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Target Specific Intracellular Delivery of siRNA/PEI−HA Complex by Receptor Mediated Endocytosis

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Abstract: Hyaluronic acid (HA) plays important biological roles in tissue integrity, angiogenesis, wound healing, and cell motility through the interaction with receptors on cell membranes. In this work, we investigated the effect of HA modification on the receptor-mediated endocytosis labeling HA derivatives with quantum dots (QDots). HA−QDot conjugates with a degree of modification less than *ca*. 25 mol % appeared to be more efficiently taken up to B16F1 cells by HA receptor mediated endocytosis than QDots alone. On the basis of bioimaging study, polyethyleneimine, PEI−HA conjugate with 24.2 mol % PEI content was developed as a target specific intracellular delivery carrier of siRNA. The siRNA/PEI−HA complex exhibited higher gene silencing efficiency in B16F1 cells with HA receptors than siRNA/PEI complex. Anti-PGL3-Luc siRNA/PEI−HA complex appeared to silence PGL3-Luc gene in the range of 50%−85% depending on the serum concentration up to 50 vol %. According to *in vivo* biodistribution test, siRNA/PEI−HA complex accumulated mainly in the tissues with HA receptors such as liver, kidney, and tumor. Furthermore, intratumoral injection of anti-VEGF siRNA/PEI−HA complex resulted in an effective inhibition of tumor growth by the HA receptor mediated endocytosis to tumor cells in C57BL/6 mice. Considering all these results, anti-VEGF siRNA/PEI−HA complex was thought to be applied successfully as target specific antiangiogenic therapeutics for the treatment of diseases in the tissues with HA receptors, such as liver cancer and kidney cancer.

Keywords: Target delivery; receptor mediated endocytosis; hyaluronic acid; quantum dot; siRNA; polyethyleneimine

Introduction

A small interfering RNA (siRNA) is a short double-stranded RNA which contains 21−23 nucleic acids with 19 duplexed region.[1,2] It shows a specific and effective gene silencing activity by the sequence-specific down-regulation of a complementary mRNA.[3,4] There have been many reports to use siRNA as potential therapeutics for the treatment of

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cancer and diseases from genetic disorder or viral infection.[5,6] However, the efficiency of gene silencing by siRNA is known to be very low because of its rapid enzymatic degradation and poor cellular uptake in the body.[7−9] Accordingly, a variety of cationic polymers, which can electrostatically bind to siRNA forming a condensed complex nanoparticle, have been explored to facilitate the intracellular delivery as well as to enhance the stability from enzymatic attack.[10,11] Among cationic polymers, polyethyleneimine (PEI) has been widely used to condense DNA plasmid, siRNA and antisense oligodeoxynucleotide (ODN).[12−14] The positive charge of PEI helps to form a complex with siRNA, and its proton sponge effect makes possible the endosomal escape of siRNA/PEI complex. However, the inherent cytotoxicity of PEI limited its applications for *in vivo* gene therapy.[15] Furthermore, siRNA/PEI complex was reported to cause the activation of complements,[16] the coagulation of blood cells,[17] and the self-aggregation with serum proteins,[18] due to the positively charged surface characteristics. In order to alleviate these problems, various kinds of polyanions have been used to coat siRNA/PEI complex without disrupting its structure.[17,18] We have chosen hyaluronic acid (HA) for the conjugation with PEI to decrease cytotoxicity, enhance serum stability and facilitate cellular uptake of siRNA/PEI−HA complex by HA receptor mediated endocytosis maintaining the endosomal escape capacity.

HA is the only non-sulfated glycosaminoglycan (GAG) that is abundant in synovial fluid and extracellular matrix (ECM).[19] It is an anionic biopolymer composed of alternating disaccharide unit of D-glucuronic acid and *N*-acetyl-D-glucosamine with $\beta(1\rightarrow4)$ interglycosidic linkage. HA plays pivotal roles in wound healing, cell motility, angiogenesis and construction of ECM. HA has different physiological functions in the body depending on its molecular weight (MW) in the range of 10^3-10^7 Da.[20] HA and its degraded products induce receptor-mediated intracellular signaling.[21] There have been many reports on HA receptors which play important biological roles such as endocytosis, degradation and signal transduction. Cluster determinant 44 (CD44),[22] receptor for hyaluronate-mediated motility (RHAMM),[7] and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)[23] have been identified as HA receptors for various biological functions. Because the carboxyl groups of HA are known to be the recognition sites for HA receptors and hyaluronidase,[24] the chemical modification of HA−COOH

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would change its biological behaviors in the body. For example, Zhong et al. reported that enzymatic degradation of HA derivatives was delayed with increasing degree of HA modification.[25]

In this work, bioimaging of HA derivatives was carried out using quantum dots (QDots) to investigate the effect of HA modification on its interaction with HA receptors. As a model system, adipic acid dihydrazide modified HA (HA−ADH) was synthesized and labeled with QDots. Previously, we have reported the synthesis, morphological analysis, and *in vivo* applications of HA−QDot conjugates to investigate the real-time distribution in the body.[26,27] QDots are semiconductor nanocrystals resisting to photobleaching with a size-tunable photoluminescence, and a broad absorption and sharp emission spectrum.[28] On the basis of bioimaging study of HA derivatives, we decided to develop HA−PEI conjugate as a target specific intracellular delivery carrier of siRNA. The binding affinity of HA, mostly in the outer layer of siRNA/PEI−HA complex, to HA receptors was thought to facilitate its intracellular uptake. The anionic property of HA might prevent siRNA/PEI−HA complex from interacting with blood serum components contributing for the serum stability. HA and modified HA have been extensively investigated and used for various medical applications.[29−31] After *in vitro* gene silencing test with anti-PGL3-Luc and antivascular endothelial growth factor (VEGF) siRNA/PEI−HA complexes, *in vivo* tumor treatment using anti-VEGF siRNA/PEI−HA complex was carried out with the analysis of biodistribution in tumor-bearing C57BL/6 mice.

