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研究成果の刊行に関する一覧表

書籍　該当なし

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Antiangiogenic gene therapy of experimental pancreatic tumor by sFlt-1 plasmid DNA carried by RGD-modified crosslinked polyplex micelles

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ABSTRACT

Disulfide crosslinked polyplex micelles with RGD peptides were formed through ion complexation of thiolated c(RGDfK)-poly(ethylene glycol)-*block*-poly(L-lysine) (c(RGDfK)-PEG-P(Lys-SH)) and plasmid DNA encoding sFlt-1 and tested for their therapeutic effect in BxPC3 pancreatic adenocarcinoma tumor bearing mice. These micelles, systemically injected, demonstrated significant inhibition of tumor growth up to day 18, as a result of the antiangiogenic effect that was confirmed by vascular density measurements. Significant therapeutic activity of the 15% crosslinked micelle (c(RGDfK)-PEG-P(Lys-SH15)) was achieved by combined effect of increased tumor accumulation, interaction with endothelial cells and enhanced intracellular uptake through receptor-mediated endocytosis. These results suggest that RGD targeted crosslinked polyplex micelles can be effective plasmid DNA carriers for antiangiogenic gene therapy.

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1. Introduction

Poly(ethylene glycol) (PEG)-polycation block copolymers have been widely investigated in the field of gene delivery as a potential non-viral vectors for systemic applications [1–7]. The complexes of plasmid DNA (pDNA) and block copolymers form self-assembling particles, termed polyplex micelles, with a core-shell structure. The outer hydrophilic shell layer, formed by PEG segment, increases micelle stability in serum, improves its pharmacokinetic properties, and reduces polymer toxicity [8–11]. Nevertheless, further stabilization and increased longevity in blood are required for polyplex micelles to achieve successful gene delivery *in vivo*.

Disulfide crosslinks were previously introduced into the polyplex micelle core to stabilize its structure in the extracellular entity, while facilitating smooth release of the entrapped pDNA in the intracellular reductive environment [12,13]. Indeed, disulfide crosslinked polyplex micelles exhibited improved transfection of the reporter gene to cultured cells and mouse liver upon systemic administration [13]. In addition, cyclic RGD peptide ligands (c(RGDfK)) were recently installed onto the surface of the disulfide crosslinked polyplex micelles to achieve specific targeting to tumor neo-vasculature [14,15]. RGD (Arg–Gly–Asp) peptide is a recognition motif in multiple ligands of α_v integrin family [16]. Moreover, cyclic RGD peptides showed increased affinity to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors [17] which are overexpressed on tumor angiogenic endothelial cells [18]. Therefore, RGD peptide ligands have been intensively investigated as an active targeting strategy in antiangiogenic gene therapy for cancer [19–22]. Consequently, we hypothesized that polyplex micelles with cyclic RGD ligands and disulfide crosslinks may be a useful system for targeting angiogenic endothelial cells by systemic administration. RGD conjugated polyplex micelles showed remarkably increased transfection efficiency in cultured HeLa cells possessing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, as a result of increased cellular uptake and intracellular trafficking of micelles toward perinuclear region via caveolae-mediated endocytosis as was previously reported [14,15]. Caveolae- mediated endocytosis is a nondigestive internalization pathway, which does not result in pH decrease, thus avoiding pDNA degradation in acidic organelles in cell. This route might be especially essential for polylysine based pDNA carriers, which do not possess "proton buffering" ability to escape endosome.

Vascular endothelial growth factor (VEGF) is a major proangiogenic molecule, which stimulates angiogenesis via promoting endothelial proliferation, survival and migration [reviewed in [23,24]]. VEGF and VEGF receptors have been found to be up-regulated in

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various types of tumors and are usually associated with tumor progression and poor prognosis (reviewed in [25]). Inhibition of VEGF or its signaling pathway has been shown to suppress tumor angiogenesis and tumor growth [reviewed in [25–27]].

The soluble form of VEGF receptor-1 (soluble fms-like tyrosine kinase-1: sFlt-1) is a potent endogenous agent for antiangiogenic therapy. The sFlt-1 binds to VEGF with the same affinity and equivalent specificity as that of the original receptor, however inhibits its signal transduction [28–30]. Therefore, exogenous sFlt-1 is considered to be an effective therapeutic agent for antiangiogenic tumor therapy [20,21,31–35]. Recently, several reports were published on *in vivo* non-viral gene therapy with sFlt-1, carried by several types of polymers, for inhibition of tumor angiogenesis [21,35]. Kim WJ et al. reported effective tumor growth suppression in CT-26 colon adenocarcinoma bearing mice by systemic injection of polyethyleneimine based polyplexes, utilizing the RGD targeting approach [21].

In this study, thiolated PEG-poly(L-lysine) (PEG-PLys) block copolymer, combining long PEG chain with optimized crosslinking degree, was designed for construction of RGD-mediated gene delivery system. Here we report the therapeutic effect of sFlt-1 expressing pDNA complexed with 15% thiolated control poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-P(Lys-SH15)) and cyclic RGD conjugated (c(RGDfK)-PEG-P(Lys-SH15)) polymers, forming crosslinked polyplex micelles, after systemic administration to BxPC3 human pancreas adenocarcinoma tumor bearing mice. Note that BxPC3 xenografts are characterized by heterogeneous vascularity and stroma-rich histology [36], which limits access of therapeutic agents to tumor cells. Thus, the accessibility of endothelial cells by bloodstream, makes antiangiogenic approach an attractive strategy against pancreatic tumor.

2. Materials and methods

2.1. Materials

N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was purchased from Dojindo Laboratories (Kumamoto, Japan). Cyclo[RGDfK(CX-)] (c(RGDfK)) peptides (X = 6-aminocaproic acid: ε-Acp) was purchased from Peptide Institute (Osaka, Japan). The PEG-PLys block copolymer (PEG, 17,000 g/mol; polymerization degree of PLys segment, 73) was synthesized as previously reported [37]. Plasmid DNA coding for luciferase (Luc) under the control of CAG promoter was provided by RIKEN Gene Bank (Tsukuba, Japan), and a fragment cDNA of sFlt-1 was inserted into the pCAcc vector having CAG promoter. The pDNAs were amplified in competent DH5α *Escherichia coli* and purified by the HiSpeed Plasmid Maxi Kit purchased from QIAGEN Sciences Co., Inc. (Germantown, MD). Luc pDNA was labeled with Cy5 by the Label IT Nucleic Acid Labeling Kit (Mirus, Madison, WI) according to the manufacturer's protocol. Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Sigma-Aldrich Co (Madison, WI) and Dainippon Sumimoto Pharma Co., Ltd. (Osaka, Japan), respectively. Rat monoclonal antibody to CD31 (platelet endothelial cell adhesion molecule 1 (PECAM1)) was purchased from BD Pharmingen (Franklin Lakes, NJ), and Alexa Fluor 488-conjugated secondary antibody to rat IgG was from Invitrogen Molecular Probes (Eugene, OR).

2.2. Preparation of block copolymers

2.2.1. Synthesis of thiolated PEG-PLys (PEG-P(Lys-SH))

Pyridyldithiopropionyl (PDP) groups were introduced to the ε-amino groups of PLys side chain as reported previously [12]. Briefly, acetal-PEG-PLys (83 mg, 2.86 μmol) was dissolved in 10 mL *N*-methyl-2-pyrrolidone containing 5 wt.% LiCl and stirred with a heterobifunctional reagent, SPDP, (10 mg, 31 μmol) in the presence of *N*,*N*-diisopropylethylamine (10 mol excess against the SPDP reagent) for 3 h at room temperature. The mixture was then precipitated into 20 times excess volume of diethyl ether. The precipitated polymer was dissolved in 10 mM phosphate buffer (pH 7.0, 150 mM NaCl), dialyzed against the same buffer and then distilled water, and lyophilized to obtain PEG-P(Lys-PDP). The degree of PDP substitution for each polymer was determined from the peak intensity ratio of the methylene protons of PEG (OCH_2CH_2, $\delta = 3.5$ ppm) to the pyridyl protons of the 3-(2-pyridyldithio)propionyl group (C_5H_4N, $\delta = 7.2$–8.3 ppm) in the ^{1}H NMR spectrum (D_2O, 25 °C). Block copolymer with X % thiolation degree was abbreviated as B-SHX%.

2.2.2. Synthesis of c(RGDfK)-PEG-P(Lys-SH)

Acetal-PEG-P(Lys-PDP) (30 mg, 1 μmol) was dissolved in 10 mM Tris–HCl buffer solution (pH 7.4) (3 mL) with 10 eq. of dithiothreitol (DTT). After 30 min incubation at room temperature, the polymer solution was dialyzed against 0.2 M AcOH buffer (pH 4.0). c[RGDfK(CX-)] (8 mg, 6.5 mmol) in AcOH buffer (3 mL) was then added to the polymer solution. After stirring for 5 days, DTT (6.67 mg, 43.9 μmol) was added and stirred at room temperature for 3 h. The reacted polymer was purified by dialysis sequentially against 10 mM phosphate buffer pH 7.0 with 150 mM NaCl and distilled water, and lyophilized to obtain c(RGDfK)-PEG-P(Lys-SH) [14].

