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TARGETING DESIGN TO THE LUNG AND PULMONARY INTRACELLULAR STRUCTURE OF ENDOGENOUS GENE BY IRQ MODIFIED NANO CARRIER

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ABSTRACT

TARGETING DESIGN TO THE LUNG AND PULMONARY INTRACELLULAR STRUCTURE OF ENDOGENOUS GENE BY IRQ MODIFIED NANO CARRIER. Inhibition of angiogenesis is a novel strategy for the treatment of lung cancer. For efficient therapy, vectors must firstly reach the target tissue and subsequently demonstrate an efficient intracellular targeting. In this study, we attempted to design a vector for in vivo pulmonary targeting which was able to deliver small interfering Ribonucleic Acid (siRNA) for endogenous gene of angiogenesis in pulmonary endothelial cells. siRNA was condensed with polycation agent and encapsulated in lipidous nano carrier. To obtain high level of lung accumulation, we controlled the surface of nano-carrier by changing the length of Polyethylene glycol (PEG) moiety. These nano carriers showed prominent Ribonucleic acid interference (RNAi) effect, when luciferase gene was used as a target. In addition, an efficient transgene knockdown of Vascular Endothelial Growth Factor Receptor 1 (VEGFR1), a responsible gene of angiogenesis, can be obtained by the Instantaneous Respiratory Exchange Ratio (IRQ) modified nano carrier with the use of Stearyl-R8 (STR-R8) peptide, known as an endosomal membrane inducer. In conclusion, pulmonary targeting of nano carrier by encapsulating siRNA can be developed by controlling the PEG length and the structure of nano carrier for efficient intracellular targeting.

Key words : Pulmonary, PEG moiety, Intracellular trafficking, Endogenous gene, Endosomal membrane inducer

ABSTRAK

DESAIN NANO *IRQ* YANG DIMODIFIKASI SEBAGAI PEMBAWA *AGENT* ENDOGEN DENGAN TARGET KE PARU-PARU DAN INTRASELULERNYA. Penghambatan angiogenesis adalah strategi baru dalam pengobatan kanker paru-paru. Untuk memberikan terapi yang efisien, vektor harus mencapai target organ dan berinternalisasi secara efisien ke dalam organ tersebut. Pada penelitian ini, telah dikembangkan vektor yang target pada paru-paru secara in vivo dan sekaligus menghantarkan *small interfering Ribonucleic Acid* (*siRNA*) gen endogen pada sel endotel paru-paru. *siRNA* dikondensasi dengan senyawa polikation dan dienkapsulasi dalam vektor lipid berukuran nano. Untuk memperoleh tingginya akumulasi di paru-paru, permukaan dari vektor nano tersebut dikontrol dengan mengubah panjang rantai dari *Polyethylene glycol* (*PEG*). Vektor nano yang dikembangkan menunjukkan efek *Ribonucleic acid interference* (*RNAi*) yang menonjol, saat gen *lusiferase* digunakan sebagai target. Selain itu, penghambatan translasi *Vascular Endothelial Growth Factor Receptor 1* (*VEGFR1*), gen penentu pada angiogenesis, dapat dicapai dengan memodifikasi permukaan vektor *Instantaneous Respiratory Exchange Ratio* (*IRQ*) dengan peptide *Stearyl-R8* (*STR-R8*) yang dikenal sebagai penginduksi fusi membran endosom. Kesimpulannya, vektor nano yang mengenkapsulasi *siRNA* target ke paru-paru dapat dikembangkan dengan mengontrol panjang rantai *PEG* dan struktur vektor nano untuk target intraselular secara efisien.

Kata kunci : *Pulmonary*, *PEG moiety*, *Intracellular trafficking*, Gen Endogen, *Endosomal membrane inducer*

INTRODUCTION

Lung cancer remains a major health problem as the leading cause of cancer-related death worldwide [1]. Non Small Cell Lung Cancer (NSCLC) includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma and represents approximately 80 % of all lung cancers. Unfortunately, the majority of

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patients present with advanced NSCLC at the time of iagnosis and die from the disease. First line therapy with cisplatin-based chemotherapy showed modest benefit with an extension in survival of approximately 2 months [1,2]. The need for more effective treatments has led to the development of new therapeutic approaches targeting tumor biology.

Investigators in non viral vector development have introduced a variety of strategies to overcome barriers for macromolecules such as small interfering Ribonucleic Acid (siRNA) delivery. These includes cellular internalization, intracellular trafficking in terms of endosomal escape and decoating process to release siRNA to the cytoplasm. In addition, for systemic in vivo applications, an important factors should be considered, including physicochemical properties that affect stability in the blood (i.e. particle size and zeta potential of the vector) and the immune system (i.e. reticuloendothelial system capture of the vector) [3].

