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Targeted Polymeric Gene Delivery for Anti-angiogenic Tumor Therapy

Won Jong Kim and Sung Wan Kim*

Center for Controlled Chemical Delivery, Department of Pharmaceutics and Pharmaceutical Chemistry, 20 S 2030 E RM 205 BPRB, University of Utah, Salt Lake City, UT 84112-5820, USA

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Abstract: Gene therapy has become a promising strategy for the treatment of genetically based diseases, such as cancer, which are currently considered incurable. A major obstacle in the field of cancer gene therapy is the development of a safe and efficient delivery system for therapeutic gene transfer. Non-viral vectors have attracted great interest, as they are simple to prepare, stable, easy to modify and relatively safe compared to viral vectors. In this review, an insight into the strategies developed for polyethylenimine (PEI)-based non-viral vectors has been provide, including improvement of the polyplex properties by incorporating hydrophilic spacer, poly(ethylene glycol) (PEG). Moreover, this review will summarize the strategies for the tumor targeting. Specifically, a targeted polymeric gene delivery system, PEI-*g*-PEG-RGD, will be introduced as an efficient gene delivery vector for tumor therapy, including its functional analysis both *in vitro* and *in vivo*.

Keywords: Gene therapy, polyethylenimine, poly(ethylene glycol), angiogenesis, targeted carrier.

Introduction

Angiogenesis is defined as the formation of new blood vessels from pre-existing microvessels, and is involved not only in physiological wound repair, but also in other pathologic processes such as diabetic retinopathy, rheumatoid arthritis, and cancer. In particular, tumor growth and metastasis is dependent on angiogenesis. Therefore, anti-angiogenic therapy is used to inhibit tumor growth and metastasis by destroying neighboring blood vessels that supply tumor cells with oxygen and nutrients and also provide an exit route for tumors to enter the bloodstream. As a result, anti-angiogenic therapy has become a promising strategy for cancer treatment.[1,2]

Among the known angiogenic growth factors and cytokines implicated in the modulation of normal and pathological angiogenesis, the vascular endothelial growth factor (VEGF)[3] family (VEGF-A, VEGF-B, VEGF-C, VEGF-D) and their corresponding receptor tyrosine kinases [VEGF-R1 (Flt-1), VEGF-R2 (KDR/flk-1), and VEGF-R3 (Flt-4)] play a paramount and indispensable role in regulating the angiogenic processes, as well as the induction of vascular permeability and inflammation.[4-6] VEGF stimulates endothelial cell proliferation, migration and tube formation via the interaction with VEGF receptors, Flt-1 (fms-like tyrosine kinase-1) and /or flk-1/KDR (fetal liver kinase-1/kinase domain).[7,8] The intervention to block VEGF action has been accomplished by a variety of methods including antibodies directed against its cognate receptors.[9-12] Another novel method to inhibit the angiogenic action of VEGF is the administration of soluble Flt-1 (sFlt-1) which is an alternatively spliced form of Flt-1 and acts as a potent, and selective inhibitor of VEGF. The sFlt-1 sequesters VEGF produced by tumor cells and forms a heterodimeric complex with a wild type VEGF receptor in a dominant negative fashion, inhibiting its signal transduction.[13-16] Recently, several reports have been published on gene therapy with the soluble VEGF receptors. These include ex vivo transfection of cancer cells with a plasmid encoding sFlt-1 receptor,[17] regional administration of an adenovirus-mediated sFlt-1 cDNA,[18] systemic administration of an adenovirus-mediated sFlt-1 cDNA,[19] and intraperitoneal transduction of a sFlt-1 cDNA using HVJ-cationic liposomes.[20] In spite of these promising advances, however, highly efficient delivery of anti-angiogenic genes to tumors remains a major obstacle. Especially in the case of the systemic administration, where targeted delivery of sFlt-1 cDNA may increase the efficiency and specificity without systemic side-effects.

In this review, we report an angiogenic endothelial cell-targeted polymeric gene delivery system, PEI-*g*-PEG-RGD, developed by incorporating the $\alpha v\beta 3/\alpha v\beta 5$ integrin-binding RGD peptide, ACDCRGDCFC (single-letter amino acid code), into the cationic polymer, polyethylenimine (PEI) via hydrophilic poly(ethylene glycol) (PEG) spacer.[21-23] In the adult human, the $\alpha v\beta 3/\alpha v\beta 5$ integrins are minimally, if at all, expressed on normal vascular endotherlial cells. However, they are significantly overexpressed on angiogenic endothe-

*Corresponding Author. E-mail: rburns@pharm.utah.edu

lial cells within the tumor environment. Because $\alpha v\beta 3$/$\alpha v\beta 5$ integrins are localized to the tumor vasculature, their ligand, RGD peptide can enable selective gene transfer to angiogenic endothelial cells when conjugated to a delivery vehicle and used as a targeting moiety for delivering anti-antiogenic gene. In addition, PEI, one of the most commonly used polymeric gene carriers, would increase the transfection efficiency by escaping DNA complexes from degradative endosome or lysosome compartments.[24,25] PEG was used to decrease the cytotoxicity and increase the solubility of the polymer/plasmid DNA complex.[26] Also, we constructed the therapeutic gene encoding sFlt-1 and evaluated its effect when complexed to PEI-g-PEG-RGD for an anti-angiogenic therapy. Details in synthesis of polymeric carrier and its promising results for tumor therapy are described in this review.