2.3. Preparation of polyplex micelles

The above polymers were dissolved in 10 mM Tris–HCl buffer (pH 7.4) containing 10% volume of 100 mM DTT. After 30 min at ambient temperature, twice-excess volume of pDNA solution (50 μg/mL) in the same buffer was added to the polymer solution to form a polyplex micelle at *N*/*P* ratio of 2. The *N*/*P* ratio was defined as the residual molar ratio of amino groups of thiolated PEG-PLys to phosphate groups of pDNA. After an overnight incubation at ambient temperature, the polyplex micelle solutions were dialyzed against 10 mM Tris–HCl (pH 7.4) containing 0.5% dimethylsulfoxide (DMSO) at 37 °C for 24 h, followed by additional 2 days dialysis for the DMSO removal. During these dialysis processes, thiol groups of the polymers in the micelles were oxidized to form disulfide crosslinks. The concentration of pDNA in each micelle solution was determined by absorbance at 260 nm. Polyplex micelles with and without cyclic RGD peptide ligands were abbreviated as RGD(+) and RGD(−), respectively.

2.4. Quantitative determination of transfection efficiency by real time reverse transcription-polymerase chain reaction (RT-PCR) for sFlt-1

HeLa cells, expressing the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors, were seeded on 24-well culture plates (10000 cells/well) and incubated for 24 h in 500 μL of DMEM medium containing 10% FBS. Micelle solutions were then added at a concentration equivalent to 1 μg of pDNA per well and the cells were incubated for 48 h. Following this incubation period, total RNA was extracted from the cells and transcripted to cDNA. The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using the following human specific primers: 5′-CCACTCCCTTGAACACGAG-3′ and 3′-CGCCTTACGGAAGCTCTCT-5′. Amplification conditions were as recommended by the manufacturer (QIAGEN Sciences Co., Inc.). Unknown and standard samples were run in triplicate. Concentrations of unknown samples were interpolated from a standard curve, established by simultaneous amplification of sFlt-1 plasmid standards.

2.5. In vivo studies

2.5.1. Mice

Five-week-old female Balb/c nude mice were purchased from Charles River Laboratories (Tokyo, Japan). Mice were maintained on ad libitum rodent feed and water. The experimental animals were allowed to acclimate for at least 1 week before tumor implantation. All studies were performed in accordance to the Guide for the Care

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and Use of Laboratory Animals as stated by the National Institutes of Health.

2.5.2. Tumor implantation

BxPC3 cell line (ATCC, Manassas, VA), derived from human pancreatic tumor was inoculated to nude mice subcutaneously to develop xenografts (100 µl of 5×10^7 cells/mL PBS suspension). Tumors were allowed to grow for 3 weeks till their size reached approximately 120–160 mm^3.

2.5.3. Blood circulation

Polyplex micelles loading Cy5-labeled pDNA (100 µg pDNA/mL, 200 µL) were intravenously injected to the mice via the tail vein at a dose of 20 µg pDNA/mouse. Blood was collected from the postcaval vein under anesthesia 15 min after injection and centrifuged to obtain blood plasma. Two microliters of 10X trypsin-EDTA were added to 20 µL of the plasma and incubated overnight at 37 °C to release pDNA from the micelle by digesting PLys segment of the block copolymer. The fluorescence intensity of the sample solution was measured at $\lambda = 670$ nm by spectrofluorometer (ND-3300, Nano Drop, Wilmington, DE), and percent of pDNA dosage in the blood was calculated according to the following equation:

$$\%\ \text{injected pDNA in the blood} = (F_{670(\text{sample})} / F_{670(\text{control})}) \times 100 \quad (1)$$

where the $F_{670(\text{control})}$ represents the fluorescence intensity of micelle solution mixed with blood sample (time 0).

2.5.4. In vivo tumor growth inhibition

Polyplex micelles, loading pDNA equivalent to 20 µg and dissolved in 10 mM Hepes buffer (pH 7.4) with 150 mM NaCl, were administered intravenously on days 0, 4, and 8. Tumor size was measured every 2 days by a digital vernier caliper across its longest (a) and shortest diameters (b) and its volume (V) was calculated according to the formula $V = 0.5ab^2$. Tumor progression was evaluated in terms of relative tumor volume (to day 0) over a period of 18 days.

2.5.5. Quantification of microvessel density

At the end of *in vivo* tumor growth studies, xenografted tumors were excised and frozen in tissue-Tek-OCT. The frozen tumors were cut into 10 µm thick slices with a cryostat maintained at −23 °C. Vascular endothelial cells were immunostained by incubation of the cryosections with anti-CD31 antibody followed by incubation with Alexa Fluor 488-conjugated secondary antibody. The tumor cryosections were observed by a confocal laser scanning microscope (CLSM), LSM 510 (Carl Zeiss, Oberlochen, Germany). Microvessel density was quantified by counting the percentage area of CD31 positive pixels per image with at least 21 images per sample (i.e., three animals per sample x 7 cryosections per tumor).

2.5.6. Micelle accumulation in tumor tissue

Polyplex micelles loading Cy5-labeled pDNA were intravenously injected at a dose of 20 µg pDNA/mouse. Mice were sacrificed after 24 h and the excised tumors were fixed in formalin for 1 h, followed by 1 h incubation periods with 10, 15 and 20% sucrose/PBS solutions at room temperature. The tumors were frozen in tissue-Tek-OCT and cryosections were prepared for CLSM visualizations as described in the previous section. The nuclei were stained with Hoechst 33342 (Dojindo Lab., Kumamoto, Japan). The CLSM observations were performed at the excitation wavelengths of 488 nm (Ar laser) for the Alexa Fluor 488, 633 nm (He–Ne laser) for Cy5, and 710 nm (MaiTai laser, two photon excitation) for Hoechst 33342, respectively. The percentage of pDNA positive pixels per image was counted to quantify the micelle accumulation inside the tumor tissue.

2.6. Data analysis

The experimental data was analyzed by Student's *t*-test. $P<0.05$ was considered as significant.

3. Results

Thiolated acetal-PEG-PLys block copolymers, composed of 17 kDa M.W. PEG and 73 lysine units, were prepared as described elsewhere [12,14,37]. SPDP was used as a thiolating reagent and conjugated to the ε-amino group of lysine unit. Conjugation of c(RGDfK) peptide ligands into the PEG terminus of acetal-PEG-P(Lys-PDP) was achieved through the formation of a thiazolidine ring between the *N*-terminal cysteine and the aldehyde group converted from the acetal group [14,15]. The targetable polyplex micelles were prepared through ion complexation of the above polymers with pDNA at $N/P = 2$ (Fig. 1), and analyzed for their size and ζ-potential by DLS and laser-doppler electrophorsis, respectively. The cumulant diameters of the B-SHX% micelles were approximately 104 ± 18 nm, with a moderate polydispersity index of 0.2. The ζ-potentials were found to be approximately 0.5 mV, as a result of the PEG palisade formation surrounding the polyplex core [8,14].

Following *in vitro* transfection in HeLa cells, the mRNA expressions of sFlt-1 were quantitatively analyzed by real time RT-PCR. From this analysis, presented in Fig. 2, it is clear that the cells were successfully transfected by the polyplex micelles. The highest transfection efficiency was achieved by RGD(+) B-SH15% crosslinked (15(+)) micelle. Worth noting, detectable protein level of sFlt-1 by ELISA, specific to human VEGF-R1/sFlt-1 (R&D Systems), could be achieved for this formulation only (1.2 ± 0.05 ng/mL) (data not shown). Other micelles, probably, resulted in sFlt-1 levels which are beyond the sensitivity of this assay (<13 pg/ml). The increased transfection efficiency of the 15(+) micelle results from the combination of crosslinked core and receptor targeting ligand, consistent with our previous studies [15].

The blood circulation experiments were carried out in BxPC3 tumor bearing mice upon intravenous injections of the Cy5-labeled pDNA (20 µg pDNA/ mouse). Blood was collected from the postcaval vein 15 min after administration and analyzed for its fluorescence intensity. Disulfide crosslinks prolonged blood circulation time, while the RGD conjugation resulted in significantly lower blood circulation period of polyplex micelles, as shown in Fig. 3. In the case of crosslinked system, 28% and 21% of injected pDNA were observed in plasma for RGD(−) and RGD(+) micelles, respectively. Significantly lower recovered doses of pDNA, 11 % and 7 % for RGD(−) and RGD(+) micelles, respectively, were found for non crosslinked system. We further evaluated micelle accumulation in tumor by iv administration of RGD-conjugated or non-conjugated 15% crosslinked micelles prepared with Cy5-labeled pDNA at a dose of 20 µg pDNA/mouse. Both micelles were found to be localized in the tumor blood vessels, 24 h after administration, as was indicated by colocalization of the Cy5-labeled pDNA (red) and the CD31 positive endothelial cells (green) (Fig. 4A). However, quantitative analysis of the pDNA positive area per image revealed significantly higher accumulation of the RGD-conjugated micelle than non-conjugated micelle inside the tumor tissue (Fig. 4B): 3.08 % and 2.44 % of red pixels per image for RGD(+) and RGD(−) micelle, respectively ($P<0.05$).