PEGylated nanoparticles with multiple components are the most promising non viral vector for systemic delivery. It was reported that nanoparticles containing Polyethylene glycol (PEG), Solid Nucleic Acid Lipid Particles (SNALPs), targeted to specific tissue and showed Ribonucleic acid interference (RNAi) mediated gene silencing in hepatitis B [4]. Thus, it is expected that the use of PEG moiety would promote to pulmonary targeting. Additionally, the multiple components used in the vector design would induce an efficient cytosolic delivery of siRNA particularly an escape from the endocytic vesicles and decoating process.

Angiogenesis, i.e. the formation of new blood vessels, plays a central role in tumor growth, invasion, metastasis, and thus represents an attractive therapeutic target [1]. To date, a number of molecules regulating angiogenesis have been described. Of these, Vascular Endothelial Growth Factor (VEGF) is the most important growth factor controlling angiogenesis in normal and tumor cells [1,5]. The Vascular Endothelial Growth Factor Receptor (VEGFR) family consists of six growth factors including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PIGF) which mediate their angiogenic effects via three receptors, VEGFR1-3. Binding of VEGF ligand to the receptor triggers downstream signaling pathways involved in enhancing vascular permeability, endothelial proliferation, invasion, migration and survival. VEGFR-1 and VEGFR-2 are important mediators of angiogenesis and VEGFR-3 is involved in lymphangiogenesis [1].

In the present study, nanoparticles designed for sophisticated lung targeting which addressed to knock down endothelial VEGFR1 gene expressed in the pulmonary endothelial cells were developed. In addition, to induce prominent gene silencing of the nanoparticles, the use of octaarginine (R8) as a fusogenic peptide enhancer was demonstrated.

EXPERIMENTAL METHOD

Preparation of IRQ Modified Liposomes

Three types of [^{3}H]CHE labeled liposomes, conventional liposomes (Conv-Lip), IRQ-modified liposomes (IRQ-Lip) 5% and IRQ-Lip 10% were prepared by the hydration method. Those liposomes were labeled with 0.03% of [^{3}H]CHE. Conv-Lip were composed of cholesteryl (Cho), Phosphatidylcoline (EPC) in a molar ratio of 3 : 7. To prepare the IRQ-Lip, two concentrations of Stearyl (STR)-IRQ were included (5 and 10 % of the total lipid in the [^{3}H]-Cho/EPC liposomes). These lipids were dissolved in 125 μL of chloroform and the solvent was then removed by nitrogen gas under reduced pressure in a glass tube to give a thin film. The lipid film was hydrated with Phosphate Buffer Saline (PBS) (pH 7.4), vortexed for 5 sec and sonicated within 1 minute by probe-type sonicator.

Synthesis of IRQ-PEG$_{900}$-DOPE and IRQ-PEG$_{2000}$-DSPE

Synthesis of IRQ-PEG$_{900}$-DOPE was performed following the procedure used for the synthesis of Cl31-PEG$_{450}$-DOPE [6]. While IRQ-PEG$_{2000}$-DSPE was synthesized as described previously [6]. To attach the IRQ to the surface of liposomes, the IRQ peptide was modified by adding cysteine residues, so that peptide can be readily conjugated to Mal-PEG-lipid to form the IRQ-PEG-lipid. In this study, two types of PEG were used, these were Mal-PEG$_{900}$-NHS and Mal-PEG$_{2000}$-DSPE. The IRQ-PEG$_{900}$-DOPE was synthesized by a two steps reaction of NHS groups of Mal-PEG$_{900}$-NHS and NH_3 primer of DOPE resulted in Mal-PEG$_{900}$-DOPE, followed by reaction between moiety of Mal-PEG$_{900}$-DOPE and cystein of IRQ peptide. The former reaction was carried out at room temperature for 2 hours in DMSO and the molar ratio of Mal-PEG$_{900}$-NHS, DOPE and triethylamine were 0.4, 0.4 and 0.8 μmol, respectively.

The latter synthesis was performed by coupling of the thiol group of IRQ peptide and the maleimide of Mal-PEG$_{900}$-DOPE. IRQ peptide and Mal-PEG-lipid were dissolved in H_2O at concentrations of 10 mM and mixed with equal ratios of 1 : 1. The reaction was carried out at room temperature overnight. The product was then identified by a Matrix-Assisted Laser Desorption/ Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry.

The IRQ-PEG$_{2000}$-DSPE (distearoylphosphatidyl ethanolamine) was synthesized by a single step reaction of maleimide moiety of Mal-PEG$_{2000}$-DSPE with thiol groups of the IRQ as described in the latter synthesis step of IRQ-PEG$_{900}$-DOPE above. The coupling between thiol group of peptide and the maleimide of Mal-PEG$_{2000}$-DSPE was performed as described in the previous study [7].

