Journal of Controlled Release
Regular Article

Ligand-based targeted delivery of a peptide modified nanocarrier to endothelial cells in adipose tissue

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Abstract

Ligand-based targeted delivery is an emerging platform in nanomedicine. We report herein on a peptide modified nanocarrier for ligand-based targeted delivery system. The liposomal surface of the carrier was first modified with a linear peptide, followed by an adipose tissue-specific circular peptide (KGGRAKD) via a polyethylene glycol (PEG) spacer. To evaluate the specificity of the carrier, we purified primary cells from the endothelium of adipose tissue. The liposomes bound only to isolated primary cultured endothelial cells derived from inguinal adipose tissue (pcEC-IWAT) and not to other endothelial cell lines, such as MBEC-4 and MFLM-4. Cellular uptake was confirmed both qualitatively and quantitatively by confocal laser scanning microscopy (CLSM) and flow cytometry. The mechanism for the intracellular uptake of tPep-PEG-LPs into pcEC-IWAT, as evidenced by three independent experiments, involves saturation of receptor binding sites by excess free peptide, the blocking of receptors by an anti-prohibitin antibody and low temperature (4°C) experiments, resulting in the inhibition of uptake of tPep-PEG-LPs into pcEC-IWAT, suggesting that receptor mediated endocytosis largely contributed to the observed cellular uptake. A co-localization study using double labeled modified liposomes (lipid membrane: NBD-DOPE and aqueous phase: Rhodamine) indicated that a predominant part of tPep-PEG-LPs was found without co-localization with lysosomes and retained their intactness. The selective delivery of tPep-PEG-LPs to endothelial cells in adipose tissue represents a potential approach for the design of diverse nanocarrier-based targeted delivery systems for targeting the vasculature in adipose tissue.

Key Words:
Adipose tissue, ligand targeting, peptide modified nanocarrier, endocytosis, intracellular uptake.

Abbreviations:
LPs: liposomes;
PEG: maleimide-PEG2000-DSPE;
R8: stearylated octaarginine;
NBD-DOPE:4-nitrobenzo-2-oxa-1,3-diazolyl-1,2-Dioleoyl-sn-glycero-3-phosphoetha-nolamine;
PEG-LPs: maleimide-PEG2000-DSPE modified liposome;
R8-PEG-LPs: R8 and maleimide-PEG2000-DSPE modified liposome;
tPep-PEG-LPs: targeted lipopeptide modified liposome;
pcEC-IWAT: primary cultured endothelial cells derived from inguinal adipose tissue;
MBEC-4: endothelial cells derived from mouse brain;
MFLM-4: endothelial cells derived from mouse fetal lung mesenchyme.
INTRODUCTION

The efficacy of the pharmacological management of anti-obesity drugs for treating obesity is limited (up to 5%) [1]. However, at this time, there are no FDA approved drugs or combinations of drugs for treating obesity that are both safe and highly effective i.e. consistently resulting in a loss of 10% of the total body weight every year [2]. In previous reports, it has been described that the use of non-specific angiogenic inhibitors may prevent obesity [3, 4] but undesirable side effects and lower efficacy necessitate the continuous development of new, more effective therapeutics. One possible approach for solving this problem is the use of ligand-based targeted delivery, involving the design of peptide modified nanocarriers and the insertion of therapeutic agents (drugs, genes encoding apoptosis promoting proteins, negative regulators of angiogenesis enhancers, suicide genes, and fluorescent/luminescent genes for molecular imaging etc) into endothelial cells and/or adjacent cells in adipose tissue during obesity and in obesity-related diseases (i.e. type 2 diabetes, hypertension, cardiovascular diseases, stroke and certain types of cancer such as colorectal, breast and prostate cancer [5-8]) which constitute the greatest threat to global human health [9].

Non-viral vectors within other vectors for ligand-based targeted delivery appear to be superior in terms of safety, lower immunogenicity and ease of use [10]. To increase the specificity of non-viral vectors, they are sometimes modified with ligands (i.e. specific to receptors), referred to as ligand modified nanocarriers that recognize and bind to target cells through ligand-receptor interactions, and the bound carriers are then internalized before the drug is released inside the cell [11]. In addition, when ligands are attached to polyethylene glycol (PEG), they have the ability to escape from reticuloendothelial system (RES) and to circulate in the blood for an extended period of time [12, 13]. This may provide space to exclude steric hindrance for recognition to the targeted receptors [14, 15]. Furthermore, several recent studies have reported that the use of peptide ligands (i.e. the RGD motif for integrins, NGR for the endothelial-associated form of amino peptidase N, and YIGSR for laminin [16, 17, and 18]) as well as antibodies (the anti-ErbB2 antibody for ErbB2 receptors [19]) could permit encapsulated drugs to be delivered to a specific site.