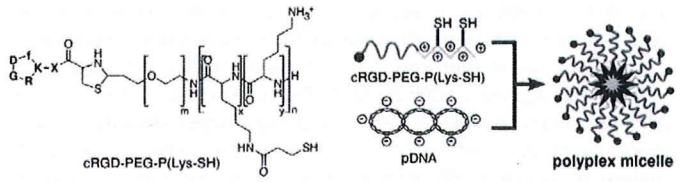

Fig. 1. Structure of cRGD-PEG-P(Lys-SH) and its polyplex micelle.

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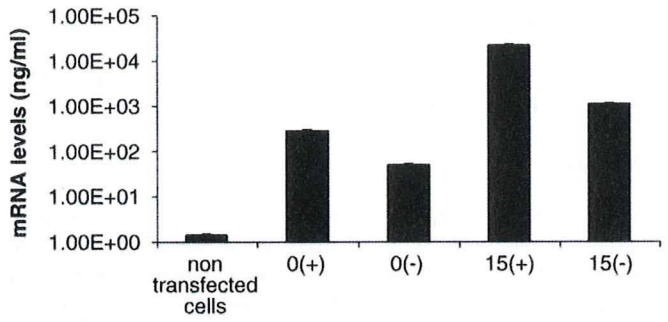

Fig. 2. *In vitro* transfection efficiency of sFlt-1 plasmid DNA in HeLa cells. The cells were transfected with RGD(+) and RGD(−) non crosslinked micelles (0(+) and 0(−)) and RGD(+) and RGD(−) 15% crosslinked micelles (15(+) and 15(−)), respectively. Non transfected cells were used as control. Each well was transfected with 1 μg of pDNA for 48 h and analyzed for sFlt-1 mRNA levels by real time RT-PCR.

The therapeutic effect of polyplex micelles following intravenous administration of the sFlt-1 expressing pDNA was evaluated by tumor growth inhibition study in BxPC3 tumor bearing mice. When tumors reached the volume of 120–160 mm^3, animals were injected with three doses of polyplex micelles containing either sFlt-1 or Luc expressing plasmid (20 μg pDNA/dose) on days 0, 4 and 8. The results of these studies, in terms of relative tumor volumes (Fig. 5), indicate the ability of RGD(+) and RGD(−) crosslinked polyplex micelles as vehicles for therapeutic gene delivery in BxPC3 tumor bearing mice. In the case of animals treated with 15(+) micelles, the tumor progression was significantly inhibited from day 6, compared to control mice. By the end of the experiment, the mean tumor volume in this group was 1.67 ± 0.18 of initial tumor volume. In the group of animals treated with pDNA encapsulated in RGD(−) micelles, significant inhibition of tumor progression was observed only from day 12, and the mean tumor volume reached 1.93 ± 0.52 of initial tumor volume by the end of the experiment. On the other hand, tumors grew much faster in the control groups, and reached 2.58 ± 0.5 of initial tumor volume.

Intravenous administration of crosslinked polyplex micelles containing sFlt-1 pDNA to BxPC3 tumor bearing mice resulted in significant reduction in the tumor neo-vasculature, as shown by CD31 immunostaining of the tumor cryosections. Representative images are shown in Fig. 6A. Increased density of blood vessels throughout the tissue was observed in control tumors. In contrast, very few blood vessels could be observed in the sFlt-1 treated groups. The quantitative results of microvessel density in tumor tissue cryosections were obtained by counting the area of stained blood vessels (green pixels) per image (Fig. 6B). Systemic administration of sFlt-1 expressing pDNA in the RGD(+) micelles resulted in the lowest average microvessel density of only 8.6% per image, whereas the RGD (−) micelle carrying pDNA led to 12.3% vessels per image. The control group had an average microvessel area of 23.7% per image, significantly higher as compared to the treated groups.

4. Discussion

In this study, we demonstrate that crosslinked polyplex micelles formed by electrostatic interaction of thiolated PEG-PLys block copolymers, modified on their surface with cRGD peptide ligand, and sFlt-1 pDNA are effective for *in vivo* tumor regression upon systemic administration. The thiolated PEG-PLys block copolymer, in this study, was further optimized by higher molecular weight PEG (17,000 Da) against 12,000 Da M.W. PEG used so far [2,3,8,12–15], to achieve enhanced shielding effect and thus higher stability in blood. Block copolymer with 15% thiolation degree, which showed the highest transfection efficiency *in vitro* and *in vivo* (data not shown), was selected for construction of RGD-mediated gene delivery vector.

The results of sFlt-1 transfection in Hela cells show higher mRNA expression levels in the cells transfected by RGD(+) crosslinked micelle relative to either RGD(−) or non crosslinked micelles (Fig. 2). This result is consistent with our previous studies, indicating the greater stability of crosslinked micelles in the medium and specific affinity of RGD ligand to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors expressed in HeLa cells [14,15]. Micelle internalization to the cell via integrin-mediated endocytosis contributes to the accelerated accumulation of pDNA in the perinuclear region through the change in its intracellular trafficking from clathrin-mediated to caveolae-mediated endocytosis, resulting in enhancement of gene expression [15].

When administrated intravenously into BxPC3 tumor bearing mice, blood levels of Cy5-labeled pDNA were significantly lower for the RGD(+) micelle compared to the RGD(−) micelle. This observation might be partly explained by enhanced accumulation of pDNA in tumor site when carried by RGD(+) micelle over RGD(−) (Fig. 4B) and other organs as well. These observations are in good agreement with other works using cyclic RGD-modified particles, which reported significantly lower blood circulation times [38–40] while higher accumulation in tumor tissue [21,38–41], liver [21,38–42] and spleen [28–31] compared to the control. Moreover, CLSM observations demonstrated colocalization of both micelles with tumor endothelial cells, confirming their potential as effective antiangiogenic gene delivery vehicles (Fig. 4A).

In vivo tumor growth assay revealed significant ($P<0.05$) tumor growth inhibition when the sFlt-1 pDNA was administrated by crosslinked micelles as compared to control groups. Compared to RGD(−), the RGD(+) micelle was more effective in suppressing tumor growth. The significant difference in relative tumor volumes between RGD(+) injected and control groups was observed from day 6 till the end of the experiment. In comparison, significant difference between RGD(−) injected and control groups was observed only from day 12. In addition, relative tumor volumes in the RGD(+) injected group were lower than those in the RGD(−). These findings may be explained by greater tumor accumulation and higher transfection efficiency of RGD-modified micelle, resulted from more effective intracellular plasmid delivery through specific receptor binding and endocytosis. The lack of significant difference in relative tumor volumes between the RGD(+) and RGD(−) injected groups might be due to the lower circulation time in blood of the RGD(+) micelle and its enhanced accumulation in organs such as liver and spleen. Accumulation in liver [21,38–42] and spleen [39–42] was shown for various cyclic RGD-modified vectors and was, in general, attributed to their accelerated clearance through the phagocytosis by macrophages located on reticuloendothelial system (RES) [39–41].

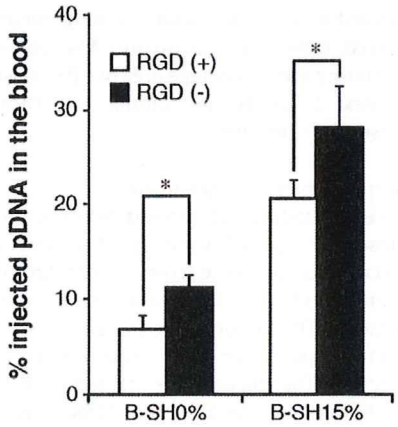

Fig. 3. Blood circulation of plasmid DNA carried by RGD (+/−) polyplex micelles. Micelles loading Cy5-labeled pDNA were intravenously administrated to the tumor bearing mice (20 μg pDNA/mouse). Blood was collected 15 min after administration and analyzed for its fluorescence intensity. $N=3$, Mean ± s.d. $^*P<0.05$ compared to RGD(−).

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The antiangiogenic effect of expressed sFlt-1 was confirmed by CD31 immunostaining of the tumor cryosections and quantification of microvessel density. From these studies, it is clear that sFlt-1 was able to significantly suppress tumor neo-vasculature formation when the pDNA was delivered in RGD(+) and RGD(−) crosslinked micelles. The most pronounced effect on microvessel density was observed with the plasmid administrated in RGD(+) micelles. This is probably due to the combined effect of tumor accumulation and increased transfection efficiency of the RGD-conjugated 15% crosslinked polyplex micelle.