Experimental Section

Materials. Sodium hyaluronate with an average molecular weight of 132,300 Da was obtained from Lifecore Co. (Chaska, MN). Adipic acid dihydrazide (ADH), branched polyethyleneimine (bPEI, 25,000 Da), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS sodium salt), 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC hydrochloride), agarose gel, sodium tetraborate decahydrate, boric acid, phosphate buffered saline tablets, methanol, paraformaldehyde, poly-D-lysine, and protease inhibitor were purchased from Sigma-Aldrich (Milwaukee, WI), and 2-mercaptoethanol and 1-hydroxybenzotriazole monohydrate (HOBt) from Daejung Chemicals and Metals Co. (Shiheung, Korea). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were obtained from Wako Pure Chemical Industries (Osaka, Japan), and dimethyl sulfoxide (DMSO) from Junsei Chemical Co. (Tokyo, Japan). Quantum dots (QDots) with an emission wavelength of 650 nm were obtained from Invitrogen Co. (Carlsbad, CA). They have carboxyl terminal ligands to be used for the conjugation with various biomolecules. Anti-PGL3-Luc siRNA with and without Cy3 labeling, anti-VEGF siRNA (siVEGF), and scrambled siVEGF (scVEGF) were purchased from Bioneer Co. (Daejeon, Korea). The sequences of anti-PGL3-Luc siRNA were 5′-UUGUUUUGGAGCGAAAdTdT-3′ (sense) and 5′-UUUCCUUCC AAAACAAdTdT-3′ (antisense). The siVEGF has the sequences of 5′-AUGUGAAUGCAGA CCAAAGAATT-3′ (sense) and 3′-TTUAACACUUACGUCUGGUUUCUU-5′ (antisense). The sequences of scVEGF were 5′-GAUAGCAAUGACAAUGCGUATT-3′ (sense) and 3′-TTCUAUCGUUACUGCUUACGCA-5′ (antisense). B16F1 cells were obtained from Korea Cell Line Bank (Seoul, Korea). Cell culture plates with 96 wells were purchased from Corning Co. (Corning, NY). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotics (penicillin) were purchased from JBI Co. (Seoul, Korea). PGL3-Luc (PGL3 Luciferase) plasmid, lysis solution and luciferase assay reagents were obtained from Promega Co. (Madison, WI), and Lipofectamine and ELISA kit from Invitrogen Co. (Carlsbad, CA).

Synthesis of HA−QDot Conjugate. HA−ADH was synthesized as reported elsewhere.[32,33] The degree of HA modification with ADH was analyzed with ^{1}H NMR (DPX300, Bruker, Germany). The carboxyl terminal ligands of QDots were activated with EDC and reacted with sulfo-NHS. Then, HA−ADH was conjugated with the activated QDots by the reaction between amine group of HA−ADH and sulfo-NHS of QDots.[26] The following are detailed descriptions for the preparation of HA−QDot conjugates. HA−ADH (10 nmol) was dissolved in 1.5 mL of phosphate buffer (0.1 M, pH = 6.4). QDots (25 μL, *ca*. 0.2 nmol) were mixed with EDC hydrochloride (0.077 mg, 400 nmol) and sulfo-NHS (0.21 mg, 1000 nmol) each dissolved in 10 μL

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of the same buffer. The solution was stirred for 20 min to modify the carboxyl ligands of QDots with sulfo-NHS. Then, 1.4 μL of 2-mercaptoethanol was added to quench the remaining EDC. After mixing for 2 min, the activated QDot solution was mixed with 1.5 mL of the HA−ADH solution for the conjugation reaction for 2 h. The final solution was buffer-changed with PD-10 column and filtered through 0.2 μm PVDF syringe filter. No large aggregates were found during the filtration. There was no difference in the emission extent and the color between QDots and synthesized HA−QDot conjugates after excitation with UV at 365 nm. The resulting HA−QDot conjugates were stored in a refrigerator before use.

Agarose Gel Electrophoresis. After conjugation of HA−ADH with QDots, aliquots of QDots and the resulting products were analyzed by agarose gel electrophoresis to confirm the successful formation of HA−QDot conjugates. Agarose gel was dissolved in borate buffer at a concentration of 1.0 wt % and used as an electrophoresis matrix. Each 4 μL of aliquot was mixed with 2 μL of dye and loaded in the well of agarose gel, which was applied to 50 V electrodes in 50 mM borate buffer (pH 8.98) for 50 min. FITC labeled HA−ADH and a mixture of FITC labeled HA−ADH and QDots were also loaded as controls. The resulting fluorescence was analyzed using a gel document system (Gelmanager, CoreBioSystem, Seoul, Korea).

Intracellular Uptake of HA−QDot Conjugate. B16F1 cells were incubated at 37 °C and 5% CO_2 in DMEM with 10% FBS and 1% antibiotics. Culture slides were coated with poly-D-lysine to improve the cellular attachment. Poly-D-lysine solution (100 μL of 0.1 mg/mL) was dropped on the wells of culture slides, kept at a CO_2 incubator for 1 h, washed with sterilized distilled water, and dried in a clean bench (JeioTech, Seoul, Korea). Cells were seeded on the poly-D-lysine coated culture slide at a density of 3×10^4 cells/well and incubated in the medium at 37 °C and 5% CO_2 for 3 days. Then, culture medium was replaced with DMEM containing 1% FBS. After incubation with and without free HA (*ca.* 7.5 μM) for an hour, HA−QDot conjugates in 100 μL DMEM were added to the wells of culture slides. The final concentration of HA−QDot conjugates was 5 nM. The cells were incubated for the predetermined time, washed with PBS, fixed with 4% paraformaldehyde in PBS, washed again with PBS two times, and observed with a confocal scanning microscope (LSM 510 META, Carl Zeiss, Germany) at a magnification of ×400. The internalized HA−QDot conjugates in the cytoplasm of B16F1 cells were excited with an Ar laser at 532 nm, and the fluorescence inside the cells was visualized through a long-pass emission filter (650 nm cutoff). For the quantification of endocytosed HA−QDot conjugates, B16F1 cells were seeded on a 96-well plate at a density of 1×10^4 cells/well and incubated at 37 °C and 5% CO_2 for 3 days. Quadruplet wells were incubated with HA−QDot conjugates under the same condition with the above confocal microscopic analysis in the absence and presence of intact HA. After incubation for the predetermined time, the cells were washed and fixed as described above. Then, the fluorescence was quantified with a well plate reader (Tecan SPECTRAFluor, ReTiSoft Inc., Canada) having a 680 nm emission filter after excitation at 360 nm.