The product of IRQ-PEG_{2000}-DSPE was structurally confirmed by a MALDI-TOF mass spectrometry. Sinapic acid was used as a matrix for mass spectrometric analysis of the peptide product. Sample solution was 5 μL product in 100 μL aqueous solution of 30% acetonitrile containing 0.1% Trifluoroacetic acid (TFA). Two μL of aliquots were spotted onto a MALDI-TOF target plate and analyzed by a Voyager-DE PRO Mass Spectrometer (Applied Biosystems, Foster City, CA).

Preparation of PEGylated Liposomes

(PEG-Lip) [^{3}H]CHE labeled PEG-Lip was prepared by hydration method. The liposomes were labeled with [^{3}H]CHE of around 0.03 %. Liposomes were basically composed of EPC and Cho in a molar ratio of 7 : 3. To prepare PEG-Lip, 10 % of Mal-PEG_{2000}-DSPE was incorporated into the liposomes.

Briefly, total lipids (1 mM) were dissolved in ethanol solution in a glass tube. The maleimide-PEG-lipid was added to the ethanol solution, followed by addition of 125 μL chloroform. The organic solvent was evaporated by nitrogen gas. To obtain a transparent and homogenous thin lipid film, 125 μL chloroform was added and evaporated. Aqueous phase of PBS was added into a dried lipid film. The vesicles were vortexed and sonicated in one minute by probe-type sonicator. Liposome were stored at 4 ^{o}C for a maximum of one week.

Preparation of IRQ-Modified PEGylated Liposomes (IRQ-PEG-Lip)

Liposomes were basically composed of EPC and Cho (7 : 3 molar ratio) and reaction mixture between Mal-PEG_{2000}-DSPE and IRQ (1 : 1) was incorporated at 5 % of the total lipid. Liposomes were prepared by hydration method. Briefly, total lipid (1 mM) was dissolved in ethanol solution. The reaction mixture was added to the ethanol solution. To increase evaporation, 125 μL of chloroform was added. The organic solvent was evaporated by nitrogen gas in a glass tube. To the obtained film, aqueous PBS 0.6 mL was added and the glass tube was sonicated for 15 sec in a bath type sonicator. The liposomes were then purified by gel filtration on Sephadex G-100 column. Liposomes were stored at 4 ^{o}C for a maximum of one week.

Biodistribution of the Liposomes

In the preliminary study, five types of liposomes, Conv-Lip, IRQ-Lip 5%, IRQ-Lip 10%, PEG_{2000}-Lip, IRQ-PEG_{2000}-Lip 5%, were individually injected into a 8 weeks Balb/c mice via the tail vein. The liposomes were fixed at 10 nmol lipid/g BW. After 6 hours, the mice were sacrificed and their lung and liver were collected. To obtain an actual uptake of liposomes in tissues, the portion of liposomes in the vascular space was removed by heart perfusion. After weighing, the samples were solubilized in Soluene-350 for 5 hours at 50 ^{o}C. To confirm lung accumulation, two types of liposomes, IRQ-PEG_{900}-Lip and IRQ-PEG_{2000}-Lip, were independently injected to each mouse.

Handling of the samples were the same as described above. Radioactivities were then determined by liquid scintillation counter, with Hionic Fluor.

Preparation of IRQ-Modified Liposomes Encapsulating siRNA

For transgene expression study, siRNA was encapsulated in IRQ-Lip, IRQ-PEG_{900}-Lip, IRQ-PEG_{2000}-Lip. These nanoparticles were prepared using a hydration method. In this study, two types of siRNA were used, each for luciferase gene and endogenous VEGFR1 gene, respectively. siRNA for luciferase gene was used in the preliminary study to screen the potential formula and it was encapsulated in all types of liposomes used. An anti-luciferase siRNA was 21 mer and it sequences were 5'-GCGCUGCUGGUGCCAACCCTT-3', 5'-GGGUUGGCACCAGCAGCAGCGCTT-3'. In the latter study, siRNA for VEGFR1 was encapsulated in the IRQ-PEG_{900}-Lip.

Firstly, siRNA and STR-R8, as a polycation agents, were firstly dissolved in RNase-free water. To condense the siRNA, the siRNA solution was added to the polycation solution while vortexing at room temperature.

The concentrations of both solutions were fixed at 0.1 mg/mL. The siRNA/STR-R8 complexes were prepared at a nitrogen/phosphate (N/P) ratio of 2.9. A volume of 0.25 mL aliquot of condensed siRNA suspension was added to the lipid film, which was formed by evaporation of a chloroform solution of 137.5 nm lipids, on the bottom of a glass tube followed by a 10-min incubation at room temperature to hydrate the lipids. In this experiment, the lipid composition used was DOPE/PA/IRQ-PEG-lipid/Cho-GALA in a molar ratios of 7 : 2 : 1 : 0.1.