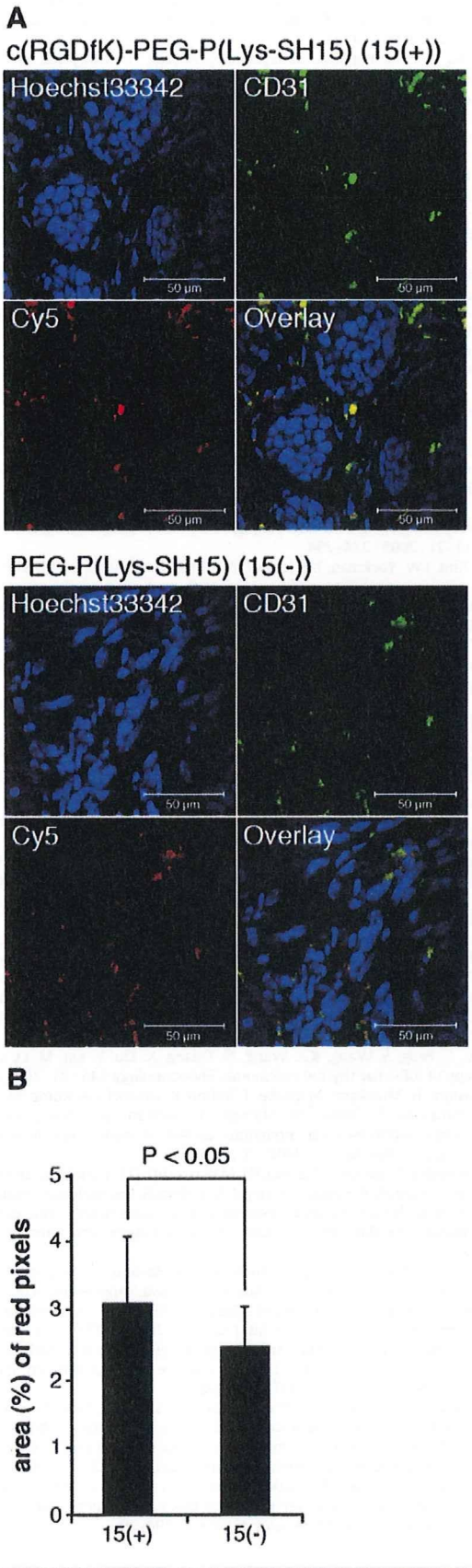

Fig. 4. Micelle localization in tumor tissue. (A) Tumor endothelium and pDNA localization. Immunostaining of CD31 (green) revealed colocalization of Cy5-labeled pDNA (red) with tumor vasculature for both RGD-conjugated (15(+)) and non-conjugated (15(−)) micelles, 24 h after administration. The cell nuclei were stained with Hoechst 33342 (blue). (B) Quantitative analysis of Cy5-labeled pDNA (red pixels). The results represent percentage areas of pDNA-positive pixels per image. Seven images were taken from each tumor tissue, from 3 mice, mean ± s.d. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

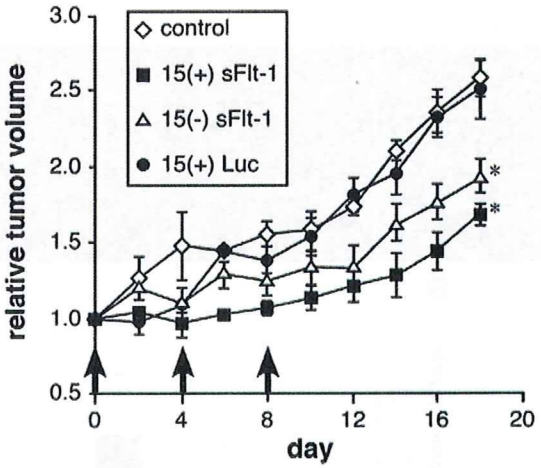

Fig. 5. *In vivo* tumor growth inhibition. RGD (+) and RGD (−) 15% crosslinked polyplex micelles loading plasmid DNA coding either sFlt-1 or Luc were administrated intravenously to BxPC3 tumor bearing mice at a pDNA dose of 20 μg on days 0, 4 and 8, as indicated by arrows. Control animals were injected with either Hepes buffer or 15 (+) micelle loading Luc expressing pDNA. Tumor volumes were measured every 2 days up to day 18 and normalized to the initial tumor volume (day 0). Results are presented in terms of relative tumor volumes, mean ± s.d., $N = 6$. $^*P < 0.05$ compared to control group.

5. Conclusion

Our data contributes to the list of successful non-viral systems for antiangiogenic cancer gene therapy utilizing sFlt-1 pDNA as VEGF sequester [21,35] and RGD targeting of tumor endothelial cells [19,21]. Worth noting, the antiangiogenic gene therapy by sFlt-1 pDNA, delivered by non-viral vector with cRGD ligand, appears to be a promising strategy to treat an intractable pancreatic tumor.

The significant inhibitory effect of tumor growth shown in this study, confirms the potential of c(RGDfK)-PEG-P(Lys-SH15) and PEG-P(Lys-SH15) polyplex micelles as effective systemic gene delivery systems to the neo-vasculature of solid tumors. Both of these formulations showed accumulation and interaction with tumor endothelial cells. The therapeutic activity of c(RGDfK)-PEG-P(Lys-SH15) was pronounced by combined effect of increased tumor accumulation and enhanced intracellular delivery. Based on these studies, c(RGDfK)-PEG-P(Lys-SH15) can be employed as an effective platform for systemic administration of therapeutic plasmid DNA for antiangiogenic therapy.

Acknowledgement

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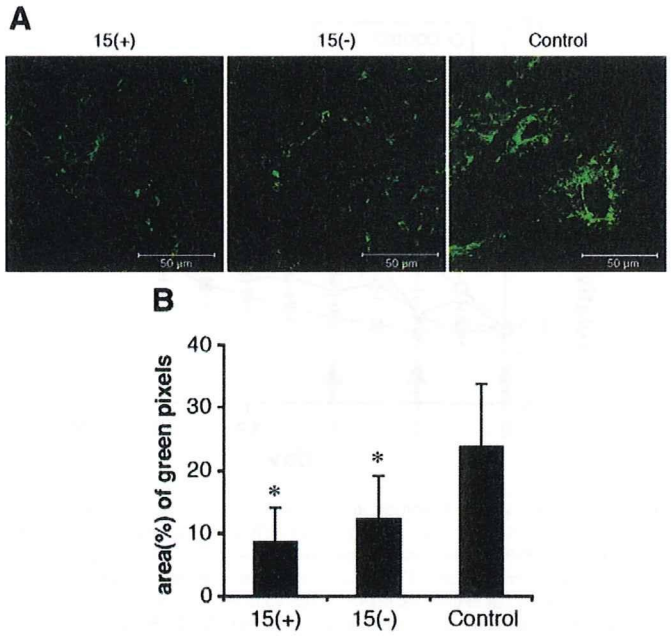

Fig. 6. Antiangiogenic effect of expressed sFlt-1 in BxPC3 tumor bearing mice. Tumor microvessels were detected by CD31 (PECAM1) antibody staining of tumor cryosections 20 days after therapy. (A) Representative CD31 immunostaining images. (B) Quantitative analysis of microvessel density in tumor cryosections. The results represent a percentage area of green pixels per image. Seven images were taken from each tumor tissue, from 3 mice, mean ± s.d. $^{*}P < 0.05$ compared to control group.

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Antiangiogenic Gene Therapy of Solid Tumor by Systemic Injection of Polyplex Micelles Loading Plasmid DNA Encoding Soluble Flt-1

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Abstract: In this study, a polyplex micelle was developed as a potential formulation for antiangiogenic gene therapy of subcutaneous pancreatic tumor model. Poly(ethylene glycol)-poly(L-lysine) block copolymers (PEG-PLys) with thiol groups in the side chain of the PLys segment were synthesized and applied for preparation of disulfide cross-linked polyplex micelles through ion complexation with plasmid DNA (pDNA) encoding the soluble form of vascular endothelial growth factor (VEGF) receptor-1 (sFlt-1), which is a potent antiangiogenic molecule. Antitumor activity and gene expression of polyplex micelles with various cross-linking rates were evaluated in mice bearing subcutaneously xenografted BxPC3 cell line, derived from human pancreatic adenocarcinoma, and polyplex micelles with optimal cross-linking rate achieved effective suppression of tumor growth. Significant gene expression of this micelle was detected selectively in tumor tissue, and its antiangiogenic effect was confirmed by decreased vascular density inside the tumor. Therefore, the disulfide cross-linked polyplex micelle loading sFlt-1 pDNA has a great potential for antiangiogenic therapy against subcutaneous pancreatic tumor model by systemic application.

Keywords: Polymeric micelle; block copolymer; antiangiogenic tumor gene therapy; sFlt-1

Introduction

Antiangiogenic tumor gene therapy is an intensively studied approach to inhibit tumor growth by destructing its neo-vasculature formation.[1,2] Vascular endothelial growth factor (VEGF) is a major proangiogenic molecule, which stimulates angiogenesis via promoting endothelial prolifera-

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tion, survival, and migration. The soluble form of VEGF receptor-1 (fms-like tyrosine kinase-1: Flt-1) is a potent endogeneous molecule, which can be used for antiangiogenic therapy.[3,4] The sFlt-1 binds to VEGF with the same affinity and equivalent specificity as that of the original receptor,[5] however it inhibits its signal transduction.