Synthesis of PEI−HA Conjugate. PEI−HA conjugate was synthesized via amide bond formation between the amine groups of branched PEI and the carboxyl groups of HA. The followings are detailed description for the synthesis of PEI−HA conjugate with a feeding weight ratio of HA to PEI of 1/20. HA (MW = 130 kDa, 20 mg) was dissolved in 10 mL of water. To the HA solution was added 10 mL of a branched PEI (MW = 25 kDa) aqueous solution at a concentration of 40 mg/mL. Then, the pH of the reaction mixture was adjusted to 6.5 by the addition of 1.0 M HCl. EDC hydrochloride (40.4 mg) and HOBt (28.5 mg) were dissolved in the mixed solvent of 500 μL water and 500 μL DMSO, and then added to the solution containing HA and PEI. After mixing for 24 h, the reaction was stopped by raising the pH of the solution to 7.0 with 1.0 M NaOH. The resulting solution was poured into the prewashed dialysis membrane tube (MWCO of 10 kDa) and dialyzed against a large excess amount of 100 mM NaCl solution for 2 days, 25% ethanol for 1 day, and pure water for 1 day. The degree of HA modification with PEI was determined by ^{1}H NMR analysis.

Preparation of siRNA/PEI−HA Complex. The siRNA/PEI−HA complex with a weight ratio of 1/3 was prepared by mixing 2 μL of anti-PGL3-Luc siRNA (0.75 μg/μL) with 4.5 μL of PEI−HA conjugate solution (1 μg/μL), followed by incubation at room temperature for 15 min. To the resulting complex solution, NaCl was added to make the final concentration to be 150 mM. As a control, siRNA/PEI complex containing the same amount of PEI was also prepared as described above. The complex formation was confirmed by agarose gel electrophoresis.

Gel Retardation Analysis of siRNA/PEI−HA Complex. Each of siRNA, siRNA/PEI complex and siRNA/PEI−HA complex was loaded in the well of 1.0 wt % agarose gel containing ethidium bromide at a concentration of 0.1 μg/mL, which was applied to 50 V electrodes in 50 mM borate buffer (pH 8.98) for 40 min. The siRNA was visualized by bromide staining, and the gel image was taken under UV.

Particle Size Analysis of siRNA/PEI−HA Complex. After dilution of the complex solution with 750 μL of water, the particle size and ζ-potential of siRNA/PEI−HA complex were measured with a particle analyzer (Zetasizer Nano, Malvern Instrument Co., U.K.). The particle size of siRNA/PEI−HA complex was also measured with an atomic force microscope (AFM, Multimode 3100, VEECO Instrument Co., New York, NJ). The AFM system was in a tapping mode with a scanning area of 8×8 μm. The siRNA/PEI−HA complex solution (50 μL) was placed on a silicon wafer and then air-dried. The average complex particle size was determined from the verticle distance of 150 sample images.

Cytotoxicity of siRNA/PEI−HA Complex. B16F1 cells were cultured at 37 °C in a humidified incubator containing

5% CO_2. DMEM was supplemented with 10 vol % FBS and 10 IU/mL of antibiotics (penicillin). The cytotoxicity was evaluated by MTT assay.[34] B16F1 cells were aliquoted to be 5×10^3 cells/well in a 96-well plate and incubated at 37 °C for 24 h. The prepared siRNA/PEI complex and siRNA/PEI−HA complex solutions (20 μL each, a weight ratio of 1/3) were incubated at room temperature for 20 min and added to the cells in the absence of FBS. PBS (pH = 7.4) without siRNA was used as a control. At the end of transfection step, 20 μL of 2 mg/mL MTT solution in PBS was added to the plate and incubated at 37 °C for additional 4 h. After removal of the medium containing MTT, 300 μL of DMSO was added to dissolve the formazan crystal formed by live cells. The optical density was measured at 540 nm with UV spectrophotometer (Biotrak II plate reader, Biochrom, Cambridge, U.K.). Cell viability (%) was calculated using the following equation: cell viability (%) = $[OD_{540(sample)}/OD_{540(control)}] \times 100$, where $OD_{540(sample)}$ represents the optical density from the wells treated with siRNA/PEI complex or siRNA/PEI−HA complex and $OD_{540(control)}$ represents that from the wells treated with PBS.

***In Vitro* Gene Silencing by siRNA/PEI−HA Complex.** B16F1 cells were aliquoted to be 5×10^5 cells/well in a 24-well plate and incubated at 37 °C for 24 h. Anti-PGL3-Luc siRNA/PEI−HA complex was cotransfected along with PGL3-Luc plasmid/Lipofectamine complex as described elsewhere.[14] Briefly, cells were pretransfected with 0.5 mL of DMEM containing 2 μg of PGL3-Luc vector and 1 mg of Lipofectamine. After incubation for 3 h and the subsequent washing, a control (PBS), anti-PGL3-Luc siRNA/PEI complex and anti-PGL3-Luc siRNA/PEI−HA complex with a weight ratio of 1/3 were transfected for gene silencing in the medium containing 10 vol % to 50 vol % serum. After incubation for 24 h, the transfected cells were lysed with a lysis buffer (1% Triton X-100) and 5 μL of the lysed solution was mixed with 25 μL of luciferase assay solution. The luciferase activity was measured with a luminometer (Luminoskan Ascent, Laboratory systems, Germany). For VEGF gene silencing experiments, B16F1 cells were grown to 80% confluency in a 24-well plate and transfected with a control, siVEGF/PEI and siVEGF/PEI−HA complexes. After incubation for 24 h, the concentration of VEGF in the medium was determined using a mouse VEGF ELISA kit (Invitrogen Co., Carlsbad, CA).