In the case of IRQ-PEG_{900}-Lip encapsulating siRNA for VEGFR1, STR-R8 of 5, 10 and 15 % of the total lipids was incorporated to make a final concentration of 0.55 mM. To coat condensed siRNA with lipids, the glass tube was sonicated approximately 1 min in a bath-type sonicator (125 W, Branson Ultrasonics, Danbury, CT). Liposomes were stored at 4 ^{o}C for a maximum of one week. The size distribution and zeta potential of each sample was determined using a Zetasizer Nano ZS ZEN3600 (MALVERN Instrument, Worchestershire, UK).

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Transgene Expression of IRQ-Modified Liposomes Encapsulating siRNA

For transgene expression study of IRQ-PEG-Lips coated anti-luciferase siRNA, 5 x 10^4 HeLa cells stably expressing luciferase (~10^8 RLU/mg protein) were seeded onto a 24-well plate in 0.6 mL of Dulbecco`s Modified Eagle`s Medium (DMEM) containing 10 % Fetal Calf Serum (FCS) and were incubated for 24 hours.

Before transfection, cells were washed with 0.5 mL PBS. IRQ-PEG-Lips corresponding to 0.4 μg siRNA were suspended in 0.25 mL OPTI-MEM containing serum and then were incubated with cells for 3 hours at 37 °C. Next, 1 mL medium supplemented with serum was added to the cells, followed by incubation for an additional 21 hours. After 24 hours, cells were washed with 0.5 mL PBS and then were solubilized with reporter lysis buffer (Promega, Madison, WI).

Luciferase activity was initiated by addition of 50 μL of luciferase assay reagent (Promega, Madison, WI) to 20 μL of cell lysate and measured by means of a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein in the cell lysate was measured usig a BCA protein assay kit (PIERCE, Rockford, IL). Results were expressed in relative light units (RLU)/mg protein.

For transgene expression study of IRQ-PEG-Lips coated anti-VEGFR1 siRNA, two days before infection, 2 x 10^5 Mouse fetal lung mesenchyme-4 (MFLM-4) cells were seeded onto a 6-well plate in 2 mL of DMEM containing 10 % FCS. Before transfection, cells were washed with 1 mL DMEM. IRQ-PEG-Lips coated siRNA corresponding to 6.4 μg siRNA were suspended in 1 mL DMEM containing 10 % FCS and were incubated with cells for 3 hours at 37 °C. Two mL medium supplemented serum was added to the cells, followed by incubation for an additional 21 hours.

Cells were solubilized with 1 mL reporter lysis buffer. mRNA was extracted from the samples and converted to complementary DNA (cDNA) by reverse transcriptase. The amount of cDNA was determined by Real Time Polymerase Chain Reaction (PCR). Results were expressed in percentage of RNAi effect.

Table 1. The size and zeta-potential of prepared liposomes for pharmacokinetics and transgene expression studies in HeLa cells

Types of liposomes	Size (nm)	Zeta-potential (mV)
Conv-Lip	81 ± 3.5	2 ± 2.6
IRQ-Lip 5%	85 ± 2.7	37 ± 3.6
IRQ-Lip 10%	83 ± 4.1	49 ± 4.4
IRQ-PEG_{900}-Lip	111 ± 2.8	30 ± 3.2
IRQ-PEG_{2000}-Lip	107 ± 3.1	7 ± 1.8
Luciferase siRNA-IRQ-PEG_{900}-Lip	117 ± 4.6	24 ± 4.1
Luciferase siRNA-IRQ-PEG_{2000}-Lip	110 ± 3.8	6 ± 2.9

RESULTS AND DISCUSSION

The present studies aimed to develop an intelligent delivery system which is able to selectively target the lung and subsequently its endothelial cells. In addition, the function of the developed nanoparticles was examined by evaluating the delivery of macromolecules siRNA to the cytosol of lung endothelial cells.

Characterization of Prepared siRNA-Coated IRQ-PEG-Lips

During preparation of the liposomes or coated particles for the use of pharmacokinetics and transgene expression studies in HeLa cells, the STR and the maleimide moeity acted as an anchor to the lipid bilayer leaving IRQ peptide freely attached to surface (IRQ-Lip) or freely attached to the terminal of PEGylated liposomal surface (IRQ-PEG-Lip). The size and ζ potential of prepared nanoparticles are summarized in Table 1. Particles for pharmacokinetics study had a size of around 80 nm. Incorporation of STR-IRQ to the Conv-Lip shifted its charges from neutral to positive one. In addition, the higher insertion density of the IRQ, the more positive their charges since the IRQ peptide is rich in arginine residues. When PEG moiety was inserted to the lipid bilayer, the size was slightly increased.