Gene therapy is becoming a promising strategy to supply consecutive expression of antiangiogenic proteins over a period of time. Indeed, a number of studies have already demonstrated the potential of therapeutic genes encoding angiogenic inhibitors to suppress tumor growth.[6,7] The major challenge in systemic gene therapy, however, is a need for a safe and effective vector system that can deliver the gene to the target tissue and cells with no detrimental side effects. In terms of safety, nonviral gene vectors are gaining popularity over viral vectors, however, their intracellular delivery and transfection potential require further optimization. Recently, several reports were published on *in vivo* nonviral gene therapy utilizing sFlt-1 for inhibition of tumor angiogenesis.[8,9]

Based on these criteria, cross-linked polyplex micelles were designed and prepared through electrostatic interaction of thiolated poly(ethylene glycol)-poly(L-lysine) (PEG-PLys) block copolymers and plasmid DNA (pDNA) encoding sFlt-1. We have previously reported that disulfide cross-links introduced into the polyplex micelle core contribute to the stabilization of its structure in the extracellular entity while facilitating smooth release of the entrapped pDNA, in response to the reductive environment, inside the cells.[10,11] The outer hydrophilic shell layer, formed by PEG segment, increases complex stability in serum, avoiding nonspecific interactions with plasma proteins and reduces polymer toxicity.[12]

In this study, cross-linked polyplex micelles were systemically administered to mice bearing subcutaneously xenografted BxPC3 human pancreatic adenocarcinoma and evaluated for their transfection efficiency. Note that BxPC3 xenografts, as some intractable solid tumors, are characterized by stroma-rich histology,[13] which limits access of therapeutic agents to tumor cells. Thus, the accessibility of endothelial cells by bloodstream makes an antiangiogenic approach an attractive strategy against this model. Here we report a potent tumor growth inhibitory effect achieved by effective antiangiogenic ability by the polyplex micelles with an optimal cross-linking degree, which enables the selective expression of loaded sFlt-1 gene in tumor tissue.

Experimental Section

Materials. pDNA for luciferase (Luc) with the pCAcc vector having the CAG promoter was provided by RIKEN Gene Bank (Tsukuba, Japan) and amplified in competent DH5α *Escherichia coli*, followed by purification using a NucleoBond Xtra Maxi (Machery-Nagel GmbH & Co. KG, Düren, Germany). Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were purchased from Sigma-Aldrich Co. (Madison, WI). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Alexa488- and Alexa647-conjugated secondary antibodies to rat IgG were obtained from Invitrogen Molecular Probes (Eugene, OR). Human soluble VEGF R1/

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Flt-1 immunoassay kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Gemcitabine was obtained from Eli Lilly and Company (Indianapolis, IN). Avastin was obtained from F. Hoffmann-La Roche, Ltd. (Basel, Switzerland). Synthesis of thiolated block copolymer, and construction and confirmation of pDNA encoding sFlt-1 are shown in the Supporting Information. A block copolymer with *X*% of thiolation degree was abbreviated as "B-SH*X*%".

Cell Lines and Animals. Human embryonic kidney 293T cells (from RIKEN CELL BANK, Tsukuba, Japan) and human pancreatic adenocarcinoma BxPC3 cells (from ATCC, Manassas, VA) were maintained in DMEM and RPMI medium, respectively, supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37 °C. 293T cells were chosen for *in vitro* experiments as cells that did not express sFlt-1.[14] Balb/c nude mice (female, 5 weeks old) were purchased from Charles River Laboratories (Tokyo, Japan). All animal experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals as stated by the National Institutes of Health.

Preparation of Polyplex Micelles. Each block copolymer was dissolved in 10 mM Tris-HCl buffer (pH 7.4), followed by the addition of 10-times-excess mol of dithiothreitol (DTT) against thiol groups. After 30 min incubation at room temperature, the polymer solution was added to a twice-excess volume of 225 μg/mL pDNA/10 mM Tris-HCl (pH 7.4) solution to form polyplex micelles with N/P ratio = 2. Note that N/P ratio was defined as the residual molar ratio of amino groups of thiolated PEG-PLys to phosphate groups of pDNA. The final pDNA concentration was adjusted to 150 μg/mL. After overnight incubation at room temperature, the polyplex micelle solution was dialyzed against 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 vol% DMSO at 37 °C for 24 h to remove the impurities, followed by 24 h of additional dialysis against 10 mM Tris-HCl buffer (pH 7.4) or 10 mM Hepes buffer (pH 7.4) to remove DMSO. During the dialysis, the thiol groups of thiolated block copolymers were oxidized to form disulfide cross-links. In the *in vivo* experiments, the polyplex micelle solution was adjusted to a concentration of 100 μg of pDNA/mL in 10 mM Hepes buffer (pH 7.4) with 150 mM NaCl.

Dynamic Light Scattering (DLS) Measurement. The size of the polyplex micelles was evaluated by DLS using Nano ZS (ZEN3600, Malvern Instruments, Ltd., U.K.). A He−Ne ion laser (633 nm) was used as the incident beam. Polyplex micelle solutions with N/P = 2 from 3 different batches were adjusted to a concentration of 33.3 μg of pDNA/mL in 10 mM Tris-HCl buffer (pH 7.4). The data obtained at a detection angle of 173° and a temperature of 37 °C were analyzed by a cumulant method to obtain the hydrodynamic diameters and polydispersity indices (μ/Γ^2) of micelles.

Zeta-Potential Measurement. The zeta-potential of polyplex micelles was evaluated by the laser-Doppler electrophoresis method using Nano ZS with a He−Ne ion laser (633 nm). Polyplex micelle solutions with N/P = 2 from 3 different batches were adjusted to a concentration of 33.3 μg pDNA/mL in 10 mM Tris-HCl buffer (pH 7.4). The zeta-potential measurements were carried out at 37 °C. A scattering angle of 173 °C was used in these measurements.

Real-Time Gene Expression. 293T cells (100,000 cells) were seeded on a 35 mm dish and incubated overnight. After replacement with fresh medium containing 0.1 mM D-luciferin, each type of polyplex micelle (N/P = 2) containing 3 μg of Luc pDNA was added. The dishes were set in a luminometer incorporated in a CO_2 incubator (AB-2550 Kronos Dio, ATTO, Tokyo, Japan), and the bioluminescence was monitored every 10 min with an exposure time of 1 min. Reproducibility was confirmed by triplicate experiments.

Antitumor Activity Assay. Balb/c nude mice were inoculated subcutaneously with BxPC3 cells (5×10^6 cells in 100 μL of PBS). Tumors were allowed to grow for 2−3 weeks to reach the proliferative phase (the size of the tumors at this point was approximately 60 mm^3). Subsequently, polyplex micelles (20 μg of pDNA/mouse), gemcitabine (100 mg/kg), or Avastin (50 mg/kg) maintained in 10 mM Hepes buffer (pH 7.4) with 150 mM NaCl were injected via the tail vein either 3 times (Figure 2a) or 5 times (Figure 2b) at 4-day intervals. Gemcitabine and Avastin doses and injection regimens were according to the previous reports published elsewhere.[15,16] A polyplex micelle containing Luc pDNA was used as a control formulation containing the nontherapeutic gene. Tumor size was measured every second day by a digital vernier caliper across its longest (a) and shortest diameters (b), and its volume (V) was calculated according to the formula $V = 0.5ab^2$.

***In Vivo* sFlt-1 Gene Expression.** Polyplex micelles loading either sFlt-1 or Luc pDNA (20 μg pDNA) were injected into the BxPC3-inoculated mice via the tail vein on days 0 and 4. Mice were sacrificed on day 6 after collecting blood, and the lungs, livers, spleens, kidneys, and tumors were excised. The excised organs were treated in 500 μL of cell culture lysis buffer (Promega, Madison, WI), homogenized, and centrifuged. The sFlt-1 concentration of supernatants was evaluated using the immunoassay kit according to the manufacturer's protocol. Note that block copolymers and polyplex micelles did not interfere with ELISA (Figure 2 in the Supporting Information).

Vascular Density in the Tumors. Polyplex micelles loading either sFlt-1 or Luc pDNA (20 μg of pDNA) and Avastin (50 mg/kg) were injected into the BxPC3-inoculated

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mice via the tail vein on days 0 and 4. Mice were sacrificed on day 6, and the tumors were excised, frozen in dry-iced acetone, and sectioned at 10 μm thickness in a cryostat. Vascular endothelial cells (VECs) were immunostained by rat monoclonal antibody antiplatelet endothelial cell adhesion molecule-1 (PECAM-1) (BD Pharmingen, Franklin Lakes, NJ) and Alexa488-conjugated secondary antibody. The samples were observed with a confocal laser scanning microscope (CLSM). The CLSM observation was performed using an LSM 510 (Carl Zeiss, Oberlochen, Germany) with an EC Plan-Neoflur 20× objective (Carl Zeiss) at the excitation wavelength of 488 nm (Ar laser). The PECAM-1-positive area (%) was calculated from Alexa488-positive pixels.