Anti-angiogenic Agents. Current available anti-angiogenic agents target endothelial cells rather than the conventional tumor cell itself. In addition, these agents seem to preferentially target tumor endothelium versus normal, since endothelial cells proliferate more rapidly in tumors than in normal tissues, activated tumor endothelial cells show higher expression of certain surface markers than normal endothelial cells. Anti-angiogenic agents work through different mechanisms, like inhibition of endothelial cell proliferation, migration, and apoptosis. Widely studied anti-angiogenic agents include naturally occurring angiogenesis inhibitors (angiostatin, endostatin, thrombospondins, platelet factor-4, etc); inhibitors of endothelial cell growth (TNP-470, thalidomide, interleukin-12), inhibitors of proangiogenic molecules (antibodies, antisense, and soluble receptors for FGF, VEGF); agents that interfere with basement membranes and extracellular matrix (tissue inhibitors of matrix metaloproteinases); antibodies to adhesion molecules ($\alpha v\beta 3$) and small inhibitors of receptor tyrosine kinases. These molecules are either stimulators or inhibitors depending on the amount, the site, the microenvironment, and the presence of other cytokines. Despite the wide variety of agents described, results have been disappointing and strategies for their optimal use are still under development.

Anti-angiogenic Gene Therapy. There are many challenges to be met before anti-angiogenesis therapies can be employed as an efficient cancer therapy even though this approach has great potential. For an efficient therapy, first, the anti-angiogenesis agent should have a half-life long enough to suppress the tumor growth and it should be effective on various kinds of tumor types. Also, it should not significantly interfere with the physiological angiogenesis. These requirements suggest that anti-angiogenesis delivers anti-angiogenic drugs into pathological sites and also releases them for a prolonged period of time until it suppresses tumor-induced angiogenesis. From this point of view, gene therapy should be considered an effective delivery for anti-angiogenic therapy. Gene therapy can also produce sustained anti-angiogenic effects as long as gene expression is persistent. Table I lists various anti-angiogenic gene therapy strategies that are categorized into two parts depending on their mechanism of action.[27-31] One part is to enhance the anti-angiogenic activity by increasing the local concentration of endogenous angiogenic inhibitors such as thrombospondin-1, platelet factor-4, angiostatin, and endostatin. The other is to suppress the angiogenic activity by directly inhibiting the VEGF expression with antisense oligonucleotides, by sequestrating endo-genous VEGF with cDNA encoding soluble VEGF receptor or by disrupting normal VEGF receptor functions with cDNA of dominant-negative Flk-1 mutant receptor.

Polymeric Gene Carriers. Generally, two different approaches have been utilized for the delivery of genes in gene therapy, namely that of viral vectors and non-viral delivery systems mainly using cationic polymers or lipids. Viral vectors show excellent transfection efficiencies. However, their use in clinical applications are often limited by several problems, including the potential of mutagenicity or oncogenesis, several host immune responses, and the high cost of production. These concerns have made nonviral vectors an attractive alternative to viral vectors.

Non-viral systems, especially cationic polymers, show significantly lower safety risks and can be tailored to specific therapeutic needs.[32] They are capable of carrying large DNA molecules and can be produced in large quantities

Table I. Anti-angiogenic Gene Therapy Strategies

Gene	Delivery System	Experimental Model
Thrpmbospondin-1	Liposome Calcium phosphate	Human prostate cancer Ex vivo transfection of breast cancer
p53	Liposome	Murine B16-F10 melanoma cells Human breast cancer 4
Endostatin	Adenovirus	Prophylactic human colon/liver metastasis xenograft murine model
VEGF antisense	Calcium phosphate	Primary glioblastoma model in nude mice
Dominant-negative Flk-1 mutant receptor	Retrovirus	Various primary tumor models in mice
Soluble Flt-1 receptor	Adenovirus	Primary & metastatic tumor models in mice

easily and inexpensively. However, the major disadvantage of these non-viral vectors is their low transfection efficiency compared by viral vectors. Non-viral vectors have great prospects concerning anti-angiogenic gene therapy, although more research is needed for optimization of these systems in order to achieve a higher transfection efficiency.

Successful nonviral polymeric carriers should have multifunctional properties to overcome many extracellular and intracellular barriers before they deliver therapeutic genes into desired cells. One of the major advantages of polymeric gene delivery carrier development is that various functional groups or molecules can be introduced to a polymer backbone, so as to get close to an ideal gene delivery carrier that should deliver therapeutic DNA into target cell with high efficiency, low toxicity and non-immunogenicity, in addition to easy production in large quantity.[33] A large variety of different polymers and copolymers of linear, branched, and dendrimeric architecture, have been tested, in terms of their efficacy and suitability for *in vitro* transfection. For instance, PEI (Figures 1(a) and (b)) and poly(L-lysine) (PLL, Figure 1(c)) have widely been investigated for polymeric gene delivery. PEI effectively condenses DNA into colloidal particles that effectively transfect DNA into a variety of cells both *in vitro* and *in vivo*.[24] These condensed particles are of spherical shape and have a narrow particle size distribution, which presumably allows high cellular uptake of the plasmids leading to high transfection efficiency. Also, PLL is a well-known polycation, which has been used to condense