For the successful ligand based-targeted delivery to adipose tissue, it is mandatory to select and verify the expression of specific receptors on targeted cells as well as the recognition of peptide modified liposomes to their targeted sites. M.G. Kolonin et al. reported on prohibitin as a vascular receptor for the circular peptide ligand (CKGGRAKDC) in white adipose tissue of wild type and lypobob mice [20]. In this regard, adipose tissue is a unique tissue whose growth is angiogenesis-dependant [3,4].Unlike a specific tissue or organ in which tumors are usually found in cancer patients, WAT is the the most highly distributed tissue in severely obese patients and can be found in all parts of the body. As a result, effective angiogenesis-targeted therapy would probably require targeting angiogenic vessels in the fat mass [9]. Furthermore, adipocytes are, in general, fat storing cells in the body and are contribute to obesity and its related diseases [21]. Endothelial cells in blood vessels are the first barrier to the passive targeting of adipocytes,. Therefore, development of a targeted drug delivery system might be a potential tool for successfully delivering therapeutics, either actively or passively, in the treatment of obesity and related diseases. In this study, we used primary cultured endothelial cells derived from inguinal
adipose tissue (pcEC-IWAT) as a model for targeted delivery. The reason for this choice was that a phage displaying CKGGRAKDC accumulated in subcutaneous/inguinal fat at a level of 150-fold higher than the background observed for a negative control phage without an insert [20].

Consistent with the goal of establishing targeted drug delivery, we report herein on a ‘peptide modified nanocarrier’ that successfully and specifically delivers the aqueous phase into the cytoplasm of pcEC-IWAT, compared to other endothelial cell lines, such as MBEC-4 and MFLM-4. Information on the quantitative expression of prohibitin on the plasma membranes of endothelial cells isolated from inguinal white adipose tissue is reported and a mechanism for the intracellular uptake of tPep-PEG-LPs is proposed.
Materials and Methods

Materials

Distearoyl-sn-Glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000 (PEG_{2000}-DSPE), 4-nitrobenzo-2-oxa-1,3-diazolyl-1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (NBD-DOPE), cholesterol (chol), Sulfo-Rhodamine (Rhodamine) and Rhodamine labeled DOPE (Rho-DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Egg yolk phosphatidylcholine (EPC) and DSPE-PEG_{2000} with a functional maleimide moiety at the terminal end of PEG: N-[(3-maleimide-1-oxopropyl) aminopropyl polyethylene glycol-carbamyl] distearoyl-phosphatidy-ethanolamine (Maleimide-PEG_{2000}-DSPE) were purchased from Nippon Oil and Fat Co. (Tokyo, Japan). Lysotracker Blue (DND-22) was purchased from Invitrogen (Carlsbad, CA, USA). Stearylated octaarginine (R8), targeting peptide (tPep: GKGRAKDGKC-NH_{2}, Purity: 93.6%, Theoretical MW: 1004.15) and two kinds of scrambled peptides (sPep1: GARKGDGKGC-NH_{2}, Purity: 80%, Theoretical MW: 1004.1 and sPep2: GDRKAGKAGGC-NH_{2}, Purity: 80%, Theoretical MW: 1018.2) were synthesized by Kurabo Industries, Osaka, Japan.

Cell culture

NIH-3T3 cells were obtained from the American Type Culture Collection (Manassas, VA) and the cells were maintained with Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA) under an atmosphere of 5% CO2/ air at 37°C.

MBEC-4 cells derived from mouse brain endothelial cells were generously supplied by Dr. T. Tsuruo and Dr. M. Naito (Tokyo University, Japan). The cells were maintained in DMEM supplemented with 10% FBS and 0.5 µg/ml heparin sulfate under an atmosphere of 5% CO2/ air at 37°C.

MFLM-4 cells derived from mouse fetal lung mesenchyme were purchased from Seven Hills Bioreagents (Cincinnati Children's Research Foundation Burnet Ave Cincinnati, Ohio ) and the cells were maintained with DMEM, 10% FBS and 2 mM glutamine; 5% CO2. To maintain EC phenotype, MFLM-4 cells are replated before reaching confluence.

pCEC-IWAT cells were isolated from blood vessel of inguinal adipose tissue from eight to nine week old male C57BL/6J mice and cultured in EGM-2MV media (Lonza, Walkersville, MD, USA) supplemented with 10% FBS and 0.1 mg / ml Kanamicin sulfate (Wako Purechemicals) as described previously [22]. The cells were seeded onto culture dishes coated with 1.5% gelatin (SIGMA) and 10 µg / ml of human fibronectin (Asahi Glass, Tokyo, Japan) under an atmosphere of 5% CO2/ air at 37°C.

Antibody

The rabbit monoclonal anti-prohibitin antibody and rabbit IgG were purchased from Abcam (Abcam inc., Cambridge, USA) and mouse FcR blocking reagent was obtained from Miltenyl Biotec (Auburn, CA, USA).