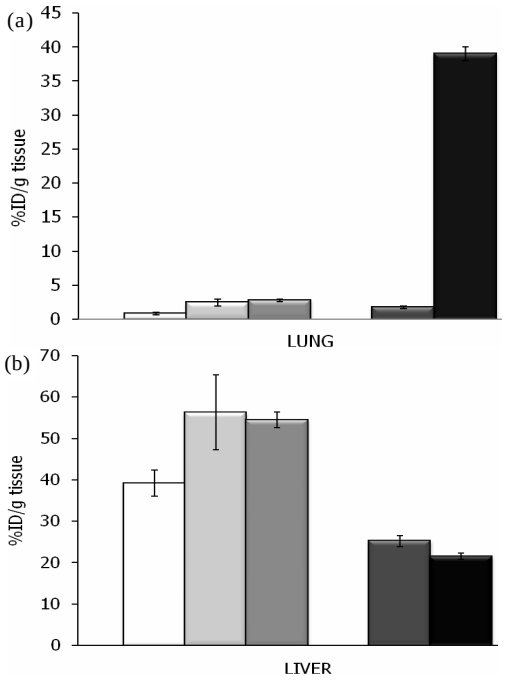

Figure 1. The tissue uptake of Conv-Lip, IRQ-Lip 5 %, IRQ-Lip 10 %, mPEG-Lip and IRQ-PEG2000-Lip 5 % in the lung and liver. The distribution was expressed as the percentage injection dose per tissue (%ID/tissue) in the lung (a) and liver (b). Open bars, light gray bars, gray bars, dark gray bars and closed bars indicate the tissue uptake at 6 hours after an i.v. injection of Conv-Lip, IRQ-Lip 5 % and IRQ-Lip 10 %

Furthermore, the use of long PEG moiety reduced its charges. These results are in line with the fact that the attachment of PEG moiety on the surface of liposomes neutralizes the effect of any charged component [7]. In general, IRQ-PEG_{900}-Lip and IRQ-PEG_{2000}-Lip had comparable sizes. Similar tendencies were observed for other coated particles between PEG_{900} and PEG_{2000} moeity, indicating that there were no significant differences in the physicochemical properties between PEG_{900}- and PEG_{2000}-modified nanoparticles.

Pharmacokinetics of IRQ-Lips and IRQ-PEG_{2000}-Lips

To determine *in vivo* distribution of IRQ-Lip, the accumulation of IRQ-Lip with the density of 5 % and 10 % in the lung were evaluated 6 hours after i.v administration (Figure 1A) and then compared to Conv-Lip as a control. The result showed that a small amount of Conv-Lip was recovered in the lung with the level of less than 1 % Injected Dose/g tissue (ID/g tissue). Incorporation of STR-R8 at 5 and 10 % to liposomal surface did not improve the uptake to the lung (Figure 1(a)). In addition, hepatic distribution was drastically increased as the result of modification with R8 (Figure 1(b)). IRQ-Lips were accumulated in the liver reaching approximately 80 %.

These results are in line with the previous study showing that the liver was the major clearance uptake of positively cationic liposomes [8]. It is possible that IRQ-Lip could be recognized by various opsonins or macrophages in the liver. When PEG_{2000} moiety was incorporated into the liposomes and the terminal of PEG moeity was conjugated with the IRQ, lung accumulation was drastically enhanced to approximately 39 % ID/g tissue at 6 hours post i.v. administration (Figure 1(a)). It is likely that the use of PEG_{2000} moiety reduced the recognition by opsonin so that liver accumulation was lower compare to that of IRQ-Lips. These results revealed that lung targeting of IRQ-modified nanoparticles could be improved by the incorporation of PEG moiety.

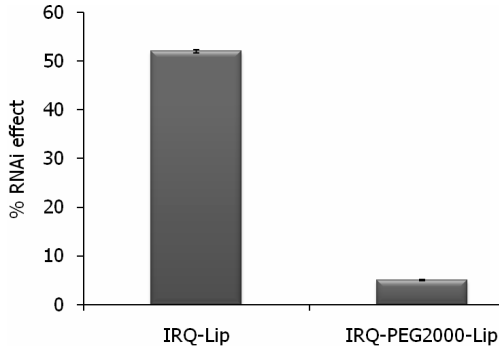

Figure 2. The effects of PEG moiety on the in vitro gene silencing of luciferase gene expression using siRNA. HeLa cells stably expressing luciferase were independently transfected with IRQ-Lip or IRQ-PEG2000-Lip containing luciferase or GFP. Luciferase activities were measured 24 hours after transfection and were expressed as percentage of silencing effect. The data represent as the mean of three data.