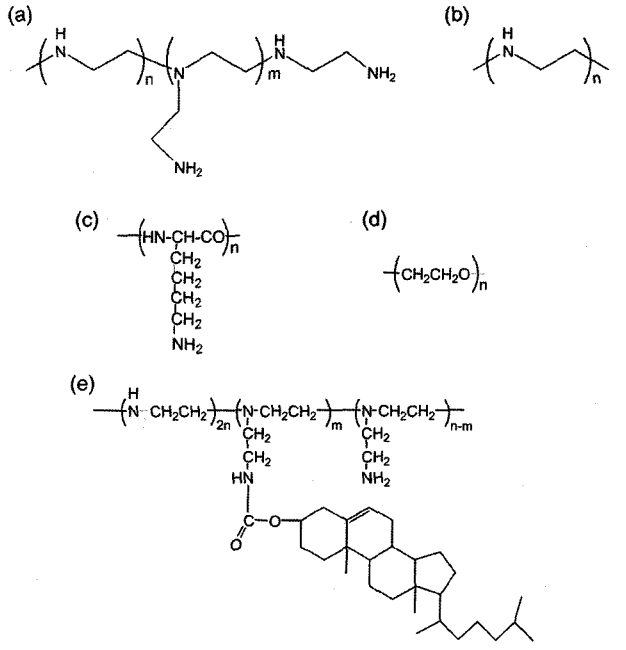

Figure 1. Polymers most frequently used for gene delivery. (a) BPEI; Branched polyethylenimine, (b) LPEI; Linear polyethylenimine, (c) PLL; Poly(L-lysine), (d) PEG; Poly(ethylene glycol), and (e) WSLP; Water-soluble lipopolymer.

DNA under various salt conditions for gene delivery[34-36] and gene analysis.[37-39] PLL has a sufficient number of primary amines to hold positive charge so as to interact with the negatively charged phosphate groups of DNA. As a result, PLL and DNA easily form the complex of electrostatic aggregation under physiological conditions.

Polyethylenimine (PEI). PEI has become the gold standard of non-viral gene delivery. PEI polymers with different molecular weights and degrees of branching have been synthesized and evaluated *in vitro* as well as *in vivo* (Figure 1). Highly branched polymer (BPEI) such as the BPEI (25 kDa) is the most frequently used (Figure 1(a)).[40] BPEI is able to effectively complex even large DNA molecules,[41] leading to homogeneous spherical particles with a size of ~100 nm or less that are capable of transfecting cells efficiently *in vitro* as well as *in vivo*. They offer significantly more protection against nuclease degradation than other polycations, such as poly(L-lysine), possibly due to their higher charge density and more efficient complexation. The large amount of positive charge, however, results in a rather high toxicity and is one of the major limiting factors for its *in vivo* application. The efficacy of BPEI-derived vectors and their cytotoxicity effects depend to a remarkable extent on material characteristics like the molecular weight, the degree of branching, the cationic charge density and buffer capacity,[42,43] polyplex properties, such as the DNA content, particle size, and zeta potential.

The high density of primary, secondary, and tertiary amino groups exhibiting protonation only on every third or fourth nitrogen at pH 7.0 confers significant buffering capacity to the polymers over a wide pH range. This property, known as 'proton sponge effect'[24] is likely one of the crucial factors for the high transfection efficiencies obtained with these polymers. Despite this recognized association, knowledge concerning the relationship between polymer structure and important biological properties such as toxicity or transfection efficiency is limited. Polymers with high molecular weight BPEI (25 KDa, 800 KDa), for example, exhibit high transfection efficiencies, though the toxicity is extensive. Polymers (BPEI) with low molecular weight around 800 Da, display low toxicity, though transfection efficiency is very low.[43] An approach to combine the advantages of high and low molecular weight BPEI has been taken recently by crosslinking small BPEIs via biodegradable bonds. This approach would enhance the transfection efficiency of crosslinked small PEIs, but with only a moderate increase in toxicity.[44] Moreover, water soluble lipopolymer (WSLP) by combining the cationic headgroup of BPEI (1.8 KDa)) with a hydrophobic lipid anchor, cholesterol chloroformate showed low cytotoxicity and enhanced transfection efficacy *in vitro* and *in vivo* (Figure 1(e)).[45-48]

More recently, several *in vitro* and *in vivo* studies have investigated the potential of linear PEI (LPEI)-derived vectors (Figure 1(b)). Most of these experiments have been done in direct comparison to the corresponding BPEI/DNA com-

plexes, revealing remarkable differences between both transfection systems in terms of DNA compaction,[24] nuclear uptake,[49,50] transfection efficiency and toxicity.[51,52] LPEI/DNA complexes exhibited improved cell viability, promote nuclear localization and increased transfection efficiency compared to BPEI-based vectors.[53]