Biodistribution of siRNA/PEI−HA Complex. In order to generate tumors, 100 μL of a single-cell suspension containing 1×10^6 B16F1 cells was injected subcutaneously to the middle of the right flank of 4- to 5-week-old female C57BL/6 mice (in total 12 heads). Tumor size was measured using a vernier caliper across its longest (a) and shortest (b) diameters, and its volume was calculated using the formula $V = 0.5ab^2$. Two types of Cy3-labeled siRNA (Cy3-siRNA, 3 nmol) formulations, Cy3-siRNA/PEI and Cy3-siRNA/PEI−HA complexes were administered via tail vein injection to the tumor-bearing mice with a tumor volume of *ca*. 250 mm^3. After anatomization in 24 h, various tissues, including liver, lung, spleen, kidney, heart, and tumor, were excised and washed with PBS. One hundred milligrams of the harvested tissues was homogenized in 0.5 mL of PBS solution. The homogenate was centrifuged at 4,000 rpm for 25 min to remove insoluble tissue debris, and the supernatant was then filtrated through a 0.45 μm filter membrane (Millipore, Bedford, MA). The fluorescent intensity of the supernatant was determined with a fluorescence spectrophotometer (CARY Eclipse, Varian, Palo Alto, CA) at an excitation and an emission wavelength of 550 and 570 nm, respectively. The amount of Cy3-siRNA in each tissue sample was determined by relating the measured fluorescent intensity to a standard curve and expressed as mean ± SD of the percentage to the injected dose per gram of tissue (% ID/g of tissue).[35]

***In Vivo* Tumor Therapy with siRNA/PEI−HA Complex.** Tumor treatment started after 5 days when the tumor size reached approximately 70 mm^3. Anti-VEGF siRNA (siVEGF)/PEI complex, scrambled siVEGF (scVEGF)/PEI−HA complex, and siVEGF/PEI−HA complex with a weight ratio of 1/3 were prepared in 5 wt % glucose solution, respectively. Each 50 μL of 5 wt % glucose solution as a control and three complex solutions at a dose of 4.5 μg of siRNA/mouse was injected intratumorally to C57BL/6 mice by daily injection for three days. The tumor volumes were measured every 3−4 days. The treated mice were examined for appearance, necrosis growth, and decreased physical activity. Tumor progression was analyzed in terms of tumor volume over a period of 17 days. The excised tumors at 17 days postinjection were homogenized in PBS with protease inhibitor. After centrifugation, the amount of VEGF in each supernatant was measured by ELISA.

Statistical Analysis. *In vivo* data are given with a mean standard deviation (SD) of three independent measurements. Statistical analysis was performed by *t*-test using a software of SigmaPlot 9.0. Statistical significance was assigned for *P* values <0.05 (95% confidence level).

Results and Discussion

Labeling HA−ADH with QDots. Several HA−ADH with ADH contents of 14, 22, 45 and 70 mol % were prepared to investigate the effect of HA modification on the receptor mediated endocytosis. The degree of HA modification with ADH, which was determined by ^{1}H NMR analysis, could be controlled simply by changing the reaction time from 5 min to 2 h. The HA−ADH was labeled with QDots for its bioimaging in B16F1 cells having HA receptors such as CD44 and LYVE-1. The carboxyl terminal ligands of QDots in excess molar amount to amine groups in HA−ADH

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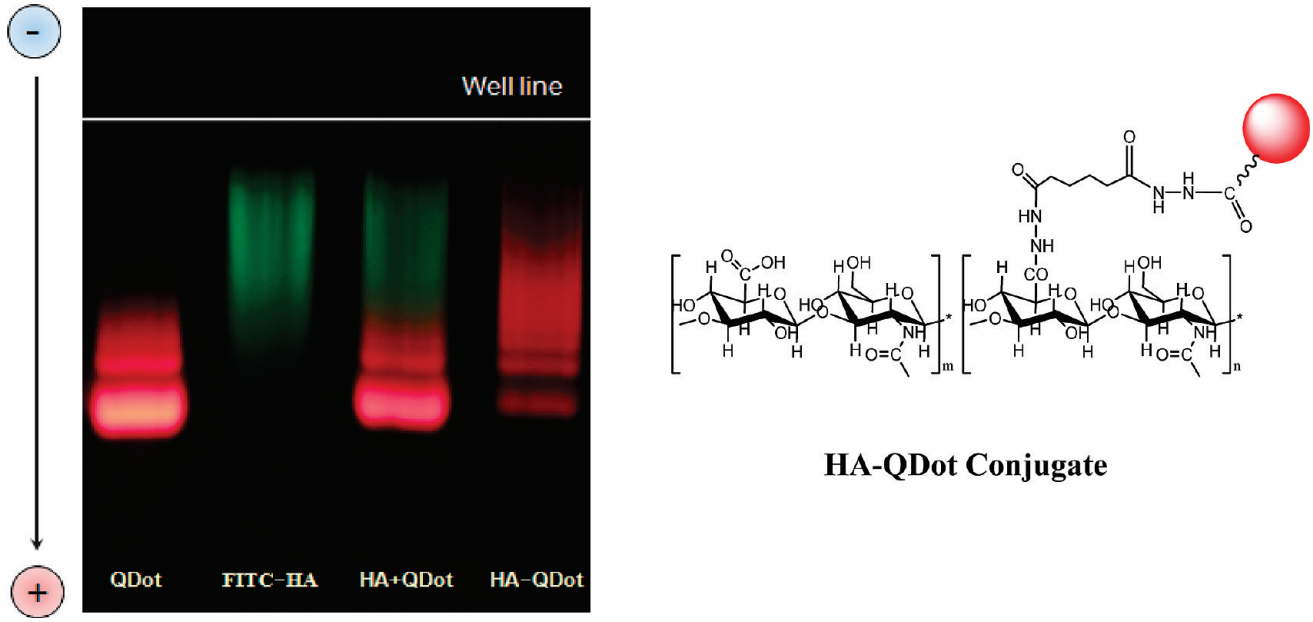