Silencing Activity of IRQ-Lips and IRQ-PEG_{2000}-Lip

For examining the efficiency of the delivery of macromolecules such as siRNA, we then compared the two liposomes, IRQ-Lip and IRQ-PEG_{2000}-Lip. When siRNA for reporter luciferase gene was encapsulated in IRQ-Lip, the transgene expression was knocked down by approximately 50% (Figure 2). This result is consistent with the previous study showing that gene silencing of IRQ-Lip was occurred. IRQ-Lip was internalized into cells via clathrin-mediated endocytosis and caveolar endocytosis. Additionally, the IRQ peptide was demonstrated to facilitate the escape from the endocytic vesicles [9].

Although, it was internalized via two cellular uptake pathways, it has been demonstrated that Clathrin-Mediated Endocytosis (CME) is a responsible pathway to induce RNA interference (RNAi) effect [10]. Therefore, after IRQ-Lip internalized through CME and entrapped in the endosomes, the IRQ peptide mediates endosomal escape. Thus, siRNA is released into the cytosol after decoating process occurred. However, when PEG moiety was inserted into the liposomes,

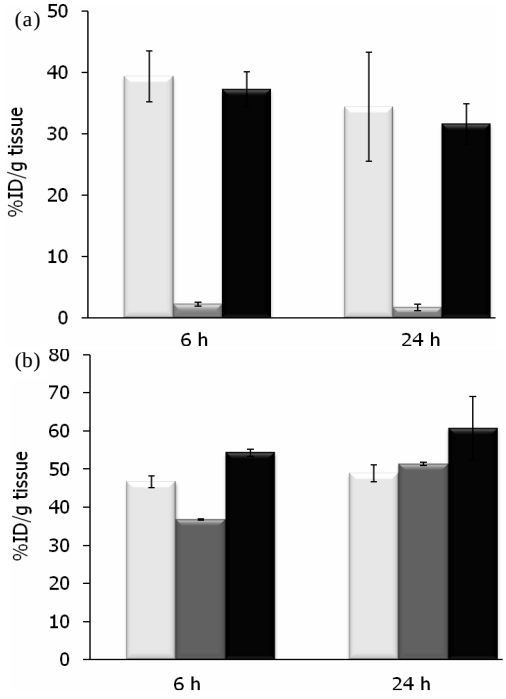

Figure 3. The tissue uptake of IRQ-PEG2000-Lip 5 %, unmodified PEG900-Lip and IRQ-PEG900-Lip in the lung and liver. The distribution was expressed as the percentage injection dose per tissue (%ID/tissue) in the lung (a) and liver (a). Light gray bars, gray bars and closed bars indicate the tissue uptake at 6 hours after an i.v. injection of IRQ-PEG2000-Lip 5 %, unmodified PEG900-Lip and IRQ-PEG900-Lip, respectively. The data represent mean SE (n = 3).

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transgene expression was severely inhibited (Figure 2). It was reported that the PEG moiety does not inhibit the endosomal escape, but it impaired the decoating process [6]. Decoating process is occurred subsequently during fusion step between liposomal membranes and endosomal membrane. It is presumable that length of PEG interrupts the interaction between the two membranes so that siRNA is still encapsulated in the lipid vesicles. Based on the results of pharmacokinetics and in vitro gene silencing studies, the use of PEG has advantages and disadvantageous that is termed as PEG dilemma.

Pharmacokinetics of IRQ-PEG$_{900}$-Lip and IRQ-PEG$_{2000}$-Lip

An alternative strategy to solve these hurdles is by modifying length of PEG moiety. The hypothesized that shorter PEG length would not only facilitate lung targeting of IRQ-Lip, but also compromise with the fusion process of the membranes to induce cytosolic release of siRNA. To confirm lung targeting by utilizing shorter PEG moiety, we first individually injected [^{3}H]-IRQ-PEG$_{900}$-Lip and [^{3}H]-IRQ-PEG$_{2000}$-Lip and specific the lung accumulation was observed at 6 hours and 24 hours post i.v. administration. The use of short PEG$_{900}$ moeity exhibited comparable results compared to that of PEG$_{2000}$ moeity. At the same indicated time, the IRQ-PEG$_{900}$-Lip was taken up by the lung of approximately 37 % ID/g tissue. Moreover, the lung accumulation of the IRQ-PEG$_{900}$-Lip and IRQ-PEG$_{2000}$-Lip were 34 % and 31 % ID/g tissue at 24 hours post i.v. administration (Figure 3(a)).

These results exhibited that the uptake of the IRQ-PEG-Lips in the lung remained after 24 hours and its accumulation was enhanced by 18.5 fold compared to that of unmodified PEG$_{900}$-Lip. This observation depicted their actual uptake in the lung because an impurity of the nanoparticles in the vascular space was removed by heart perfusion technique. These results indicated that lung accumulation of IRQ-modified liposomes can be achieved by shortening the PEG moiety approximately PEG$_{900}$. As to the accumulation of the nanoparticles in the liver, it is likely that modification of PEG moiety did not affect in liver uptake. The nanoparticles were taken up by the liver in a range of approximately 50-60 % ID/g tissue (Figure 3(b)). High liver accumulation can not be avoided since the positive charges of IRQ-nanoparticles were also responsible for the uptake.