Poly(ethylene glycol) (PEG) as a Stabilizing Agent for Gene/Polymer Complexes. Steric stabilization involves the attachment of hydrophilic polymers to complexes in order to shield positive surface charges and create a steric barrier against aggregation with plasma proteins such as albumin, complement factors or cellular components in the bloodstream. The modification of such complexes may reduce the potential of non-specific interactions, resulting from the cationic surface charges. PEG is a common biocompatible shielding agent, widely used for drug delivery (Figure 1(d)). Two different strategies using PEG for steric stabilization of polyplexes have been developed. The first strategy is based on the formation of copolymers from cationic polymers and PEG,[54-56] whereas the second approach relies on the initial formation of polymer/DNA complexes with subsequent attachment of PEG to free amino groups.[57] PEG has been widely used in the polymeric gene carriers because of its excellent characteristics. First, it reduces the cytotoxicity of the polymer/DNA complex. Second, PEG increases the water-solubility of the DNA/polymer complex. Third, PEG shields excess positive charges of polymer/DNA complex, resulting in the reduction of interaction between the complex and blood components as mentioned before. Using PEG, small particles with a size of ~100 nm and surface charges close to neutrality could be obtained under the appropriate conditions. Additionally, the *in vivo* application of PEG conjugation displayed a prolonged circulation time compared to unmodified complexes. A decrease in gene expression in the lung and a lower initial toxicity were observed as well, when compared to unmodified complexes. This is most likely due to decreased interactions with blood constituents. Fourth and last, PEG can be used as a spacer between a targeting ligand and a polymeric carrier, which facilitates the access of the ligand to its receptor. The major disadvantage of using PEG-conjugation to polymeric gene carrier is the reduced capacity of efficient DNA complexation. This effect has been moderated by using higher molecular weight BPEI.

A modified and rather elegant PEGylation method led to the formation of shielded nanometer-sized polyplexes by mixing DNA with ligand-PEG-PEI conjugates, PEG-PEI copolymers and PEI, forming a targeting unit, shielding agent, and DNA condensing agent, respectively. These trans-fection systems were based on either branched or linear PEI, shielded by linear or branched PEG derivatives of varying molecular weights.

Conjugation of Targeting Moiety for Cell Specific Administration of Therapeutic Gene. In order to reach distant organs or tumors, systemic application of polymeric carrier/DNA complexes through the blood circulation provides several advantages over local administration. The directing of therapeutic systems to the targeted cell or tissue can be accomplished by simply taking advantage of special physiological conditions, e.g. the irregular fenestration in the liver, spleen, bone marrow or certain tumors, which facilitates passive accumulation. Tumors often show irregular endothelial fenestration, which makes it possible to reach the tumor tissue via blood vessels. Furthermore, polymeric carrier/DNA complexes may even accumulate at this site, due to the enhanced permeation and retention (EPR) effect and/or electrostatic interactions,[58,59] thus enabling passive tumor targeting. After intravenous (i.v.) injection, both unmodified LPEI and BPEI-derived vectors deliver the transgene into the heart, spleen, liver and kidney, with highest gene expression levels predominantly in the vascular endothelial cells of the lung, most likely due to the accumulation within the fine lung capillary beds.[60,61] At increasing N/P ratios, gene expression levels are enhanced in all organs. However, the biodistribution pattern largely depends on the method of polyplex administration and material characteristics. Receptor-targeting via the incorporation of receptor ligands like carbohydrates,[62] transferrin,[63] folate,[64] ligands for the low density lipoprotein receptor,[65,66] or RGD peptide[21-23] into the polyplex seems to be most favorable means to achieve site-directed gene delivery towards a preferred or specific cell type.

The $\alpha v\beta 3/\alpha v\beta 5$ integrins have an interesting expression pattern on endothelial cells during angiogenesis. In the adult human, the $\alpha v\beta 3/\alpha v\beta 5$ integrins are minimally, if at all, expressed on normal vascular endothelial cells, but they are significantly overexpressed on angiogenic endothelial cells within tumors. The $\alpha v\beta 3/\alpha v\beta 5$ integrin-selective peptide RGD peptide, ACDCRGDCFC, was found in the phage display library.[67] This peptide contains two disulfide bonds that restricts the peptide conformation more than a single disulfide bond. Thus, the peptide has much higher binding affinity to $\alpha v\beta 3/\alpha v\beta 5$ integrins than similar peptides with one disulfide bond or linear RGD peptide.[68] Also, this peptide was known to be selective for the $\alpha v\beta 3/\alpha v\beta 5$ integrins among others. Both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins may mediate the internalization of this peptide through arginine-glycine-aspartate (RGD) recognition because many integrins, including $\alpha v\beta 3$, bind to their ligands by recognizing the RGD sequence.[69] The bound $\alpha v\beta 3/\alpha v\beta 5$ integrins are endocytosed and recycled to the cell membrane.[70] Thus, this localized tissue distribution of $\alpha v\beta 3/\alpha v\beta 5$ integrins can enable selective gene transfer into angiogenic endothelial cells by using $\alpha v\beta 3/\alpha v\beta 5$ integrin-binding RGD peptide as a targeting moiety for delivering anti-antiogenic gene. Here, we report an angiogenic endothelial cell-targeted polymeric gene delivery system, PEI-*g*-PEG-RGD, developed by incorporating the $\alpha v\beta 3/\alpha v\beta 5$ integrin-binding RGD peptide into the BPEI via PEG spacer. This targeted gene carrier is able to transfer therapeutic genes encoding sFlt-1 into angiogenic endothe-

lial cells in a site-specific manner. Upon internalization and subsequent expression of the therapeutic anticancer gene, sFlt-1, into angiogenic endothelial cells, tumor angiogenesis was disrupted by blocking the interaction between VEGF and VEGFR resulting in tumor reduction. This mechanism of inhibition is shown in Figure 2.