Figure 1. Gel electrophoresis bands of quantum dots (QDot), FITC labeled adipic acid dihydrazide modified HA (HA−ADH), a mixture of QDots and FITC labeled HA−ADH, and HA−QDot conjugates, respectively.

were activated with EDC and reacted with sulfo-NHS. Then, HA−ADH was conjugated with the activated QDots by the reaction between amine group of HA−ADH and sulfo-NHS of QDots.[27] In order to confirm the successful conjugation of HA−ADH with QDots, aliquots of QDots and the resulting products were analyzed by agarose gel electrophoresis. Gel electrophoresis is a simple way to support the surface modification of QDots.[36,37] As shown in Figure 1, there was a big difference among their gel electrophoresis bands. Due to the negative charge of QDots with carboxyl groups, QDots moved down toward the positive electrode during the gel electrophoresis (lane 1). As a control, FITC labeled HA−ADH showed a green color with a broad band due to the polydispersity of HA molecules (lane 2). Interestingly, a mixture of FITC labeled HA−ADH and QDots showed two separate bands of a green color of FITC labeled HA−ADH and a red color of QDots at their positions (lane 3). In contrast, HA−QDot conjugates with relatively bigger particle sizes and weaker negative charges moved down slowly, resulting in a broad band depending on the degree of HA modification (lane 4). They showed the same red color with QDots. The broad band of HA−QDot conjugates might be also ascribed to the polydispersity of HA molecules with a different content of QDots. The gel electrophoresis results confirmed the successful conjugation of HA−ADH with QDots. The morphologies of QDots and HA−QDot conjugates were analyzed with TEM and reported in detail elsewhere.[26] QDots appeared to be coupled in a group possibly along the HA molecules with an extended length of *ca.* 300 nm. Considering all these results, we could conclude the successful synthesis of HA−QDot conjugates.

Receptor Mediated Endocytosis of HA−QDot Conjugates. In order to investigate the effect of HA modification on its receptor mediated endocytosis, B16F1 cells were incubated with HA−QDot conjugates with various ADH contents of 14, 22, 45, and 70 mol %. Figure 2-A shows a confocal microscopic image of B16F1 cells containing HA−QDot conjugates with 14 mol % ADH content. A set of images was obtained by confocal microscopy using the Z-stack method changing the focal length from bottom to top of the cells. The HA−QDot conjugates appeared to be present clearly inside the cells on their cross-sectional images. However, there was apparent decrease in fluorescent intensity with increasing ADH modification in HA−ADH. HA−QDot conjugates with 45 mol % and 70 mol % ADH contents were much less internalized into the cells compared to those with 14 mol % and 22 mol % ADH contents (Figures 2-B). For the quantification of previous results, the fluorescent intensity measured with a well plate reader was averaged for quadruplet wells containing endocytosed HA−QDot conjugates with 14, 22, 45, and 70 mol % ADH contents, respectively. Graphical representation of the normalized values in Figure 3 clearly shows the decrease of endocytosed HA content with increasing degree of HA modification. Interestingly, when the degree of HA modification increased by 2 times from 22 mol % to 45 mol %, the fluorescent intensity for internalized HA−QDot conjugates decreased by four times from *ca.* 6472 to 1658 (Figure 3). The result may be explained by the fact that more than three carboxyl groups (hexasaccharide) in the HA molecule are related with

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(37) Liu, H. Y.; Vu, T. Q. Identification of quantum dot bioconjugates and cellular protein co-localization by hybrid gel blotting. *Nano Lett.* **2007**, *7*, 1044–1049.