Synthesis of GKGRAKDGGC-NH_{2} -PEG_{2000}-DSPE
GKGRAKDGGC-NH₂ and maleimide-PEG₂₀₀₀-DSPe were separately dissolved in distilled water, using bath sonicator at room temperature for 1 min. A maleimide-PEG₂₀₀₀-DSPe solution (5 mM) was then added to the GKGRAKDGGC-NH₂ (Targeted peptide), GARKGDGKGGC-NH₂ and GDRKAGKAGGC-NH₂ (Non-targeted scrambled peptides) solution (5 mM) at a molar ratio of 1:1 with gentle stirring. The reaction was allowed to proceed for 24 h at 30°C with continuous shaking on a Bio-shaker. The synthesized targeting lipopeptide (GKGRAKDGGC-NH₂-PEG₂₀₀₀-DSPe) and two kinds of scrambled lipopeptides (sPep1: GARKGDGKGGC-NH₂-PEG₂₀₀₀-DSPe and sPep2: GDRKAGKAGGC-NH₂-PEG₂₀₀₀-DSPe) were analyzed by MALDI-TOF mass spectrometry and the data compared with mass of the reactants.

**Nanocarrier preparation and characterization**

The PEG-, R8-PEG-, tPep-PEG-, sPep1-PEG- or sPep2-PEG-LPs were prepared by the REV (Reverse phase evaporation) method [23]. For the preparation of these liposomes, lipid containing EPC and chol at a ratio of 2:1 (total lipid content: 5 μmol) separately dissolved in chloroform, 500 μl of 10 mM HEPES buffer (pH 7.4) and 500 μl of lipid solution were mixed and the solution sonicated with a probe-type sonicator for 15 sec at 4°C. For the preparation of PEG-, tPep-PEG-, sPep1-PEG- or sPep2-PEG-LPs, maleimide-PEG₂₀₀₀-DSPe (5 mole% of total lipids), targeting lipopeptide or scrambled lipopeptides (5 mole% of total lipids) were added to the lipid solution. For the preparation of R8-PEG-LPs, we separately added maleimide-PEG₂₀₀₀-DSPe (5 mole% of total lipids) and R8 (5 mole% of total lipids) to the lipid mixture. The fluorescent labeling of lipid membranes, Rho-DOPE or NBD-DOPE (1 mole% of total lipids) was also added. The mixture was evaporated under a stream of N₂ gas followed sonication for 1 min with a bath-type sonicator.

We also prepared double labeled liposomes using NBD-DOPE (1 mole% of total lipids) and 0.5 mM Rhodamine in 10 mM HEPES as described above and, for the removal of free rhodamine, ultracentrifugation was carried out two times for 30 min at 82000 x g in each wash. The sizes and zeta potentials of the PEG-, R8-PEG-, tPep-PEG-, sPep1-PEG- or sPep2-PEG-LPs were measured by photon correlation spectroscopy on a Malvern Zetasizer (Malvern instruments, Malvern, UK). The resulting PEG-, R8-PEG-, tPep-PEG-, sPep1-PEG- or sPep2-PEG-LPs were stored at 4°C and used within 3 h of preparation.

**Quantification of cellular uptake of tPep-PEG-LPs by flow cytometry**

The cellular uptake of the liposomes was assessed by flow cytometry. pcEC-IWAT cells were seeded at a density of 2 x 10⁵ cells per well in 6 well plates coated with gelatin (1.5%) and fibronectin (10 μg / ml). After 24 h, the cells were washed with EBM-2 media (serum containing media) and were incubated with PEG-LPs and tPep-PEG-LPs (final concentration: 62.5 nmol lipids/ml) in EBM-2 media for 1 h. The cells were washed once with FACS buffer (PBS supplemented with 0.5% bovine serum albumin and 0.1% NaN₃) and then trypsinized and collected in a microtube, followed by washing two additional times by repeated precipitation of the cells by centrifugation (200x g, 4°C, 10 min) and resuspending in 1 ml of PBS including heparin (20 units/ml). Finally, the cells were suspended in 500 μl of FACS buffer. The cell suspension was then filtered through a nylon mesh to remove cell aggregates and dust, and the
cells were then analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

**Intracellular distribution of PEG-, R8-PEG-, tPep-PEG-, sPep1-PEG- or sPep2-PEG-LPs by confocal laser scanning microscopy**