Synthesis of PEI-*g*-PEG-RGD Gene Carrier. RGD peptide was conjugated to BPEI by a heterobifunctional PEG, *N*-hydroxysuccinimide-vinyl sulfone poly(ethylene glycol) (NHS-PEG-VS) as shown in Figure 3(a). In the first step, the NHS group of heterobifunctional PEG reacted with the amino terminal primary amine of a peptide that was preactivated by the excess of TEA in anhydrous DMF. Then, the conjugated RGD-PEG-VS was mixed with PEI in buffer (pH 9). The conjugation ratio of PEI-*g*-PEG-RGD conjugates, expressed as a molar ratio of RGD to PEI, were determined by NMR spectrum analysis. The molar ratio of RGD to PEI of conjugate was 1.3.

To identify the formation of PEI-*g*-PEG-RGD/DNA complexes, agarose gel electrophoresis was performed at different N/P ratios, i.e., the ratio of concentrations of total nitrogen atoms (N) of the polycation to the phosphate groups (P) of DNA, as the characteristic of the complex composition. The movement of the plasmid in the gel was retarded as the amount of the PEI-*g*-PEG-RGD conjugate was increased, demonstrating that the conjugate binds to the DNA, neutralizing its charge. At N/P ratios exceeding the neutralization composition, the complexes migrated slightly toward the anode, suggesting that they have a small positive charge. Complete complex formations were achieved at N/P ratios from approximately 2 to 5 (data not shown). Size and Zeta potential of complexes were measured to study the physicochemical properties of complexes. While PEI/DNA complex showed the dependence of their size on the charge ratio, PEI-*g*-PEG-RGD/DNA complex was much less affected by charge ratio and their size was around 100-200 nm, in the

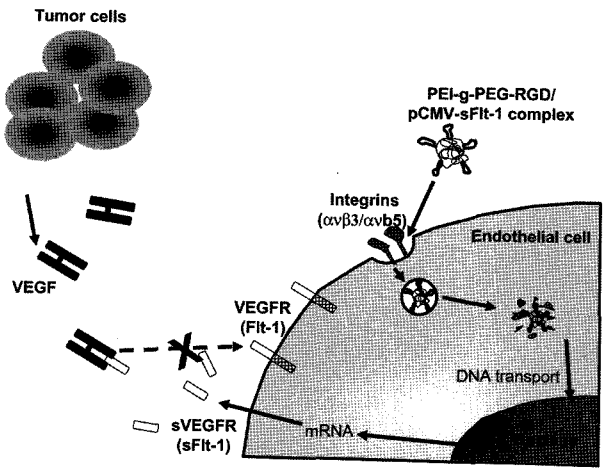

Figure 2. Scheme of anti-angiogenesis by PEI-*g*-PEG-RGD/pCMV-sFlt-1 complex.

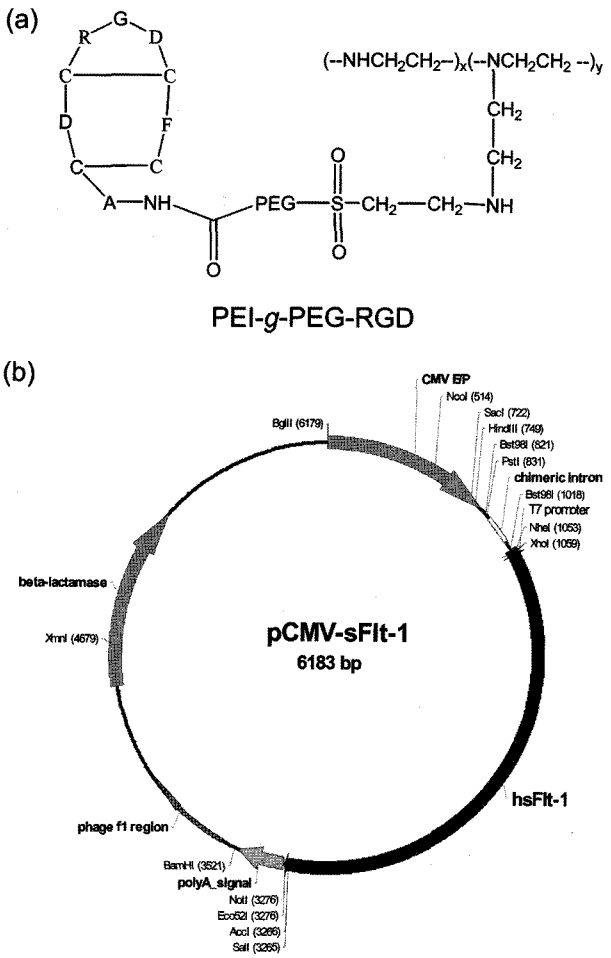

Figure 3. Structure of PEI-*g*-PEG-RGD conjugate (a) and map of pCMV-sFlt-1 plasmid DNA (b).

entire range of charge ratio (5 to 20). At N/P=10, the zeta potential of the PEI/DNA complex was around 35 mV, whereas that of PEI-*g*-PEG-RGD/DNA complexes was 32 mV (data not shown).