***In Vivo* EGFP Gene Expression in the Tumors.** Polyplex micelles loading EGFP pDNA (20 μg of pDNA) were injected into the BxPC3-inoculated mice via the tail vein. Mice were sacrificed on either day 3 or day 7. Tumors were excised, fixed with 10% formalin, frozen, and sectioned. VECs were immunostained by anti-PECAM-1 antibody and Alexa647-conjugated secondary antibody. After nuclear staining with Hoechst 33342, CLSM observation was carried out using the LSM 510 with the EC Plan-Neoflur 20× objective at the excitation wavelength of 488 nm for EGFP expression, 633 nm (He−Ne laser) for Alexa647, and 710 nm (MaiTai laser, two photon excitation; Spectra-Physics, Mountain View, CA) for Hoechst 33342, respectively. The representative images of tumors excised on day 3 are shown in Figure 5. Note that images of tumors excised on day 7 showed similar patterns to those on day 3, however with lower intensity of EGFP expression.

Results

Formation of Polyplex Micelles. No free pDNA was detected by agarose gel electrophoresis, confirming that all pDNA was entrapped in disulfide cross-linked polyplex micelles, which were prepared as previously reported through ion complexation of block copolymers with pDNA at the N/P ratio = 2. Free thiol groups in polyplex micelles were estimated to be less than 2% by Ellman's test (data not shown), which is consistent with our previous report.[10] Weight−weight % ratios of pDNA/micelle in each formulation were as follows: 32.8% in B-SH0% formulation; 31.0% in B-SH5%; 29.2% in B-SH11%; 26.4% in B-SH20%; and 21.0% in B-SH36%. The mean size of the micelles was between 100 and 150 nm, with a moderate polydispersity index between 0.17 and 0.2 (Figure 3 in the Supporting Information), while zeta-potential revealed approximately neutral values, confirming the formation of PEG palisade surrounding the polyplex core (Table 1).

Real-Time Gene Expression. *In vitro* real-time Luc gene expression of polyplex micelles was evaluated using Kronos

Table 1. Sizes and Zeta-Potentials of Polyplex Micelles with Various Cross-Linking Rates at N/P = 2[a]

thiolation degree (%)	cumulant diameter (nm)	polydispersity index (μ/l^{-2})	zeta-potential (mV)
0	107 ± 2	0.195 ± 0.021	1.66 ± 0.28
5	117 ± 2	0.184 ± 0.011	1.25 ± 0.40
11	116 ± 2	0.171 ± 0.013	1.02 ± 0.30
20	139 ± 6	0.182 ± 0.050	0.40 ± 0.07
36	147 ± 2	0.192 ± 0.061	−0.96 ± 0.02

[a] The results reported were expressed as mean ± SEM (n = 3).

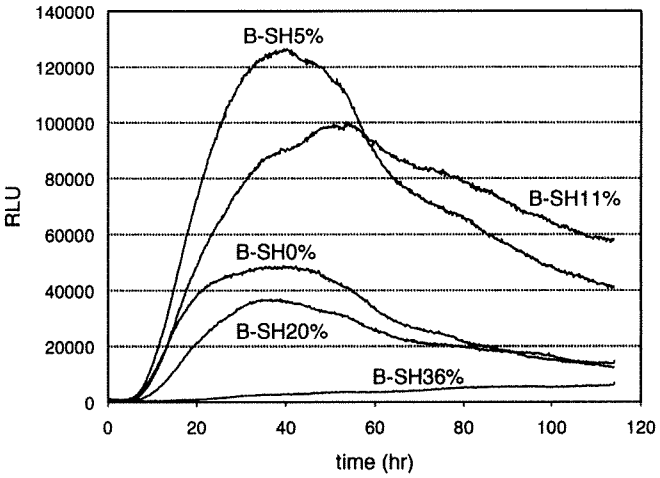

Figure 1. Real-time luciferase gene expression of the polyplex micelles with varying thiolation degrees at N/P = 2 against 293T cells.

Dio for a prolonged period (Figure 1).[17,18] The B-SH5% cross-linked polyplex micelle showed the highest gene expression among all micelles until 60 h. Worth mentioning is that the transfection efficiency of the B-SH11% micelle continued to exceed that of the B-SH5% micelle after 60 h. Disulfide cross-links in the polyplex core are believed to contribute not only to enhanced stability of the micelles in the medium but also to sustained release of complexed pDNA inside the cells with a reductive environment, resulting in polyplex micelles with higher cross-linking rates that can maintain an appreciable transfection efficiency over a longer time scale. Note that the B-SH36% micelle showed an increasing trend in gene expression with time.

Antitumor Activity. Polyplex micelles containing sFlt-1 pDNA were injected iv into mice bearing pancreatic adenocarcinoma BxPC3, followed by evaluation of tumor volume (Figure 2). All the micelles were injected three times on days

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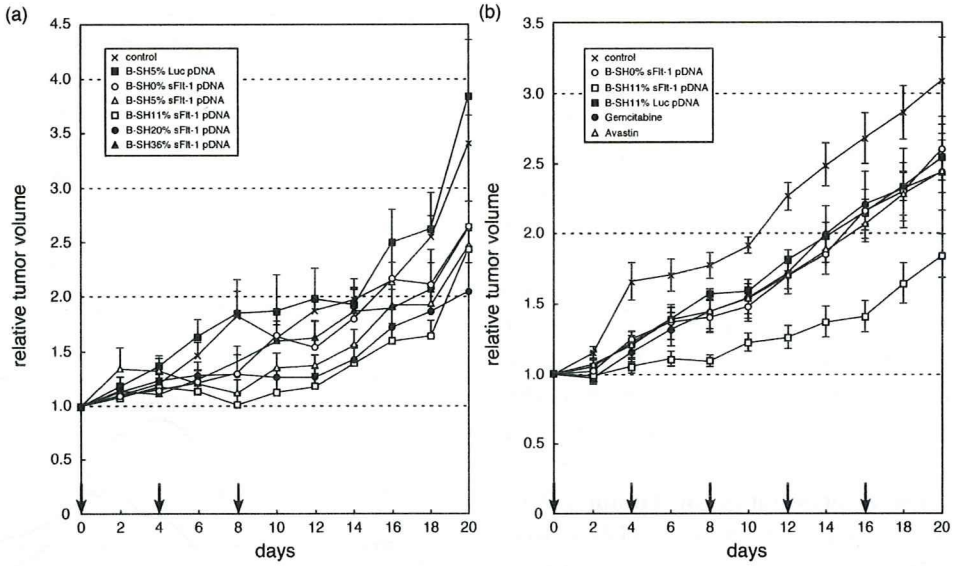

Figure 2. Antitumor activity of polyplex micelles with sFlt-1 pDNA in subcutaneously BxPC3-inoculated mice. (a) Effect of thiolation degree. Hepes buffer (control) was used as a negative control. Polyplex micelles were injected iv on days 0, 4, and 8 at 20 μg pDNA/mouse, and mice were monitored for the relative tumor volume every second day. Error bars represent the SEM ($n = 6$). Only the B-SH11% polyplex micelles exhibited significant retardation of tumor growth compared to the control ($P < 0.01$). (b) Growth curve study with an increased dose of the B-SH11% polyplex micelles compared to commercially available drugs. Polyplex micelles (20 μg pDNA/mouse), gemcitabine (100 mg/kg), and Avastin (50 mg/kg) were injected iv on days 0, 4, 8, 12, and 16. Relative tumor size was measured every second day. Hepes buffer (control) was used as a negative control. Error bars represent the SEM ($n = 5$). Only the B-SH11% polyplex micelles exhibited significant retardation of tumor growth compared to the control ($P < 0.001$). P values were calculated by multivariate ANOVA study.