NIH-3T3, MFLM-4, MBEC-4 and pcEC-IWAT cells were seeded on sterile 35 mm glass-base dishes in the presence of their respective media described above. The cell density was 2 x 10^5 cells/well. The cells were incubated for 24 h to 50% confluence and then incubated with RhoDOPE labeled liposomes (PEG-, R8-PEG-, tPep-PEG-, sPep1-PEG-, or sPep2-PEG-LPs) (final concentration: 10 nmol lipids/ml) in serum free medium for 3 h at 37°C under an atmosphere with 5% CO_2_. Before the final washing, the cell nuclei were stained with Hoechst 33342 (final conc. 2.5 µg/ml) for 15 min and then washing with medium, followed by heparin (20 U/ml) in 5 mM HEPES was performed. After the washing procedures, 5 mM HEPES buffer supplemented with KCl 5.4 mM, MgCl2.6H2O 1 mM, CaCl2.2H2O 1.8 mM, NaCl 138 mM, pH 7.3 was added and the cells were observed by confocal laser scanning microscopy by means of an oil-immersion objective lens (plan-apochromat x 63/NA (Carl Zeiss Co. Ltd., Jene, Germany).

**Flow cytometric analysis of prohibitin expression on the cell surface of pcEC-IWAT**

To confirm the expression of prohibitin on isolated endothelial cells from inguinal adipose tissue, we collected pcEC-IWAT cells at 6 passages by brief tripinsinization. For the determination of prohibitin, the collected pcEC-IWAT cells were incubated with mouse FcR blocking reagent for 10 min at 4°C and washed with fresh FACS buffer followed by incubation with the primary antibody against prohibitin at a 1:20 dilution for 30 min at 4°C. After washing, the cells were incubated with a secondary antibody, Alexa488-labeled goat anti-rabbit IgG at the same dilution (Invitrogen, Grand Island, NY, USA) for a further 15 min at 4°C and, after washing, the cells were analyzed using a FACS Calibur flow cytometer.

**Influence of free peptides, anti-prohibitin and temperature on cellular uptake of tPep-PEG-LPs**

pcEC-IWAT cells were prepared as described above. The cells were incubated with free targeting peptide solution (25.0 µM) or anti-prohibitin antibody (5 µg/ml) respectively for 60 min at 4°C followed by washing with EBM2 medium and were then incubated with NBD-DOPE labeled tPep-PEG-LPs (final concentration: 10 nmol lipids/ml) in serum free medium for 3 h at 37°C. As negative controls for these competitive assays, two kinds of scrambled peptides (sPep1 and sPep2) and rabbit control IgG were also utilized. To confirm endocytosis, the cells were incubated with NBD-DOPE labeled tPep-PEG-LPs (10 nmol lipids/ml) in serum free medium for 3 h at 4°C. Finally, the washed cells were examined by confocal laser scanning microscopy, as described above.

**Intracellular trafficking and delivery of aqueous phase marker**

pcEC-IWAT cells, prepared as described above, were incubated with double labeled R8-PEG-LPs and tPep-PEG-LPs (final concentration: 20 nmol lipids/ml) in serum free medium for 3 h at
37°C under an atmosphere with 5% CO₂. The cells were stained by LysoTracker Blue (DND-22) (3 µg/ml) for 30 min at 37°C. After washing, the cells were examined by confocal laser scanning microscopy, as described above.
Results
Synthesis of GKGRAKDGCC-NH$_2$-PEG$_{2000}$-DSPE, GARKGDGKGGC-NH$_2$-PEG$_{2000}$-DSPE and GDRKAGKAGGC-NH$_2$-PEG$_{2000}$-DSPE

Maleimide-activated reagents are effective for modifying protein sulphydryl groups. Maleimide groups react efficiently and specifically with free (reduced) sulphydryl residues at pH 6.5-7.5 to form stable thioether bonds [24]. Here we successfully covalently conjugated the sulphydryl group (-SH) of cysteine within targeting or scrambled peptides (tPep: GKGRAKDGGC-NH$_2$, Theoretical MW: 1004.15, sPep1: GARKGDGKGGC-NH$_2$, Theoretical MW: 1004.1 and sPep2: GDRKAGKAGGC-NH$_2$, Theoretical MW: 1018.2) (Fig 1 and Supplementary Fig 1) and maleimide-PEG$_{2000}$-DSPE (Theoretical MW: 2936.25 and 3104.29) under the condition of the reaction (24 h with continuous shaking by Bioshaker at 30°C, pH 7.2, and a 1:1 molar ratio) and compared the molecular mass of targeting and scrambled lipopeptides (tPep-, sPep1- and sPep2-PEG-DSPE) with the original tpeptide and peptides by MALDI-TOF. As a result, the targeting or scrambled lipopeptides (tPep: GKGRAKDGGC-NH$_2$-PEG$_{2000}$-DSPE, Theoretical MW: 3940.40; sPep1: GARKGDGKGGC-NH$_2$-PEG$_{2000}$-DSPE, Theoretical MW: 4108.1; sPep2: GDRKAGKAGGC-NH$_2$-PEG$_{2000}$-DSPE, Theoretical MW: 4132.29) (Fig 1 and Supplementary Fig 1) simulated the exact molecular mass of reactant’s total mass.