Construction and Confirmation of Therapeutic Gene Encoding sFlt-1. Using derivatives of the primer sequences described in Mahasreshti *et al.*'s literature,[71] we generated PCR products from human placenta cDNA (Spring Bioscience). The PCR products were run on 1% agarose gel to separate any non-specific product and demonstrated a band at 2214 bp. The 2214 bp band was excised, purified and digested to ligate into the pCI plasmid at the XhoI and SalI restriction sites to produce the plasmid pCMV-sFlt-1 (Figure 3(b)). Following endonuclease digestion and sequencing qualification, reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to confirm transgene/protein expression. We used the 293T cell line as a control cell line as this cell line does not produce sFlt-1. ELISA demonstrated significant level of sFlt-1 production following transfection with PEI-*g*-PEG-

RGD/pCMV-sFlt-1 complexes. The expression level was more than 8-fold higher than non-transfected and plasmid only controls at 8 ng/mL (data not shown).

Cytotoxicity. The cytotoxicity of PEI-*g*-PEG-RGD/DNA complex or PEI/DNA complex was investigated using the MTT assay on human dermal microvascular endothelial cells (CADMEC) (Figure 4). The cationic polymer/DNA complexes were formed at N/P ratios of 10:1 and 20:1. The amount of plasmid DNA was fixed at 0.5 μg. The viability of the cells incubated with PEI was 60% at an N/P ratio of 20:1. In contrast, the viability of the cells incubated with PEI-*g*-PEG-RGD/DNA was over 90% at a 20:1 N/P ratio. Therefore, PEI-*g*-PEG-RGD/DNA complex was proven to be less toxic to CADMEC than the PEI/DNA complex.

***In vitro* Transfection with Angiogenic and Angiostatic CADMEC.** Transfection efficiencies of PEI and PEI-*g*-PEG-RGD were evaluated with a tissue culture model of angiogenesis developed by incubating CADMEC with human vascular endothelial growth factor (VEGF). The PEI/DNA complex exhibited significant expression of pCMV-luciferase reporter gene. Further enhanced transfection efficiency was achieved by conjugating an integrin-binding peptide, RGD to PEI via PEG spacer in angiogenic CADMECs (Figure 5(a)). This specificity of RGD-mediated gene transfer was also confirmed by a transfection experiment with angiostatic CADMECs. Unconjugated PEI/DNA complex showed higher transfection efficiency than PEI-*g*-PEG-RGD/DNA complex to angiostatic CADMECs (Figure 5(b)). This result could be explained as PEG-RGD moiety shields the electrostatic interaction of the cationic polymer and negatively charged DNA and PEG shields the surface charge of the complex, reducing the interaction of the positively charged complex and negatively charged cell membrane.

Inhibition of CADMEC Proliferation with Transfected sFlt-1. To confirm the efficacy of the transgene, the transfected sFlt-1 should suppress the VEGF-driven proliferation

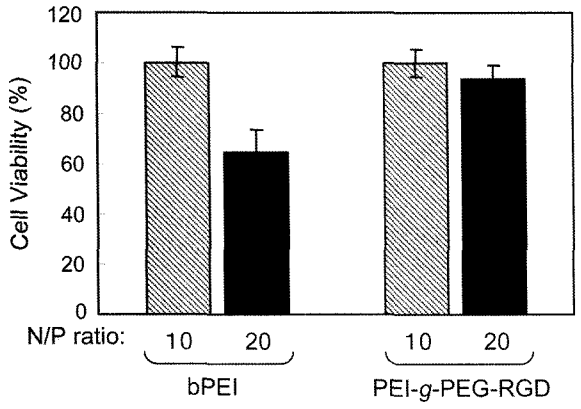

Figure 4. Cytotoxicity of PEI and PEI-*g*-PEG-RGD for CADMEC. The data are expressed as mean values (±standard deviation) of three experiments. (Reprinted from Ref. 22, with permission from Elsevier).

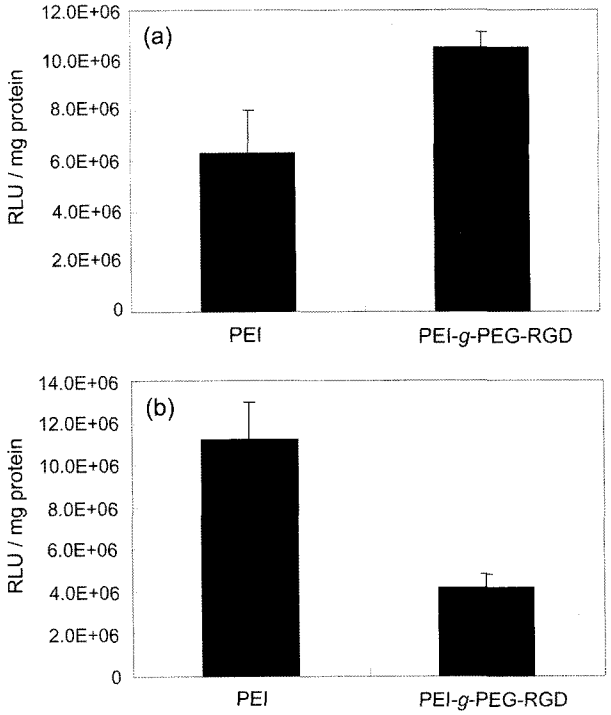

Figure 5. Transfection efficiency in angiogenic (a) and angiostatic CADMEC (b). Each data point represents the mean±standard deviation (n=3). (Reprinted from Ref. 22, with permission from Elsevier).

