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

Makoto Oba,[†] Yelena Vachutinsky,[‡] Kanjiro Miyata,[§] Mitsunobu R. Kano,[||,⊥] Sorato Ikeda,[#] Nobuhiro Nishiyama,[*,§] Keiji Itaka,[§] Kohei Miyazono,[||,⊥] Hiroyuki Koyama,[†] and Kazunori Kataoka[*,‡,§,||,#]

Department of Clinical Vascular Regeneration, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan, Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-0033, Japan, Center for NanoBio Integration, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan, Department of Molecular Pathology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan, and Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan

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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-

* To whom correspondence should be addressed. K.K.: tel, +81-3-5841-7138; fax, +81-3-5841-7139; e-mail, kataoka@bmw.t.u-tokyo.ac.jp; The University of Tokyo, Department of Materials Engineering, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan. N.N.: tel, +81-3-5841-1430; fax, +81-5841-7139; e-mail, nishiyama@bmw.t.u-tokyo.ac.jp.

† Department of Clinical Vascular Regeneration, Graduate School of Medicine.

‡ Department of Bioengineering, Graduate School of Engineering.

§ Center for Disease Biology and Integrative Medicine, Graduate School of Medicine.

|| Center for NanoBio Integration.

⊥ Department of Molecular Pathology, Graduate School of Medicine.

Department of Materials Engineering, Graduate School of Engineering.

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 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

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/Γ^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).

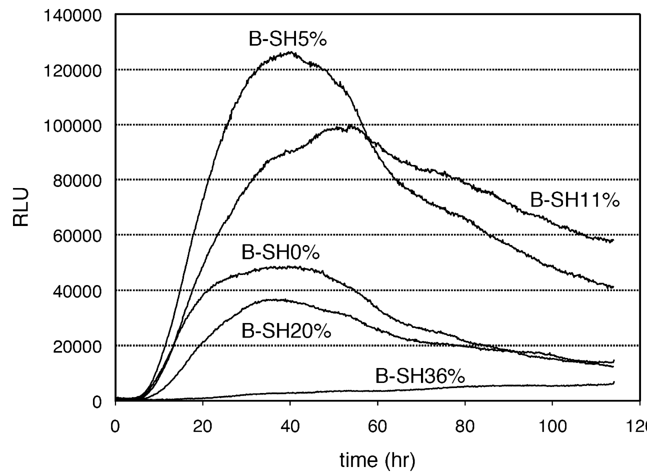

Figure 1. Real-time luciferase gene expression of the polyplex micelles with varying thiolation degrees at N/P = 2 against 293T cells.

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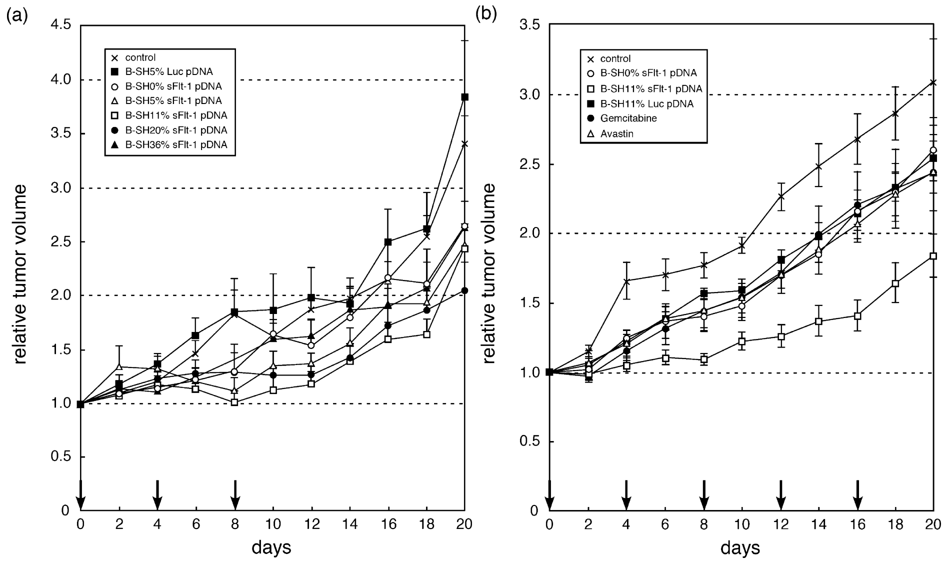