Physicochemical characterization of PEG-, R8-PEG-, tPep-PEG-, sPep1-PEG or sPep2-PEG-LPs

The average particle sizes and polydispersity index (PDI) of PEG-, R8-PEG-, tPep-PEG-LPs were similar (around 100 nm, PDI<0.3) (Table 1). It has been reported that a liposome with maleimide-PEG$_{2000}$-DSPE and Pep-PEG$_{2000}$-DSPE, with a diameter of around 100 nm, is likely to be the optimal size, not only for the extravasation of liposomes but also for their extended retention by the blood for delivering the drug to its targeted cells [25]. As shown in Table 1, the liposomes (PEG-LPs, R8-PEG-LPs, tPep-PEG-LPs) had negative (-22.4±3.4 mV), positive (31.3±6.7 mV) and almost neutral (1.5±5.5 mV) zeta potentials, respectively. In addition, sPep1- and sPep2-PEG-LPs exhibited similar characters with tPep-PEG-LPs (Supplementary Table 1). Due to the slight positive charge of the peptide, the targeting or scrambled peptides modified liposomes neutralized the negativity of the maleimide-PEG$_{2000}$-DSPE.

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Size (nm)</th>
<th>$\zeta$-potential (mV)</th>
</tr>
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<tbody>
<tr>
<td>PEG-LPs</td>
<td>94.4±15.7</td>
<td>-22.4±3.4</td>
</tr>
<tr>
<td>R8-PEG-LPs</td>
<td>99.5±19.3</td>
<td>31.3±6.7</td>
</tr>
<tr>
<td>tPep-PEG-LPs</td>
<td>105.6±13.9</td>
<td>1.5±5.5</td>
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Cell specificity for internalization of modified liposomes by Confocal Laser Scanning Microscopy (CLSM)

To examine the cell type specificity for tPep-PEG-LPs, pcEC-IWAT and NIH-3T3 cells were treated with tPep-PEG-LPs and PEG-LPs. As shown in Fig. 2, although the PEG-LPs were not internalized by either of the both cell types, the tPep-PEG-LPs were highly incorporated by pcEC-IWAT but not NIH-3T3 cells. These results suggest that the tPep-PEG-LPs specifically associated with and were subsequently internalized by pcEC-IWAT cells, but that this was not the case of NIH-3T3 cells.

Quantification of cellular uptake of modified liposomes in pcEC-IWAT by flow cytometry

To quantify the cellular up-take of tPep-PEG-LPs by pcEC-IWAT cells, we measured fluorescent intensity of the internalized PEG-LPs (control) and tPep-PEG-LPs by FACS. pcEC-IWAT cells treated with PEG-LPs showed a low level of background fluorescence. On the other hand, the intensity of the cells treated with tPep-PEG-LPs were around six fold (according to geometric means) higher than that of PEG-LPs (Fig.3). Therefore, these results indicate that tPep-PEG-LPs are highly internalized, as the result of the ligand (KGGRAKD motif) grafted to the liposomes via a PEG spacer.

Tissue specificity for the uptake of modified liposomes by confocal laser scanning microscopy (CLSM)

In order to further verify the association and uptake of tPep-PEG-LPs, the internalization of rhoDOPE labeled PEG-LPs, R8-PEG-LPs and tPep-PEG-LPs was studied using MBEC-4 and MFLM-4 cell lines derived from brain and lung tissue and the results compared with respect to corresponding pcEC-IWAT cells. R8-PEG-LPs was internalized by all cells, whereas no PEG-LPs was detectable (Fig 4). As shown in Fig 4, high levels of tPep-PEG-LPs were internalized by pcEC-IWAT cells but not the other cells. These results serve to validate the conclusion that tPep-PEG-LPs clearly
bound to the specified target determinant on endothelial cell surfaces derived from inguinal adipose tissue and other endothelial cells derived from lungs and brain did not express moieties that had affinities for tPep-PEG-LPs.

**Prohibitin expression of pcEC-IWAT cells**

To determine the selective expression of surface prohibitin, we investigated the expression of prohibitin on pcEC-IWAT and MFLM-4 cells using flow cytometry. We detected a high level of expression of surface prohibitin on pcEC-IWAT cells where as MFLM-4 cells showed no expression of prohibitin on cell surfaces (Fig 5). These results suggest that prohibitin is a reliable vascular receptor in inguinal adipose tissue.

**Prohibitin mediated endocytosis**

To disclose the inherited mechanism by which tPep-PEG-LPs are internalized by pcEC-IWAT cells, we examined whether this uptake was inhibited at low temperatures (4°C) and pre-treatment of the free targeting peptide and an anti-prohibitin antibody. At 37°C, the uptake of tPep-PEG-LPs by pcEC-IWAT was considerably higher, while that was entirely inhibited at 4°C (Fig 6). The saturation and blocking of prohibitin on the cell surface also blocked this uptake, whereas pretreatment of scrambled peptides or control IgG did not affect the uptake of tPep-PEG-LPs into pcEC-IWAT cells (Figs 7 and 8). In addition, sPep1- and sPep2-PEG-LPs could not be incorporated into pcEC-IWAT (Supplementary Fig 2). Collectively these results strongly suggest that the uptake of tPep-PEG-LPs is mediated by affinity moieties on prohibitin and that prohibitin mediates endocytosis.