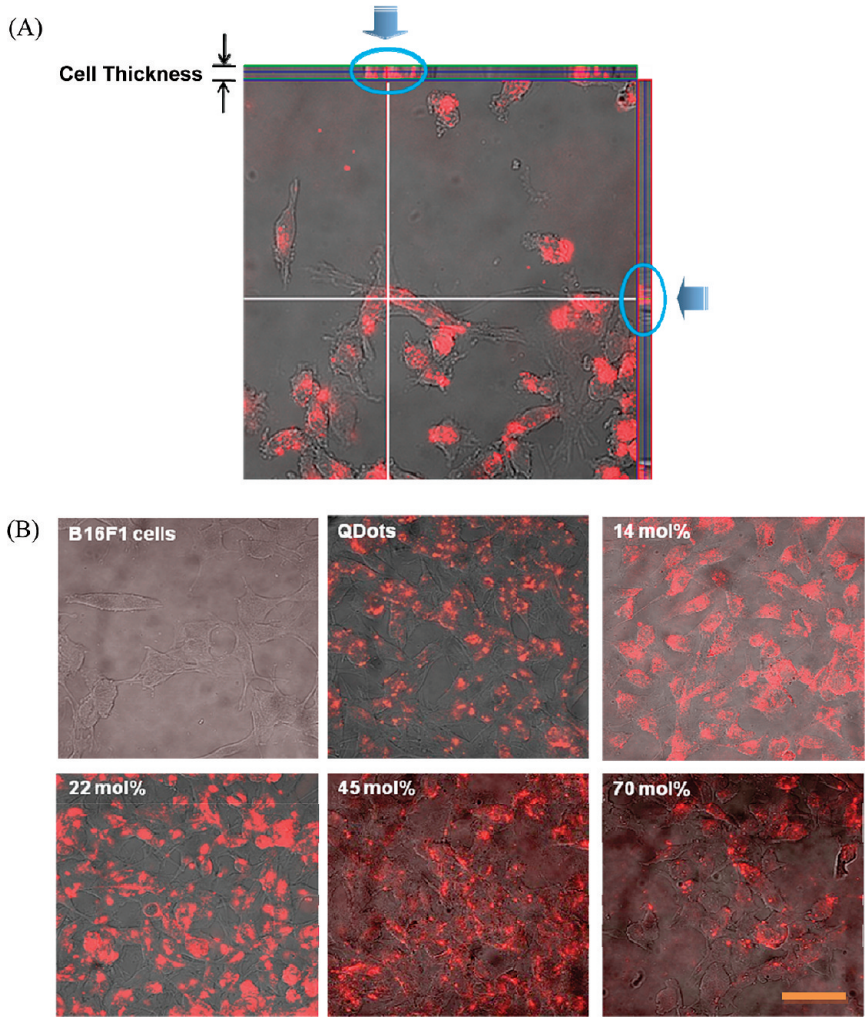

Figure 2. (A) An integrated image of B16F1 cells after incubation with HA−QDot conjugates (14 mol % modification) for 2 h by confocal microscopy using the Z-stack method. The top of the image shows a cross-section of cell thickness on the *x*-axis while the right side of the image presents that on the *y*-axis. Fluorescence is shown clearly inside the cells as arrows indicate. (B) Confocal microscopic images of B16F1 cells incubated for 2 h with QDots and HA−QDot conjugates with 14 mol %, 22 mol %, 45 mol %, and 70 mol % HA modifications, respectively. Scale bar corresponds to 15 μm.

its binding to HA receptors.[24,38] It has been reported that the more biotinylation of HA caused the less cellular uptake into cells with LYVE-1.[38] Furthermore, when the cells were preincubated with intact HA, the fluorescent intensity of endocytosed HA−QDot conjugates decreased reflecting the HA receptor mediated endocytosis. The preincubation of intact HA appeared to be less effective for HA−QDot conjugates with 45 and 70 mol % HA modifications than those with 14 and 22 mol % HA modifications (Figure 3). The results supported that HA modification affected the receptor mediated endocytosis significantly. Overall, the

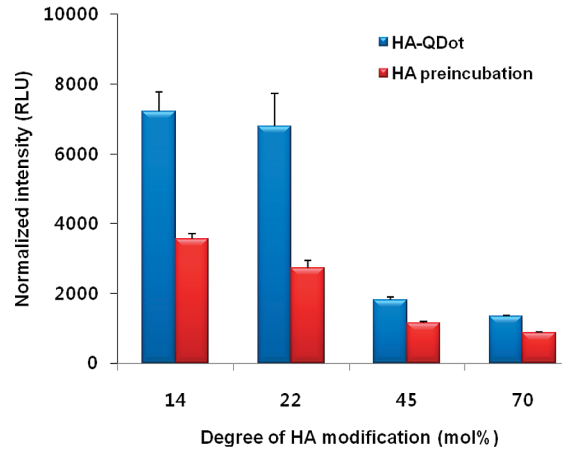

Figure 3. Quantification of the fluorescent intensity of endocytosed HA−QDot conjugates into B16F1 cells after incubation for 2 h in the presence and absence of intact HA.

(38) Schledzewski, K.; Falkowski, M.; Moldenhauer, G.; Metharom, P.; Kzhyshkowska, J.; Ganss, R.; Demory, A.; Falkowska-Hansen, B.; Kurzen, H.; Ugurel, S.; Geginat, G.; Arnold, B.; Goerdt, S. Lympathic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macropahages in malignant tumours and wound healing tissue in vivo and in bone marrow cultures in vitro: Implications for the assessment of lymphangiogenesis. *J. Pathol.* **2006**, *209*, 67–77.