Figure 2. Antitumor activity of polyplex micelles with sFlt-1 pDNA in subcutaneously BxPC3-inoculated mice. (a) Effect of thiolation degree. Hepes buffer (control) was used as a negative control. Polyplex micelles were injected iv on days 0, 4, and 8 at 20 μg pDNA/mouse, and mice were monitored for the 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.

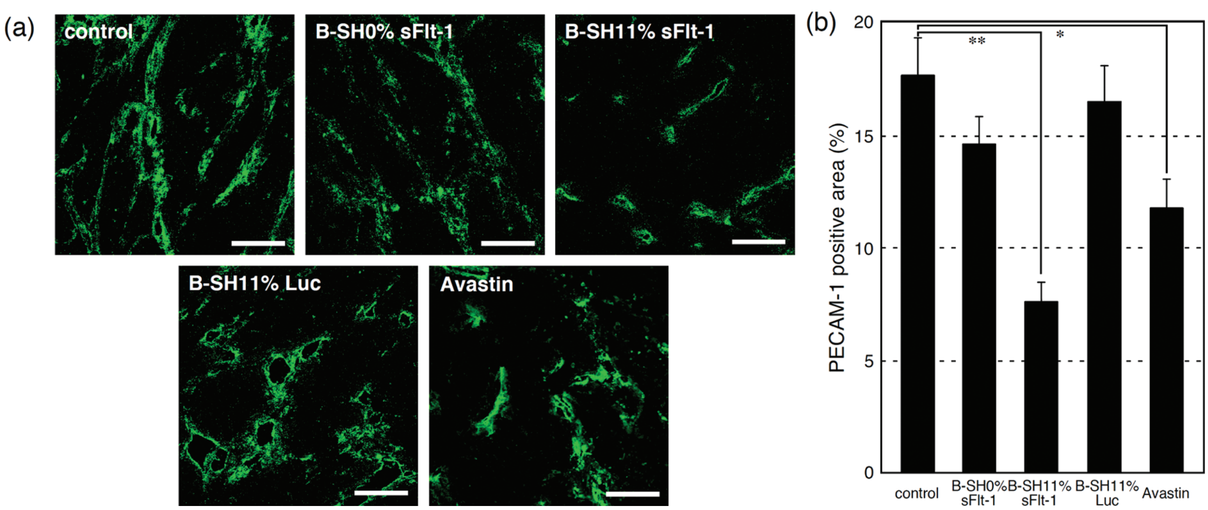

Figure 3. Immunostaining of the VECs in the BxPC3 tumor tissue by PECAM-1 antibody. Hepes buffer (control), three types of polyplex micelles (20 μg of pDNA/mouse), and Avastin (50 mg/kg) were injected into the BxPC3-inoculated mice via the tail vein on days 0 and 4. Mice were sacrificed on day 6, and 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, 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.

