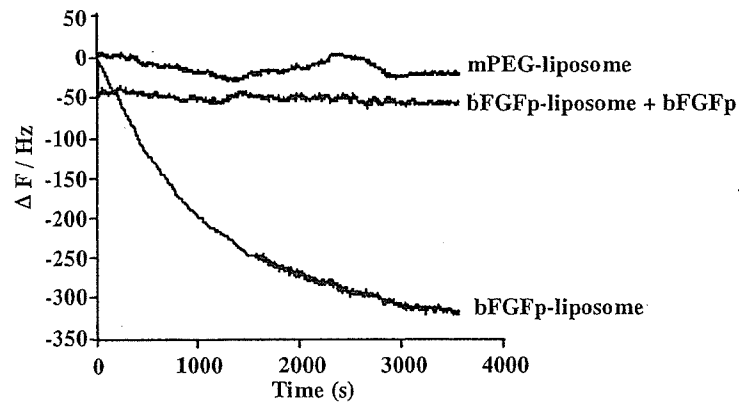


Fig. 2

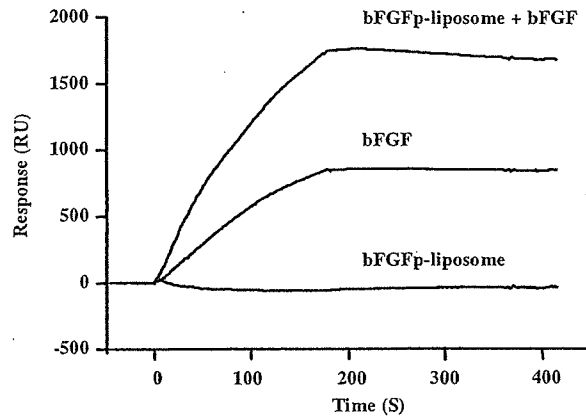


Fig. 3

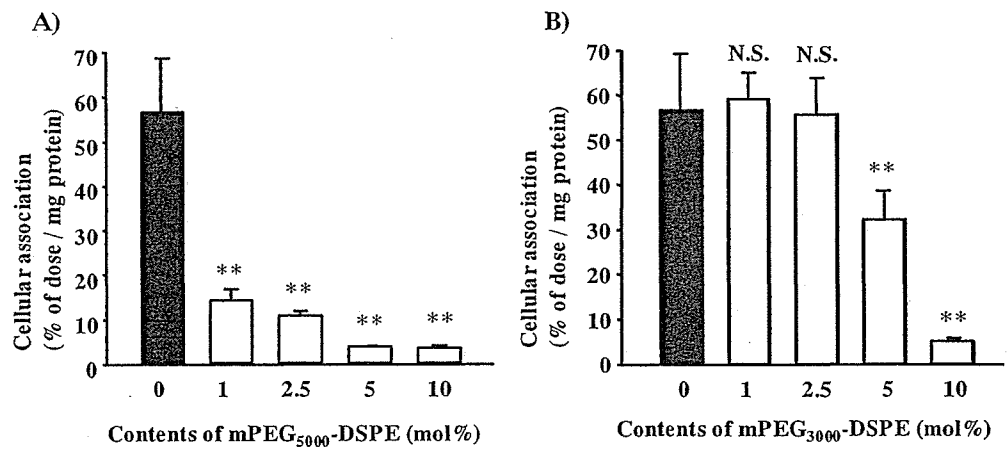


Fig. 4