Thus, it is hypothesized that flexibility of the IRQ to interact with its receptor was enhanced by modifying the PEG moiety. It is probable that an interaction between the IRQ with its receptor on the surface of lung endothelial cells is more prominent when the IRQ was attached to the tip of PEG moiety rather than attachment to liposomal surfaces.

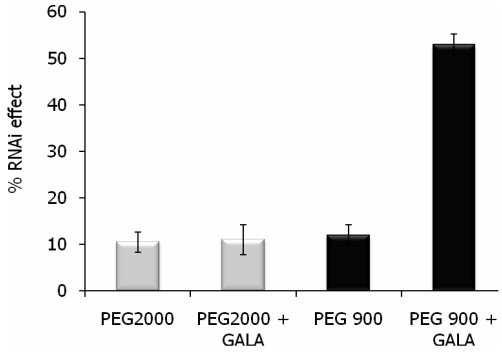

Figure 4. The effects of different PEG length on the in vitro gene silencing of luciferase gene expression using siRNA. HeLa cells stably expressing luciferase were independently transfected with IRQ-PEG-Lips containing luciferase or GFP. The cells were treated with IRQ-PEG2000-Lip, IRQ-PEG2000-Lip containing GALA, IRQ-PEG900-Lip and IRQ-PEG900-Lip containing GALA. Luciferase activities were measured 24 hours after transfection and were expressed as percentage of silencing effect. The data are represented as the mean of three data.

Therefore, the IRQ peptide would more freely reach the receptor compared to that of direct attachment to surface of the liposomes.

Transgene Expression of IRQ-PEG-Lips Coated Anti Luciferase siRNA

To investigate the function of IRQ-PEG-Lips, firstly siRNA for luciferase gene was encapsulated in the nanoparticles. After 24 hours post transfection to the HeLa stably expressing luciferase, the RNAi effect was determined. When siRNA was encapsulated in IRQ-PEG$_{2000}$-Lip, transgene knocked down was limited approximately to 11 % (Figure 4). This result was consistent with the previous study whose results showed that the use of PEG moiety inhibited the decoating of lipid vesicles [6]. When PEG$_{2000}$ moeity attached was replaced with the shorter PEG$_{900}$ moiety, RNAi effect was also inhibited in the absence of fusogenic peptide GALA. However, with incorporation of GALA to the surface of liposomes, transgene expression was drastically enhanced to approximately 53 % (Figure 4). GALA is a pH sensitive fusogenic peptide (WEAALAEALAEALAEHLAEALAEALE- ALAA) in the form of Cholesteryl-GALA (Cho-GALA) to modify the lipid vesicles. It was reported that it improved

Table 2. The size and zeta-potential of IRQ-PEG900-Lips coated anti-VEGFR1 siRNA for transgene expression studies in MFLM-4 cells.

Types of liposomes	Size (nm)	Zeta-potential (mV)
IRQ-PEG$_{900}$-Lip	84 ± 4.8	19 ± 5.3
IRQ-PEG$_{900}$-Lip + STR-R8 5%	116 ± 3.9	43 ± 2.6
IRQ-PEG$_{900}$-Lip + STR-R8 10%	122 ± 3.2	51 ± 3.4
IRQ-PEG$_{900}$-Lip + STR-R8 15%	117 ± 4.3	44 ± 4.9

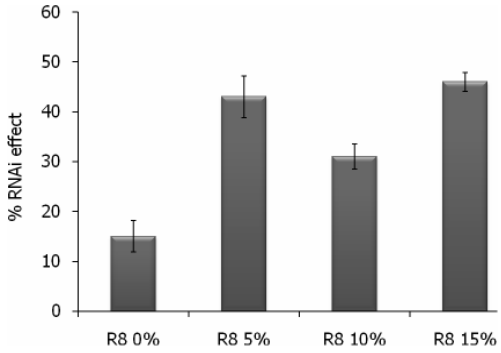

Figure 5. The effects of incorporation of STR-R8 on the in vitro silencing of VEGFR1 gene expression using siRNA. MFLM-4 cells were independently transfected by IRQ-PEG900-Lips containing VEGFR1 or GFP. The cells were treated with IRQ-PEG900-Lips without incorporation of R8 (R8 0 %), incorporation of R8 5 %, 10 % and 15 %.VEGFR1 activities were measured 24 hours after transfection and were expressed as percentage of silencing effect. The bars represent the mean of three data.

transfection activity of encapsulating plasmid DNA due to an enhanced endosomal release [11]. It is presumably that during the IRQ mediates escape nanoparticles from the endosomes, this synthetic pH-responsive pore-forming peptide at the N-terminus of the vectors assisted the escape of the cargo into cytosol by effectively fused with the endosomal membranes and disrupted their integrity [12].