**Intracellular trafficking and delivery of aqueous phase marker**

To investigate whether the intracellularly delivered tPep-PEG-LPs remain intact and escape from endosomes, double labeled tPep-PEG-LPs and R8-PEG-LPs were used (Fig 9). The incubation of pcEC-IWAT cells with double labeled tPep-PEG-LPs resulted in the intracellular
appearance of co-localized NBD-DOPE and rhodamine fluorescence, i.e. both the liposome bilayer membrane and the internal cargo markers are delivered to the cell cytoplasm. Interestingly, a predominant colocalization with lysosomal compartment was found in the case of R8-PEG-LPs at 3h after the incubation, whereas major part of tPep-PEG-LPs was observed without colocalization with lysosomes (Fig 9 and Supplementary Data 1 and 2).

Fig 9 Intracellular trafficking and delivery of aqueous phase of double labeled tPep-PEG-LPs within pcEC-IWAT cells by confocal laser scanning microscopy (CLSM).
Discussion

We recently described a method for isolating highly pure microvascular endothelial cells from murine adipose tissue, including inguinal fat [22]. The challenge for delivering nanocarriers modified with peptide ligands to target endothelial cell receptors in adipose tissue is the ability of the cells to recognize and selectively internalize these ligand modified nanocarriers, while sparing off-target cells. It is widely accepted that targeting ligands that direct nanocarriers can be used to deliver therapeutics within targeted cells for a given receptor [26–28]. For the development of ligand-based targeted delivery system for a specific receptor, prohibitin, which was reported as a vascular receptor in white adipose tissue [20], we synthesized a targeting lipopeptide (tPep-PEG-DSPE) [Fig 1] containing a peptide motif (KGGRAKD) that has a specific affinity for prohibitin [20], by covalently attaching the peptide to maleimide-PEG$_{2000}$-DSPE through a stable thio-ether bond under physiological conditions [29]. Therefore, the ligand would not dissociate from the liposome in the systemic circulation [30] meeting another important requirement for a stable targeted delivery system.

The targeting peptide modified nanocarrier for a ligand-based targeted delivery system was prepared by attaching ligands at the PEG terminus on the liposome. This is more effective than directly attaching ligands to the surface of a PEG-containing liposome, since PEG chains interfere with both the coupling of ligands to the lipid bilayer and the interaction of these ligands with the intended biological targets. Therefore, we attached ligands to the distal end of PEG chains to overcome this drawback. These ligands coupling to the PEG terminus does not cause any interference with the binding of ligands to their respective recognition molecules [30]. It was previously reported that the binding sites of circular peptides (CKGGRAKDC) was located in the adipose tissue vasculature [20]. We initially grafted linear targeting peptide (tPep: GKKGGRADGGC) and two kinds of scrambled peptides (sPep1: GARKGDGKGGC and sPep2: GDRKAGKAGGC) to the liposome surface via a PEG spacer and characterized the product (Table1 and Supplementary Table 1), since it is likely to remain accessible for unperturbed interactions with the target molecule on the cell surface. Thus, the tPep-PEG-LPs contain an effective targeting binding site. We first examined cell type specificity of the uptake of tPep-PEG-LPs. As a result, tPep-PEG-LPs were highly associated and internalized by pcEC-IWAT cells but not NIH-3T3 cells as the peptide motif was designed so as to to recognize the vascular endothelial cells in adipose tissue (Fig 2) [20]. Both cells were negative to PEG-LPs (Fig 2) since the steric repulsions due to the PEG polymer layer on the PEG liposome weakens the interaction of liposomes with the target cells [31, 32]. Therefore, modification of the peptide on the distal end of the PEG polymer is crucial for the association and internalization of liposomes into pcEC-IWAT cells. We next quantified the successive ligand-receptor interactions by measuring intensity of the fluorescence uptake of tPep-PEG-LPs and compared these findings with that of PEG-LPs i.e. about a six fold higher uptake than that of PEG-LPs within pcEC-IWAT cells was found (Fig 3). A previous report indicated that CKGGRAKDC-diplaying phage bound to immobilized prohibitin at an eight fold higher level than that of a control phage with no insert [20]. This finding strongly suggests that the uptake of tPep-PEG-LPs into pcEC-IWAT cells was very efficient.
For specific association as well as internalization of tPep-PEG-LPs with their target cells we determined whether tPep-PEG-LPs were specifically bound to the target cells. Pep-PEG-LPs were associated and internalized to pcEC-IWAT at relatively high levels, whereas other no affinity was found for the other two endothelial cell lines, MFLM-4 and MBEC-4 (Fig 4). It was assumed that the high and selective expression of surface prohibitin on pcEC-IWAT cells rather than MFLM-4 cells (Fig 5) could result in the specific association and internalization of tPep-PEG-LPs. Although it has been reported that prohibitin is an inner mitochondrial protein that is present in all cells and nuclear expression in some types of cells [33, 34], it is expressed on the plasma membrane only by vascular endothelial cells in adipose tissue [22]. These findings prompted us to hypothesize that the binding of tPep-PEG-LPs to surfaced-expressed prohibitin is a factor in determining the extent of uptake. Since binding occurs only in the case of pcEC-IWAT, number of targeting peptide modified liposomes taken up by the cell is dependent on its target determinant (prohibitin) on the cell surface.