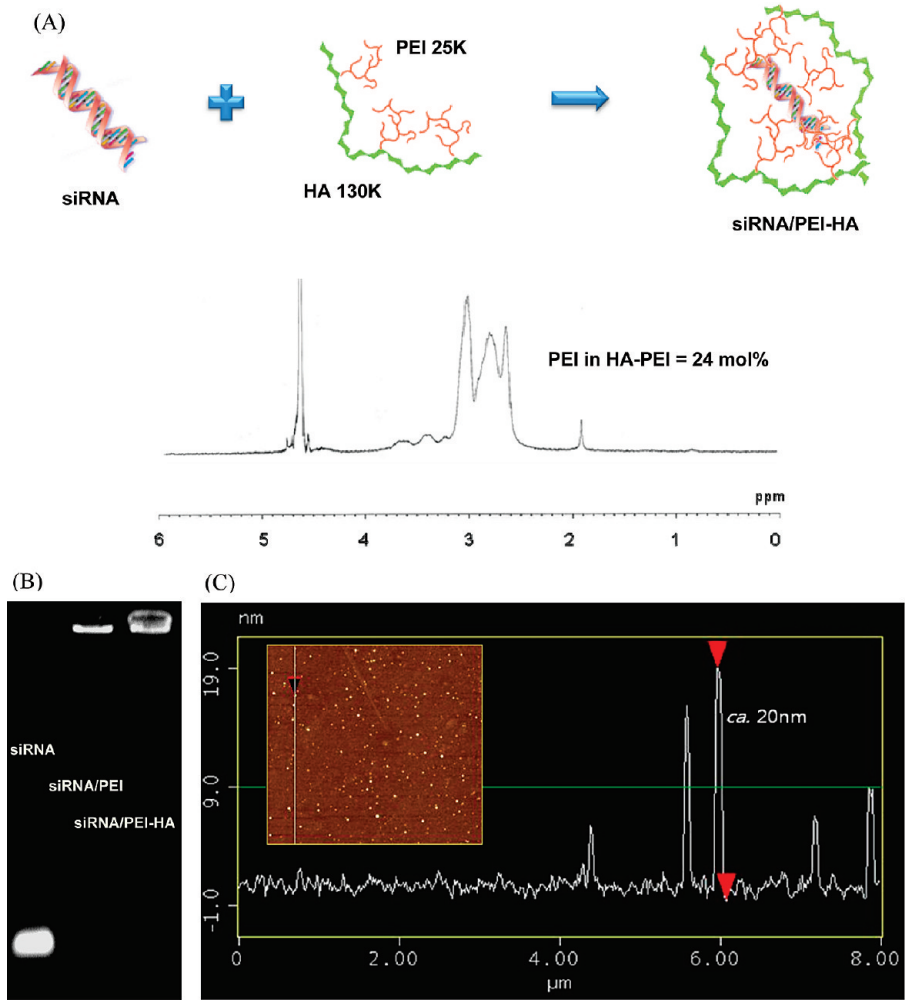

Figure 4. (A) Schematic representation of siRNA/polyethyleneimine (PEI)−hyaluronic acid (HA) complex. The molecular weight of PEI was 25K, and that of HA was 130K. ^{1}H NMR analysis confirmed the successful formation of PEI−HA conjugate with *ca.* 24 mol % PEI content. (B) Agarose gel electrophoresis of siRNA, siRNA/PEI complex and siRNA/PEI−HA complex. (C) Atomic force microscopic particle size analysis of siRNA/PEI−HA complex. The particle size of siRNA/PEI−HA complex was *ca.* 20 nm with slightly negative surface characteristics.

results from the quantification of fluorescent intensity using a well plate reader were well matched with those from the confocal microscopic analyses. Considering all these results, we concluded that HA−QDot conjugates with a degree of HA modification less than 25 mol % could be taken up to B16F1 cells with HA receptors more efficiently than QDots alone by the receptor mediated endocytosis. In other words, HA modification less than 25 mol % was thought not to affect its receptor mediated endocytosis.

Preparation and Characterization of siRNA/PEI−HA Complex. On the basis of bioimaging study of HA derivatives using QDots, we decided to develop a PEI−HA conjugate with a PEI content less than 25 mol % as a novel target specific intracellular delivery carrier of siRNA by HA receptor mediated endocytosis. The carboxyl groups of HA were activated with EDC and sulfo-NHS, and then conjugated with amine groups of PEI. The weight ratio of HA to PEI in the reaction solution was 1/20. ^{1}H NMR analysis confirmed the successful conjugation of PEI to the activated HA (Figure 4-A). There were both a methyl peak of acetoamide group of HA at 1.9 ppm and a PEI peak at 2.5−3.2 ppm shifted from 2.5 ppm as reported elsewhere.[39] The synthesized PEI−HA conjugate with 24.2 mol % PEI content was successfully applied for the preparation of siRNA/PEI−HA complex (Figure 4-A), which could be confirmed by agarose gel electrophoresis (Figure 4-B). As shown in Figure 4-B, siRNA/PEI and siRNA/PEI−HA complexes with a weight ratio of 1/3 exhibited retarded movement during the gel electrophoresis compared to naked siRNA. According to ζ-size analysis, a hydrated diameter of siRNA/PEI−HA complex was estimated to be 42.7 ± 0.32 nm with a slightly negative ζ-potential value. In contrast, AFM analysis revealed that siRNA/PEI−HA complex had an average particle size of *ca.* 20 nm in the dried state (Figure

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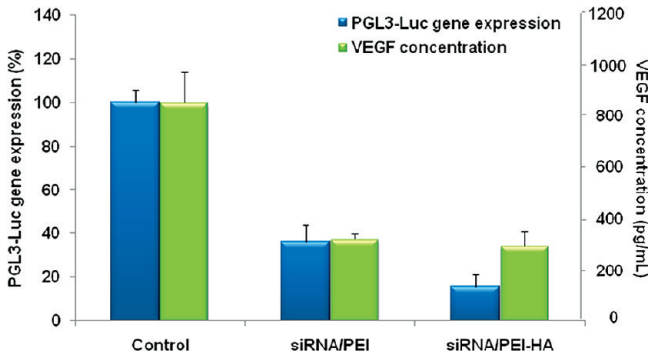

Figure 5. *In vitro* gene silencing efficiency of siRNA/PEI−HA complex: (Left) Comparative PGL3-Luc gene silencing of a control (PBS), anti-PGL3-Luc siRNA/PEI complex, and anti-PGL3-Luc siRNA/PEI−HA complex in B16F1 cells, respectively. (Right) Comparative vascular endothelial growth factor (VEGF) concentration after treatment of B16F1 cells with a control, anti-VEGF siRNA/PEI complex, and anti-VEGF siRNA/PEI−HA complex, respectively.

4-C). All these results were thought to reflect the core/shell structure of siRNA/PEI−HA complex formed by the electrostatic interaction between siRNA and PEI part of PEI−HA conjugate. Prior to gene silencing tests, a comparative MTT cytotoxicity test for siRNA/PEI and siRNA/PEI−HA complexes was performed in B16F1 cells. Compared to the control of PBS, cell viability for siRNA/PEI−HA complex with a weight ratio of 1/3 was 92.3% whereas that for siRNA/PEI complex was only 61.2%. Despite of the wide use of siRNA/PEI complex for nonviral gene delivery, its serious cytotoxicity has been an obstacle to *in vivo* applications.[15] The relatively less cytotoxicity of siRNA/PEI−HA complex than siRNA/PEI complex might be ascribed to the electrostatic neutralization of positively charged PEI by the negatively charged HA. In addition, when siRNA is complexed with PEI−HA conjugate, the biocompatible HA on the shell of siRNA/PEI−HA complex would greatly contribute to the reduction of cytotoxicity. We previously reported the morphological analysis and cytotoxicity of siRNA/PEI−HA complex in detail elsewhere.[39]

In Vitro **Gene Silencing of siRNA/PEI−HA Complex.** The post-transcriptional gene silencing efficiency of anti-PGL3-Luc siRNA/PEI complex and anti-PGL3-Luc siRNA/PEI−HA complex with a weight ratio of 1/3 was assessed in B16F1 cells after the pretransfection of PGL3-Luc gene with Lipofectamine. As shown in Figure 5 (left *y*-axis), the down-regulation by siRNA/PEI−HA complex in B16F1 cells having HA receptors, such as LYVE-1[38] and CD44,[40] was *ca.* 84% whereas that by siRNA/PEI complex was only *ca.* 64%. The results were well matched with those of bioimaging of HA derivatives using QDots. From the results, we concluded that anti-PGL3-Luc siRNA/PEI−HA complex with the outer layer of HA was more efficiently delivered to B16F1 cells with HA receptors than anti-PGL3-Luc siRNA/PEI complex without HA, which might be ascribed to enhanced cellular uptake of the anti-PGL3-Luc siRNA/PEI−HA complex by HA receptor mediated endocytosis.