Transgene Expression of IRQ-PEG-Lips Coated AntiVEGFR1 siRNA

Since the IRQ-PEG_{900}-Lip exhibited high lung accumulation and prominent gene silencing in vitro, it is then addressed to deliver its content of siRNA to the primary barrier of the lung endothelial cells. Thus, siRNA for marker luciferase gene was replaced by siRNA for specific endothelial gene marker. In this study, expression of several endothelial gene markers in the lung endothelial MFLM4 cells, such as CD31, Tie-2, VEGFR-1 and VEGFR-2, were examined.

The results showed that VEGFR-1 and VEGFR-2 were expressed in high level in MFLM4 cells (unpublished data). We then focused on the VEGFR-1 gene since it plays an important role in angiogenesis. To examine silencing activity in the endothelial cells, nanoparticles encapsulating synthetic double-stranded anti-VEGFR-1 siRNA (21 base pairs) were tested for their ability to deliver siRNA to the cytosol and to elicit an RNA interference effect. Physical characteristics of prepared nanoparticles was shown in Table 2. Inclusion of STR-R8 to the liposomes resulted in increasing size and charge of nanoparticles. However, similar tendencies in size and charge of coated particles modified R8 indicating that there were no significant changes in their physicochemical properties. As a result, siRNA-IRQ-PEG_{900}-Lips equivalent to 6.4 μg of siRNA resulted in 15% silencing of VEGFR1 activity (Figure 5). However, when stearyl-R8 was incorporated to the liposomal surfaces, RNAi effects was improved. Particularly, inclusion of

STR-R8 15 % to the total lipid vesicles resulted in drastically enhancement in transgene knocked down to approximately 46%. The enhancement of RNAi effect by the use of STR-R8 15 % was 3-fold compared to that without STR-R8. It was very clear the effects of STR-R8 on gene silencing of IRQ-PEG_{900}-Lip in the MFLM4 cells. Principally, the nanoparticles have designed to overcome hurdles in intracellular targeting. They were equipped with fusogenic lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), the IRQ peptide and the Cho-GALA. The presence of the DOPE affects an acidification in the endocytic vesicles so that fusion occurs between the liposomal membrane and endosome during escape [13].

Secondly, the use of IRQ promotes the endosomal escape by fusion-independent mechanism. Lastly, the Cho-GALA is expected to induce pore-formation of endocytic vesicles at low pH compartment so that decoating process is induced. Collectively, siRNA would be released in high extent to the cytoplasm. However, novel strategy should be implemented to improve the cytosolic release due to the low RNAi effect.

In the present study, we utilized the R8 which is incorporated to the surface of liposomes by the use of stearyl moiety as an anchor. Fusion to endosome was postulated as the main mechanism of escape. For fusion to take place between different membranes, three steps are required specific recognition of the site of fusion, close apposition of membranes and fusion or coalescence of membranes [13,14].

At decreasing endosomal pH, the total positive charge on IRQ-PEG-Lips containing R8 remains high due to full protonation of all arginine moieties [13,15]. In addition, R8 interact efficiently with amphoteric components embedded in the endosomal membrane even at acidic pH, not only through electrostatic interactions, but more importantly through bidentate hydrogen bonding. R8 seems to play an additional role in enhancing fusion through insertion into the endosomal membrane after being neutralized by negatively charged membrane components.

The formed ion pair may insert into the membrane and move inwards, driven by the transmembrane potential. This is expected to bring the lipid film of the liposome into intimate contact with the endosomal membrane, resulting in their fusion. This action is thought to increase at acidic pH when the potential across the endosomal membrane is high [13,16]. Oligo and polyarginines were reported to bind to anionic membrane-bound fatty acid salts, cholesterol derivatives, or phosphatidyl glycerol to form hydrophobic ion pairs that are soluble in chloroform or octanol [13,15,17].

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CONCLUSION

The use of long PEG_{2000} moiety drastically improved lung accumulation. However, it provided poor transgene expression. The use of short PEG_{900} moiety exhibited a high pulmonary uptake, comparable to long PEG_{2000} moiety. In addition, the use of IRQ-PEG_{900}-Lip containing siRNA for VEGFR1 in combination with the R8 showed prominent gene silencing in pulmonary endothelial cells. Collectively, the use of PEG_{900} moiety and its incorporation along with the R8 into the IRQ-modified nanoparticles is promising vector design for efficient pulmonary targeting as well as the subsequent pulmonary endothelial cells targeting.

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