We next explored the mechanism that controls the uptake of tPep-PEG-LPs into pcEC-IWAT by three independent methods. The free peptide ligands and an anti-prohibitin antibody were associated to the level of simple specific interactions with their affinity moieties on cells resulting in an inhibition of uptake (Figs 7 and 8). Furthermore, as expected, sPep1- and sPep2-PEG-LPs were not incorporated into pcEC-IWAT (Supplementary Fig 2). We also confirmed endocytosis by blocking the uptake at a low temperature (4°C), at which cellular activity is nearly completely inhibited [35] whereas at 37°C uptake was found to be high (Fig 6). These findings provide evidence to show that tPep-PEG-LPs are associated with surface prohibitin to a higher degree and that this association is mainly through linear peptide ligand mediated specific interactions and operates via prohibitin mediated endocytosis.

Lastly we examined the intactness as well as the intracellular trafficking of tPep-PEG-LPs after internalization by pcEC-IWAT cells and compared the results to that for R8-PEG-LPs mediated by either clarithrin or macropinocytosis in a concentration dependant manner in endothelial cells [36, 37]. Major part of R8-PEG-LPs was co-localized with lysosomes, whereas a predominant part of tPep-PEG-LPs was found without co-localization with lysosomes and retained their intactness (i.e. co-localization of lipid membrane and aqueous phase) (Fig 9 and Supplementary Data 1 and 2). The mechanism for the escape from lysosomes remains to be clarified. Further studies about the intracellular trafficking of tPep-PEG-LPs would be necessary for proper elucidation.

In conclusion, several qualitative and quantitative evaluations were conducted, which confirm that prohibitin is selectively expressed at high levels by the inguinal adipose tissue vasculature. This is the first report to demonstrate that liposomes modified with a peptide containing a KGGRAKD motif can be specifically taken up into the EC from IWAT via prohibitin-mediated endocytosis. The co-localization of double labeled liposomes contributed to the successful delivery of the aqueous phase to the cytoplasm of pcEC-IWAT cells using a system that mimics the delivery of low molecular drugs, peptides, proteins and nucleic acids.
Acknowledgments

This study was supported by grants from the Special Education and Research Expenses of the Ministry of Education, Culture, Sports, Science and Technology of Japan. We also thank Dr. Milton Feather for editing this manuscript. We also thank him for his kind advice in preparing this manuscript.
References


[9]. Y. Cao, Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases, Nature Reviews 9(2) (2010) 107-115


Tables

Table 1. Physiochemical property of PEG-LPs, R8-PEG-LPs and tPep-PEG-LPs.

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Size (nm)</th>
<th>ζ-potential (mV)</th>
</tr>
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<tbody>
<tr>
<td>PEG-LPs</td>
<td>94.4±15.7</td>
<td>-22.4±3.4</td>
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<tr>
<td>R8-PEG-LPs</td>
<td>99.5±19.3</td>
<td>31.3±6.7</td>
</tr>
<tr>
<td>tPep-PEG-LPs</td>
<td>105.6±13.9</td>
<td>1.5±5.5</td>
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Data are represented as means ± SD for three separate experiments.
Figure legends

Fig 1 Mass spectral (MALDI-TOF) data for the synthesized lipopeptide (tPep-PEG-DSPE).

The targeting lipopeptide (Theoretical MW: 3940.40) was synthesized at 30°C for 24 h continuous reaction between original targeting peptide (GKGGRAKDGCC, Theoretical MW: 1004.15) and Maleimide-PEG2000-DSPE (Theoretical MW: 2936.25) in the molar ratio, 1:1. The mass of conjugated targeting lipopeptide and original peptide was analyzed by Mass spectrometer (MALDI-TOF).