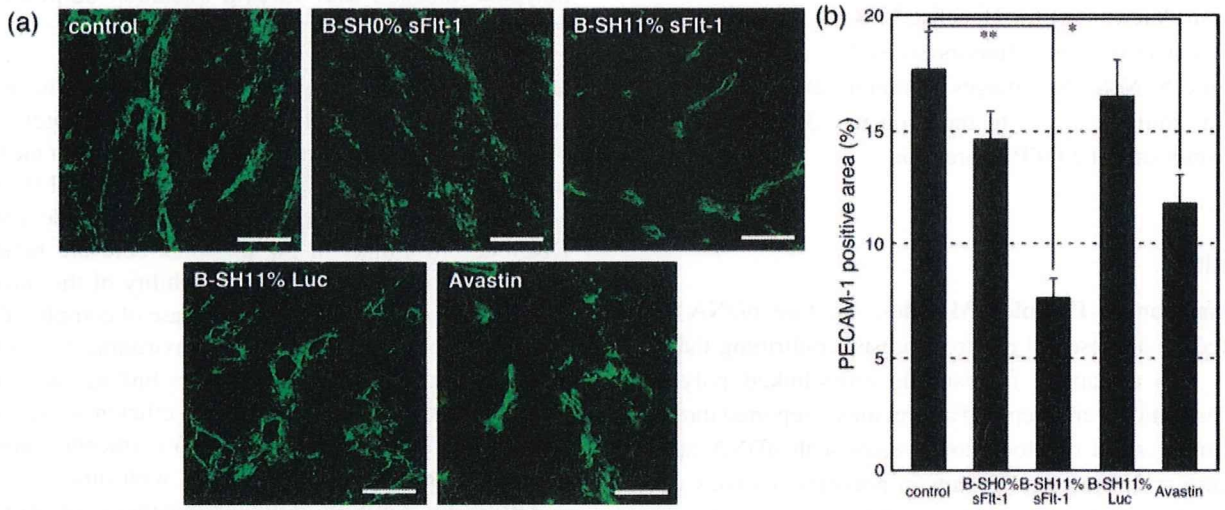

Figure 3. Immunostaining of the VECs in the BxPC3 tumor tissue by PECAM-1 antibody. Hepes buffer (control), three types of polyplex micelles (20 μg of pDNA/mouse), and Avastin (50 mg/kg) were injected into the BxPC3-inoculated mice via the tail vein on days 0 and 4. Mice were sacrificed on day 6, and tumors were excised and immunostained. (a) CLSM images of immunostained tumors. PECAM-1-positive regions are green. Bars represent 100 μm. (b) Areas of PECM-1-positive endothelium were quantified. Error bars represent the SEM ($n = 15$). P values were calculated by Student's t test. $^{*}P < 0.01$ and $^{**}P < 0.001$.

0, 4, and 8 (Figure 2a). The B-SH11% micelle significantly suppressed tumor growth compared to control mice treated with Hepes buffer ($P < 0.01$). There was no significant change in tumor growth after injection of other polyplex micelles, implying that an optimal cross-linking rate is required to achieve an effective expression of the gene. Encouraged by these results, the tumor growth suppression activity of B-SH11% micelle was further evaluated, implying a regimen with enhanced number of injections. The effect of the micelles was compared to commercially available drugs, gemcitabine, a standard chemotherapeutic agent for pancreatic tumor, and bevacizumab (Avastin), a monoclonal antibody against VEGF (Figure 2b). The doses of gemcitabine and Avastin implied in our study were based on

previous reports published elsewhere.[15,16] The administration of B-SH11%/sFlt-1 micelle resulted in significant suppression of tumor growth ($P < 0.001$), while gemcitabine and Avastin, under the reported experimental regimen, showed no remarkable therapeutic effect. Note that the difference observed in tumor volumes between the B-SH11%/Luc micelle-treated group and the control group was not significant.

Tumor Vascular Density. The antiangiogenic effect of expressed sFlt-1 was confirmed by immunostaining of VECs using PECAM-1 (Figure 3). Vascular density of tumors treated with either B-SH11%/sFlt-1 micelle or Avastin was significantly lower than that of the other groups. The most pronounced and significant effect on neo-vasculature suppression was achieved by B-SH11%/sFlt-1 micelle (7% PECAM-1 positive area) over Avastin (12% PECAM-1 positive area) ($P < 0.05$). These results suggest that the expressed sFlt-1 may entrap VEGF secreted in the tumor tissue, thereby suppressing the growth of VECs.

***In Vivo* sFlt-1 Gene Expression.** Expression levels of sFlt-1 in the body were then evaluated by measuring the amount of sFlt-1 in lung, liver, spleen, kidney, tumor, and blood plasma using enzyme-linked immunosorbent assay (ELISA) (Figure 4). Injection of B-SH11%/sFlt-1 micelle resulted in significantly higher expression of sFlt-1 selectively in tumor tissue compared to the control. On the other hand, injection of B-SH0%/sFlt-1 micelle or B-SH11%/Luc micelle did not result in any difference in sFlt-1 expression compared to the control. These results strongly support that tumor-specific elevation in sFlt-1 expression led to the significant growth suppression of VECs in the tumor tissue and, eventually, the suppression of tumor growth.

***In Vivo* Enhanced Green Fluorescence Protein (EGFP) Gene Expression in Tumors.** The location of gene expression in BxPC3 tumors after administration of the micelles was analyzed histologically using pDNA encoding EGFP (Figure 5). As previously reported,[13,19,20] thick fibrotic tissue was formed around blood vessels (red) inside the stroma of BxPC3 tumors, and nests of tumor cells (region T) were scattered in the stroma (Figure 5a). The expression of EGFP (Figures 5b and 5c) was observed mainly in the VECs and cells in stromal regions adjacent to some vasculature, indicating that VECs and fibroblasts near some vasculature in the stroma, but not the tumor cells, were transfected. As seen in Figure 5a, there were thick fibrotic tissues around blood vessels in the BxPC3 xenograft,

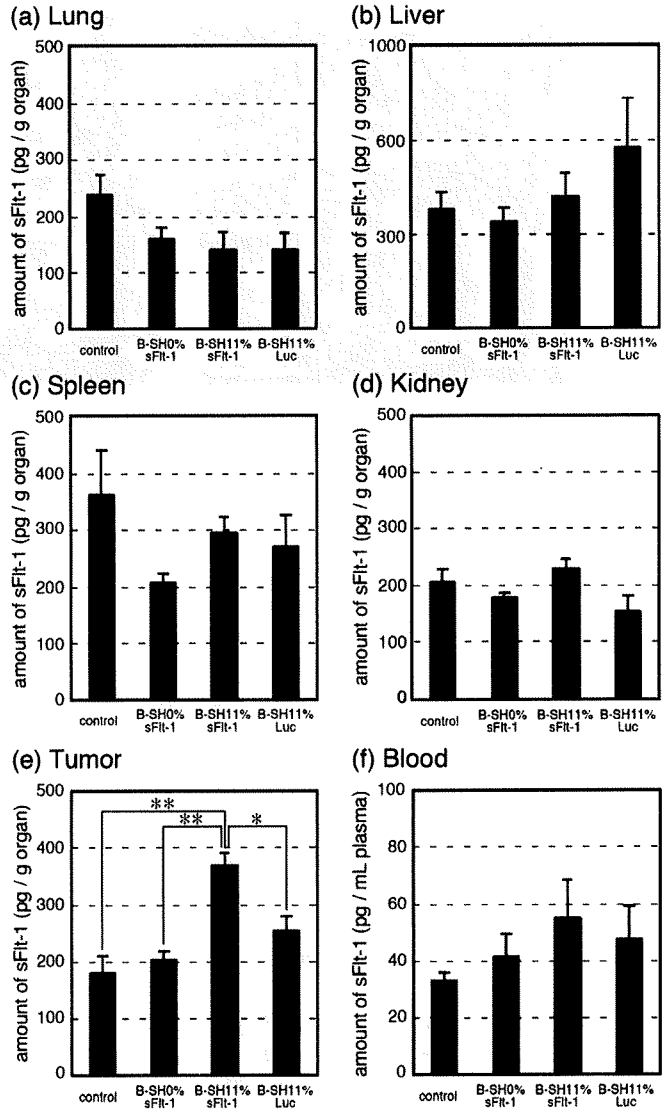

Figure 4. Evaluation of sFlt-1 gene expression in organs by ELISA. Hepes buffer (control) and three types of polyplex micelles (20 μg pDNA/mouse) were injected into the BxPC3-inoculated mice via the tail vein on days 0 and 4. Mice were sacrificed on day 6 after collecting blood (f), and the lungs (a), livers (b), spleens (c), kidneys (d), and tumors (e) were excised, followed by evaluation of sFlt-1 concentration by ELISA according to the manufacturer's protocol. Error bars represent the SEM ($n = 6$). P values were calculated by Student's t test. $^{*}P < 0.01$ and $^{**}P < 0.001$.

indicating that the penetration of polyplex micelles deep into the stroma or into the tumor nest was interrupted and the gene expression was limited in the VECs and some of the fibroblasts in the stroma. Higher levels of EGFP expression were observed for B-SH11% micelle, confirming their enhanced ability to accumulate inside tumor tissue compared to B-SH0% micelle.