of endothelial cells. As shown in Figure 6(a), the PEI-*g*-PEG-RGD/pCMV-sFlt-1 complex inhibited the CADMEC proliferation by 63% compared with non-treated control, thus confirming the PEI-*g*-PEG-RGD-mediated secretion of functionally active soluble Flt-1. When used alone, PEI-*g*-PEG-1.3RGD was shown to be cytotoxic and reduced cell numbers by up to 50% (unpublished data). This is most likely the cause of the lowered number of cells in the PEI-*g*-PEG-RGD only transfected cells. While statistically insignificant, it is interesting to note that a small amount of sFlt-1 (produced by pCMV-sFlt-1 alone) produced a 20% reduction in cell numbers. However, this may also be attributed to inconsistencies inherent in cell passaging. In contrast, no inhibitory effects of the PEI-*g*-PEG-RGD conjugate with or without pCMV-sFlt-1 were observed in angiostatic CT-26 colon adenocarcinoma cells (Figure 6(b)).

Biodistribution. The biodistribution experiments were performed on CT-26 subcutaneous tumor-bearing mice by the intravenous injection of PEI-*g*-PEG-RGD/pCMV-sFlt-1 or PEI-*g*-PEG/pCMV-sFlt-1 complexes. The polymer/pDNA complexes were intravenously injected into mice, and tumors as well as lung, heart, kidney, liver, and spleen were extracted at 36 h post injection. In order to achieve a high degree of sensitivity, reproducibility, and accuracy in quantitating gene transfer efficiency, we established a real-time PCR assay for quantification of pCMV-sFlt-1 copies in various organs. The

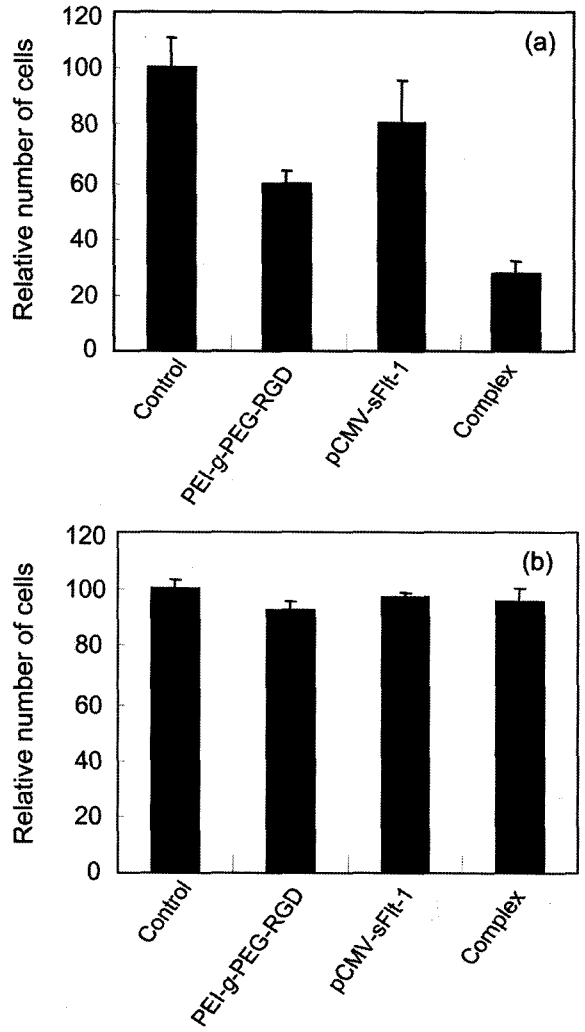

Figure 6. *In vitro* inhibition of CADMEC proliferation (a) and CT-26 murine adenocarcinoma cells (b) by PEI-*g*-PEG-RGD/pCMV-sFlt-1 complexes. Results indicate mean and standard deviation of experiments performed in triplicates. (Reprinted from Ref. 22, with permission from Elsevier).

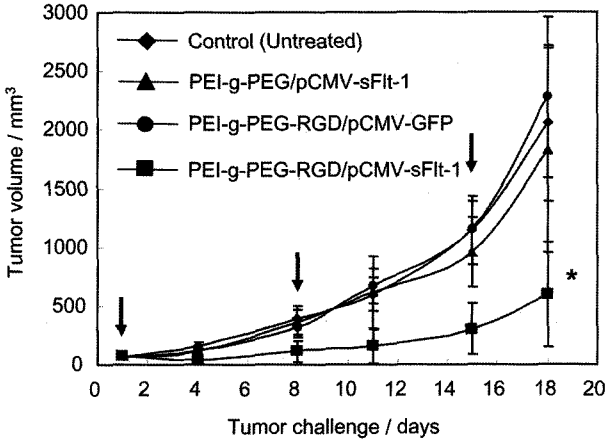

Figure 7. Tumor growth curves. PEI-*g*-PEG-RGD/pCMV-sFlt-1, PEI-*g*-PEG/pCMV-sFlt-1, or PEI-*g*-PEG-RGD/pCMV-GFP complexes were injected into mice via tail vein weekly as indicated by arrows. Results represent the means ± standard deviation (n=5 tumors). *P<0.05 compared to other groups. (Reprinted from Ref. 23, with permission from Elsevier).