Fig 2 Cell specificity for internalization of tPep-PEG-LPs to pcEC-IWAT cells.

pcEC-IWAT and NIH-3T3 cells were incubated with Rho-DOPE labeled PEG-LPs and tPep-PEG-LPs for 3 h at 37°C. Nuclei were stained with Hoechst-33342. Cells were observed by means of Confocal Laser Scanning Microscopy (CLSM). Scale bar indicated 10 µm.

Fig 3 Quantification of cellular uptake of tPep-PEG-LPs to pcEC-IWAT cells.

pcEC-IWAT cells were treated with NBD-DOPE labeled PEG-LPs and tPep-PEG-LPs for 1 h at 37°C. Histograms indicated the resultant fluorescence intensity of internalized PEG-LPs (red) and tPep-PEG-LPs (green) analyzed 10,000 cells by FACS.

Fig 4 Tissue specificity for uptake of tPep-PEG-LPs to MBEC-4, MFLM-4 cell lines and pcEC-IWAT cells.

MBEC-4, MFLM-4 and pcEC-IWAT cells were incubated with Rho-DOPE labeled PEG-LPs, R8-PEG-LPs and tPep-PEG-LPs for 3 h at 37°C. Cells were also treated with Hoechst 33342 (blue) for nuclei staining before final washing. Images were captured by CLSM. Scale bars indicated 20 µm.

Fig 5 Prohibitin expression on the surface of pcEC-IWAT cells.

pcEC-IWAT and MFLM-4 cells were treated with anti-prohibitin antibody for 30 min at 4°C and then incubated with FITC-conjugated goat anti-rabbit secondary antibody (IgG) for 15 min at same condition. After washing with several times the cells were analyzed by FACS. Histograms represented the fluorescence of non-treated MFLM-4 and pcEC-IWAT cells whereas others, the fluorescence of anti-prohibitin antibody treated both cells. The count cells are 10,000.

Fig 6 Effect of temperature on uptake of tPep-PEG-LPs.

pcEC-IWAT cells were incubated with NBD-DOPE labeled tPep-PEG-LPs and kept one for 3 h at 4°C and another for 3 h at 37°C. Fluorescence images were captured by means of CLSM. Scale bars denoted 10 µm.

Fig 7 Competitive inhibition of uptake of tPep-PEG-LPs by free targeting peptide.
pcEC-IWAT cells were incubated with free targeting or scrambled peptides for 1 h at 4°C and then treated with NBD-DOPE labeled tPep-PEG-LPs followed incubation for 3 h at 37°C. Fluorescence images were captured by means of CLSM. Scale bars denoted 10 µm.

**Fig 8 Inhibition of uptake of tPep-PEG-LPs by antibody bloking of targeting protein.**

pcEC-IWAT cells were incubated with free rabbit IgG and anti-prohibitin antibody for 1 h at 4°C and then treated with NBD-DOPE labeled tPep-PEG-LPs followed incubation for 3 h at 37°C. Fluorescence images were captured by means of CLSM. Scale bars denoted 10 µm.

**Fig 9 Intracellular trafficking and delivery of aqueous phase of double labeled tPep-PEG-LPs within pcEC-IWAT cells by CLSM.**

pcEC-IWAT cells were incubated with double labeled R8-PEG-LPs and tPep-PEG-LPs for 3 h at 37°C. The cells were stained by lysotracker blue (DVD-22) for 30 min and observed by CLSM. Yellow arrows indicated the intactness of tPep-PEG-LPs as the merge of lipid marker (green: NBD-DOPE) and aqueous maker (red: rhodamine) and remained separated from lysosomal compartments (blue). White arrows denoted the co-localization of R8-PEG-LPs with lysosomal compartments. Scale bars indicated 5 µm.
Fig 1 Mass spectral (MALDI-TOF) data for the synthesized tipopeptide, tPep-PEG-DSPE.
Fig 2 Cell specificity for internalization of tPep-PEG-LPs to pcEC-IWAT cells.
Fig 3 Quantification of cellular uptake of tPep-PEG-LPs to pcEC-IWAT cells
Fig 4  Tissue specificity for uptake of tPep-PEG-LPs to MBEC-4, MFLM-4 and pcEC-IWAT cells.
Fig5 Prohibitin expression on the surface of pcEC-IWAT cells
Fig 6 Prohibitin mediated endocytosis
Fig 7 Prohibitin mediated endocytosis

Free peptide pretreatment

<table>
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Merge

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Scale bars indicate 50 μm.
Fig 8 Prohibitin mediated endocytosis

Antibody pretreatment

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<th>Anti-prohibitin</th>
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<tr>
<td>Merge</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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</table>
Fig9 Intracellular trafficking and delivery of aqueous phase of double labeled tPep-PEG-LPs within pcEC-IWAT cells.

- **Lysosomes** (LysoTracker)
- **Lipid membrane** (NBD)
- **Aqueous phase** (Rhodamine)
- **Merge** (without DIC)

**tPep-PEG-LPs**

**R8-PEG-LPs**