Discussion

Since all solid tumors need angiogenesis for their growth, antiangiogenic therapy is a promising strategy for treating

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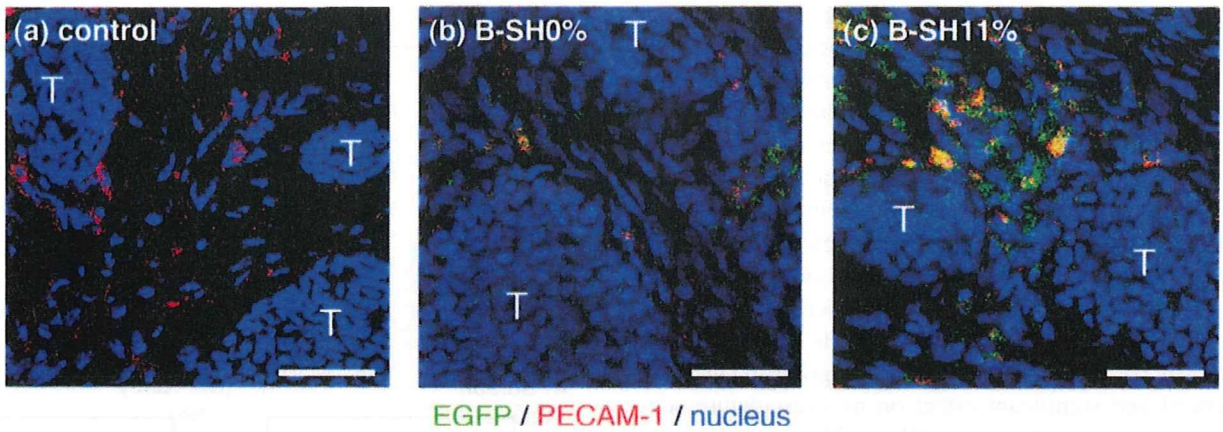

Figure 5. EGFP gene expression by polyplex micelles in the inoculated BxPC3 tumors. Hepes buffer (a) was used as a negative control. B-SH0% (b) and B-SH11% (c) polyplex micelles containing EGFP pDNA (20 μg pDNA/mouse) were injected into the BxPC3-inoculated mice via the tail vein. Mice were sacrificed on day 3, and tumors were excised and immunostained. "T" indicates nests of tumor cells in tumor tissues. Bars represent 50 μm.

tumor patients. In fact, Avastin, the recombinant humanized monoclonal antibody against VEGF, has been widely used as an antiangiogenic drug, and its application range is spreading to the various types of solid tumors.[16] Other antiangiogenic proteins,[21,22] e.g., angiostatin, endostatin, and soluble forms of VEGF receptor, have also received great attention. Meanwhile, antiangiogenic gene therapy represents an attractive alternative to antiangiogenic proteins for reasons such as low dose, continuous expression of the therapeutic protein, and low cost. Therefore, development of an effective and safe gene vector is a key to successful antiangiogenic gene therapy.

In this study, thiolated PEG-PLys block copolymers were applied in the formation of disulfide cross-linked polyplex micelles for delivery of pDNA encoding sFlt-1, and tested for their antiangiogenic effect on mice bearing xenografted BxPC3 cell line, derived from human pancreatic adenocarcinoma. Disulfide cross-links in the polyplex core were designed to increase blood stability of the polyplex micelles and effectively release pDNA in the intracellular milieu.[10,11,18] PEG palisade of the polyplex micelle is expected to cover the polyplex core to shield the positive charge as well as to decrease interfacial free energy.[12,23] The formation of the PEG palisade surrounding the polyplex core was confirmed by the neutral zeta-potential of the polyplex micelles (Table 1). B-SH36% micelle showed an approximately 10 times higher concentration of pDNA in the blood at 60 min after iv injection than that of the micelle without core cross-linking (B-SH0%) (Figure 4 in the Supporting Information). The disulfide cross-links in the polyplex core apparently contribute to the enhanced stability of the micelles in the bloodstream. Note that the size of polyplex micelles is between 100 and 150 nm (Table 1), which may be in a suitable range for accumulation in solid tumors due to the enhanced permeability and retention (EPR) effect,[24] although the size may be too large to allow the micelles to penetrate into the stroma in pancreatic tumors.[13] Nevertheless, there is a concern that excessive disulfide cross-links interfere with the smooth release of entrapped pDNA in the core, resulting in decreased transfection efficiency.[10] Accordingly, optimal cross-linking density should be determined to balance the stability and maintain high transfection efficiency. The results of *in vitro* real-time gene expression showed that B-SH5% micelle possessed the highest efficiency among the evaluated samples up to 60 h after transfection. It is noteworthy that B-SH11% micelle exerted sustained Luc expression and kept an appreciably high efficiency beyond 60 h (Figure 1). Apparently, gene expression is prolonged with an increase in cross-linking rates, although excess cross-links induced overstabilization of polyplex micelles, resulting in decreased transfection efficiency in the case of the B-SH20% and B-SH36% micelles. Eventually, the B-SH36%/sFlt-1 micelle had no *in vivo* efficiency, even though they showed the highest stability in the bloodstream among the evaluated samples (Figure 4 in the Supporting Information). It is also noteworthy that the B-SH11%/sFlt-1 micelle achieved an appreciably high therapeutic efficiency, even though it showed only limited improvement in blood circulation time compared to the B-SH0% and B-SH5% systems. Presumably, a sustained

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profile in gene expression may have been the key to this achievement. Note that no change in body weight of the mice was observed during the experiment (data not shown), indicating few serious side effects of polyplex micelles.

Comparison with the commercially available agents, gemcitabine and Avastin, confirmed the encouraging tumor growth suppression effect of the B-SH11% polyplex micelle (Figure 2b). Gemcitabine continues to be the standard therapy in the treatment of pancreatic tumors; however, its objective response rate is limited in patients with advanced disease.[25] Avastin is a recombinant humanized monoclonal antibody against human VEGF, which may neutralize tumor-cell-derived VEGF in the model used here. In humans, Avastin is the first clinically available antiangiogenic drug, and it has been efficient when used in combined chemotherapy for metastatic colorectal cancer[26] and non-small-cell lung cancer.[27] However, it showed no benefit in patients with pancreatic tumors.[25] The B-SH11%/sFlt-1 micelle significantly suppressed tumor growth compared not only to the control ($P < 0.001$) but also to the B-SH11%/Luc micelle, gemcitabine, and Avastin ($P < 0.01$) (Figure 2b). Xenografted BxPC3 was reported not to respond to gemcitabine,[28] probably due to its inability to penetrate through the tumor thick fibrotic tissue and target tumor cells, which is consistent with our results. Evaluation of vascular density in BxPC3 tumor (Figure 3) clearly showed that the B-SH11%/sFlt-1 micelle decreased vascular density compared to the control ($P < 0.001$), the B-SH11%/Luc micelle ($P < 0.001$), and Avastin ($P < 0.05$) treated tumors.

Inhibitory effect on tumor growth (Figure 2) is consistent with the result of decreased vascular density. There are several studies on antiangiogenic gene therapy for subcutaneously inoculated tumors in mice by systemic expression of sFlt-1 using viral vectors, including im injection of adeno-associated viral vectors[29] and iv injection of adenoviral vectors to target livers.[30] In these studies, however, sFlt-1 was expressed mainly in organs rather than tumor tissue. What was worse, the excess expression of sFlt-1 in the liver led to unacceptable hepatotoxicity.[31] Thus, tumor-specific expression of sFlt-1 is essential for a safe and efficient antiangiogenic gene therapy. However, any nonviral gene vectors loading sFlt-1 gene have failed to exhibit selective gene expression in the tumor tissue, although they achieved certain inhibition of tumor growth.[8,9] In this regard, the B-SH11%/sFlt-1 micelle system might be promising, since sFlt-1 expression was significantly increased selectively in the tumor tissue compared not only to the control ($P < 0.001$) but also to the B-SH11%/Luc micelle ($P < 0.01$), as shown in Figure 4, without any significantly enhanced expression in other normal tissues. Note that no significant increase of sFlt-1 expression was observed in any normal organs treated with B-SH0%/sFlt-1 micelle or B-SH11%/Luc micelle. Histological analyses revealed that EGFP expression of the B-SH11%/EGFP micelle was located mainly around VECs but not in the tumor cells (Figure 5), probably due to restricted permeation of micelles by thick fibrotic tissues and pericyte-covered vasculature of the BxPC3 tumors. These results suggested the ability of expressed sFlt-1 molecule to entrap excess VEGF in the tumor tissue and to inhibit tumor growth by an antiangiogenic effect. Xenografted BxPC3 tumors in mice are characterized by stroma-rich histology,[20] which might explain the only slight inhibitory effects on BxPC3 growth achieved by gemecitabine[28] targeting tumor cells.

Conclusions

In conclusion, antiangiogenic gene therapeutic study was carried out by iv administration of polyplex micelles with sFlt-1 pDNA to mice bearing pancreatic adenocarcinoma BxPC3 xenografts, and the results demonstrated the ability of B-SH11% sFlt-1 micelle as a safe and effective gene delivery system. The optimal disulfide cross-linking rate of polyplex micelles was found to show significant suppression of tumor growth. Gene expression of sFlt-1 by iv injection of polyplex micelles was observed in tumor tissue only, followed by decreased vascular density and significant suppression of tumor growth. Based on these results, the B-SH11% disulfide cross-linked polyplex

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micelle with sFlt-1 pDNA is interesting and worthy to develop further for antiangiogenic gene therapy of solid tumors.

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Supporting Information Available: Synthesis of thiolated block copolymer and Supporting Figures 1, 2, 3, and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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