biodistribution of complexes are summarized in Table II. No pDNA copies were found in the blood for either polymer complexes (data not shown). Heart and lung samples contained fewer copies than the tumor in the PEI-*g*-PEG-RGD group, while the liver, spleen, and kidney had more copies. This observation coincides with other work on cationic polyplexes.[72,73] On the other hand, in the PEI-*g*-PEG group, all of the organs possessed more copies than the tumor, except for the kidney (Table II). These results therefore suggest that tumor accumulation of PEI-*g*-PEG-RGD/pCMV-sFlt-1 complexes may be due to the effect of the RGD peptide on tumor endothelial cells.[74-76] This association may be responsible for the decreased accumulation in the organs of the PEI-*g*-PEG-RGD complexes through the reticuloendothelial system.[77-80] While the PEG conjugated to the polymer shields the cationic polymer and reduces the overall charge of the complex, the RGD moiety and excess positive charge can influence targeting efficiency by affecting internalization.[81] Therefore, relative tumor accumulation of PEI-*g*-PEG-RGD conjugates is much higher than PEI-*g*-PEG conjugate. This suggests that the RGD peptides are effective for tumor neovasculature homing and transfection.

Inhibition of Tumor Growth. To evaluate *in vivo* efficacy of PEI-*g*-PEG-RGD and pCMV-sFlt-1, polymer/plasmid complexes were administrated to tumor-bearing mice intravenously through the tail vein. PEI-*g*-PEG-RGD/pCMV-sFlt-1, PEI-*g*-PEG/pCMV-sFlt-1, and PEI-*g*-PEG-RGD/pCMV-GFP complexes were prepared at a N/P ratio of 10, and injected into mice every 7 days. As an additional control, 5% glucose solution was also used. PEI-*g*-PEG/pCMV-sFlt-1 complexes did not have a RGD moiety. The PEI-*g*-PEG-

Table II. Percentile Copy Numbers of pCMV-sFlt-1 Distributed in Organs (%)

	Heart	Lung	Liver	Spleen	Kidney	Tumor
PEI-*g*-PEG-RGD	5.73 ± 2.09	2.12 ± 0.18	40.1 ± 5.16	27.3 ± 2.78	17.7 ± 1.26	7.33 ± 4.09
PEI-*g*-PEG	0.99 ± 0.34	2.77 ± 0.71	19.16 ± 10.8	76.6 ± 15.1	0.17 ± 0.08	0.30 ± 0.10

Mean ± SEM were calculated from n=5. The copy numbers of pCMV-sFlt-1 were calculated by real-time PCR.

RGD/pCMV-GFP complexes did not have the therapeutic gene. As shown in Figure 7, tumors grew rapidly in the control, PEI-*g*-PEG/pCMV-sFlt-1 or PEI-*g*-PEG-RGD/pCMV-GFP groups. These three groups did not show any significant difference in tumor growth. On the contrary, the tumor growth was significantly inhibited in the PEI-*g*-PEG-RGD/pCMV-sFlt-1 group compared with the other groups, suggesting that the RGD moiety of the carrier and therapeutic effect of the plasmid were responsible (Figure 7). The mean tumor volume at 18 days was less than 500 mm^3 for PEI-*g*-PEG-RGD/pCMV-sFlt-1 complex, while the mean tumor volumes of other groups were over 1500 mm^3. This may be due to the increased presence of pCMV-sFlt-1 in the tumor, which was mediated by the delivery of PEI-*g*-PEG-RGD as demonstrated by the biodistribution assay (Table II). We believe that the increased presence of pCMV-sFlt-1 plasmid at the tumor site lead to higher sFlt-1 expression. This is indirectly evidenced by the lack of tumor growth in animals receiving PEI-*g*-PEG-RGD/pCMV-sFlt-1 therapy only.

Conclusions

In this report, we recapitulated current development of non-viral gene delivery carrier and its using for anti-angiogenesis. We used a targeted polymeric carrier for expression of sFlt-1 that is an endogenously expressed, potent VEGF antagonist. We have evaluated the function of therapeutic gene carrier with an inhibition of endothelial cell proliferation assay. The complex of therapeutic gene and PEI-*g*-PEG-RGD conjugate efficiently inhibits proliferation of endothelial cells by blocking the binding of VEGF to the membrane bound Flt-1 receptor. The PEI-*g*-PEG-RGD gene carrier delivers pCMV-sFlt-1 to tumors more efficiently than PEI-*g*-PEG after systemic administration. In addition, the expression of sFlt-1 by pCMV-sFlt-1 delayed the tumor growth and increased the survival rate. Taken together, PEI-*g*-PEG-RGD/pCMV-sFlt-1 complexes may be useful to eventually develop tumor-specific anti-angiogenic gene therapy. The use of a non-viral gene carrier to deliver an anti-angiogenic gene can demonstrate a low continuous dosage through repeated injections that other vectors cannot thereby allowing for future clinical use.

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