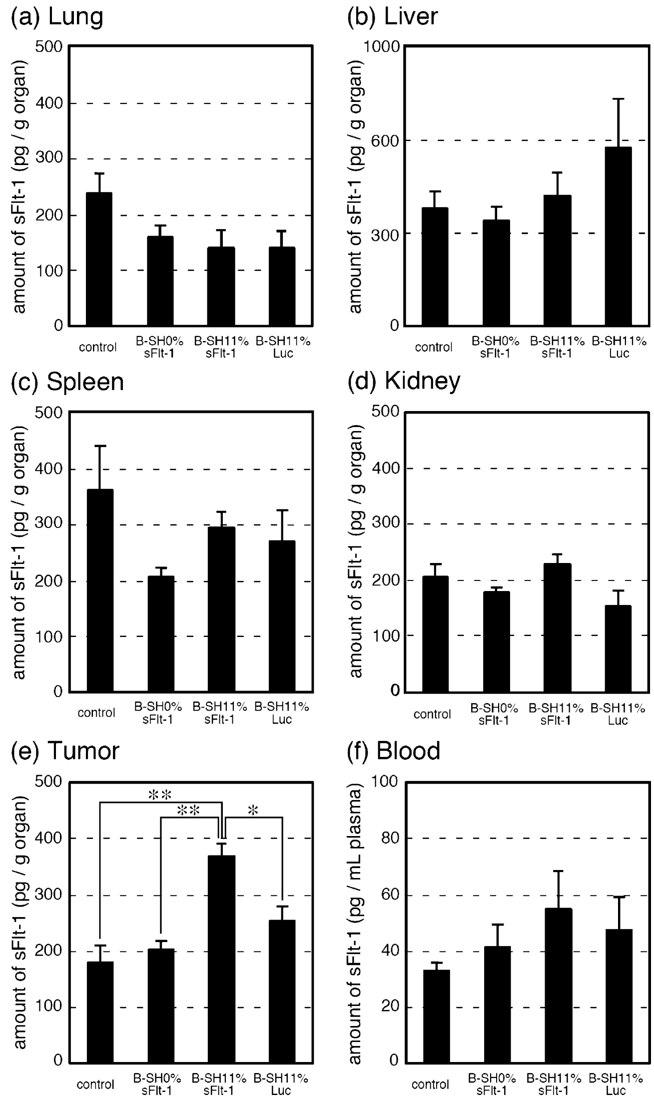

Figure 4. Evaluation of sFlt-1 gene expression in organs by ELISA. Hepes buffer (control) and three types of polyplex micelles (20 μg pDNA/mouse) were injected into the BxPC3-inoculated mice via the tail vein on days 0 and 4. Mice were sacrificed on day 6 after collecting blood (f), and the lungs (a), 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 **$P < 0.001$.

Discussion

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

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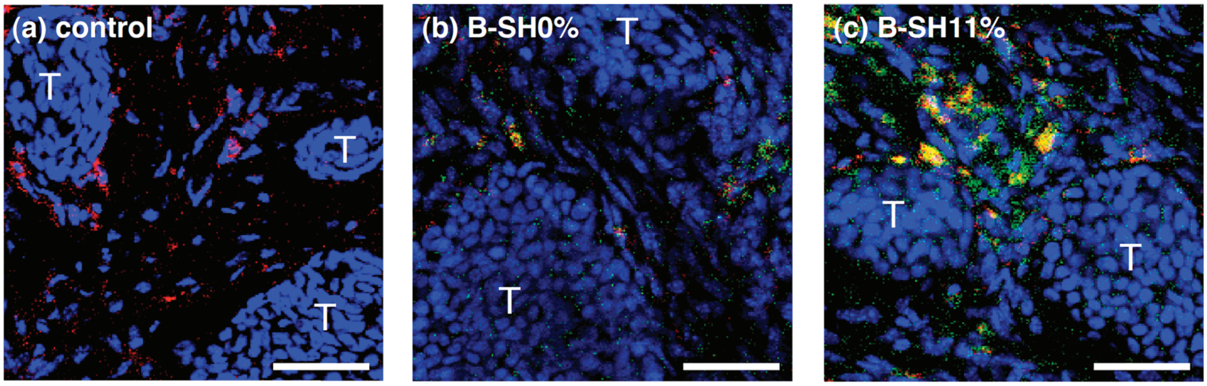

EGFP / PECAM-1 / nucleus

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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