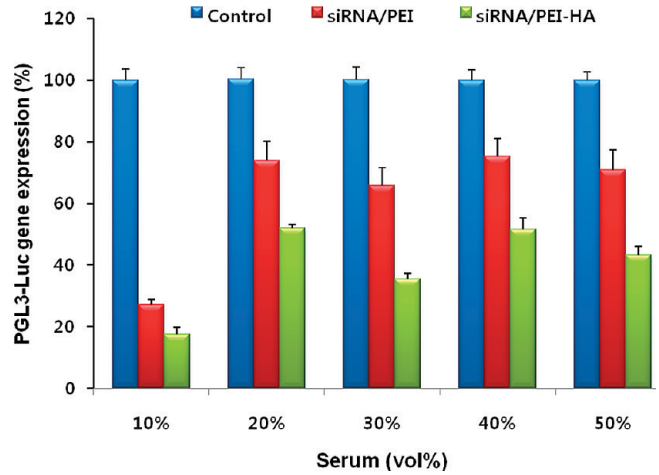

Figure 6. Effect of serum concentration up to 50 vol % on PGL3-Luc gene silencing of anti-PGL3-Luc siRNA/PEI complex and anti-PGL3-Luc siRNA/PEI−HA complex in B16F1 cells.

HA−PEI conjugate was also applied to make a complex with antivascular endothelial growth factor siRNA (siVEGF) for the inhibition of tumor angiogenesis. A number of growth factors have been identified as potential positive regulators of angiogenesis. Among them, VEGF has been widely investigated as a target for antiangiogenic therapy.[41] Figure 5 (right *y*-axis) shows the inhibitory effect of siVEGF/PEI−HA complex on the production of VEGF. According to VEGF ELISA, the gene silencing efficiency of siVEGF/PEI−HA complex with a weight ratio of 1/3 appeared to be slightly higher than that of siVEGF/PEI complex suppressing the VEGF production by 65% compared to the untreated control (Figure 5, right *y*-axis).

Before *in vivo* applications, we investigated the effect of fetal bovine serum (FBS) in the transfection medium on *in vitro* gene silencing of anti-PGL3-Luc siRNA/PEI−HA complex in B16F1 cells. Figure 6 shows the comparative PGL3-Luc gene silencing efficiency between siRNA/PEI and siRNA/PEI−HA complexes in the presence of serum up to 50 vol %. While the PGL3-Luc gene silencing efficiency of siRNA/PEI complex decreased significantly in the presence of 20 vol % FBS, that of anti-PGL3-Luc siRNA/PEI−HA complex was maintained in the range of 50%−70% for the serum concentration up to 50 vol % in the medium. The reduced gene silencing efficiency of anti-PGL3-Luc siRNA/PEI complex in the presence of serum might be attributed to nonspecific adsorption of positively charged complexes to the serum proteins, resulting in the formation of interparticular aggregates and the decrease of cellular uptake of anti-PGL3-Luc siRNA/PEI complex.[42] In contrast, siRNA/PEI−HA complex having a slightly negative surface charge was not likely to have such deleterious protein adsorption problems and could maintain their stability even in the presence of serum. The ζ-potential analysis clearly showed the negative surface characteristics of siRNA/PEI−HA complex.[39] The high serum stability as well as the enhanced cellular uptake of siRNA/PEI−HA complex by HA receptor

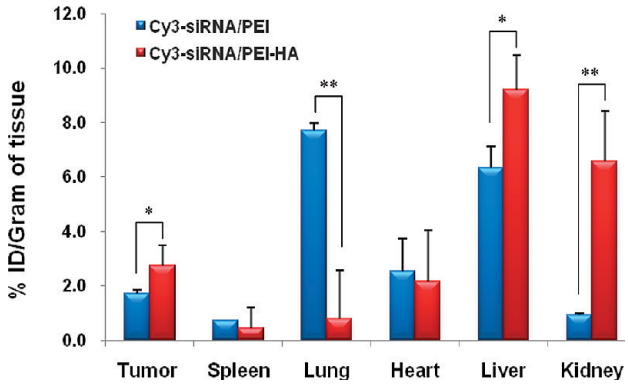

Figure 7. Biodistribution of Cy3-labeled siRNA (Cy3-siRNA)/PEI and Cy3-siRNA/PEI−HA complexes after tail-vein injections to tumor-bearing C57BL/6 mice. After anatomization in 24 h, the amount of Cy3-siRNA in dissected tissues was determined by spectrofluorometer and expressed as a percentage to an initial dose per gram of tissue (% ID/g of tissue). Data are represented as a mean ± SD ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ compared to Cy3-siRNA/PEI.

mediated endocytosis encouraged us to perform the following *in vivo* applications.

***In Vivo* Biodistribution of siRNA/PEI−HA Complex.** On the basis of the results from *in vitro* tests, we investigated *in vivo* biodistribution of siRNA/PEI−HA complex in B16F1 melanoma tumor-bearing mice using Cy3-labeled siRNA (Cy3-siRNA). At 24 h postinjection, the siRNA formulations were distributed mainly in the tumor, spleen, lung, heart, liver, and kidney (Figure 7). While Cy3-siRNA/PEI complex was accumulated mainly in the lung, Cy3-siRNA/PEI−HA complex was observed in the tissues with HA receptors such as liver, kidney, and tumor. The results might be ascribed to the enhanced cellular uptake of Cy3-siRNA/PEI−HA complex by HA receptor mediated endocytosis. Previously, we have reported real-time bioimaging of HA derivatives in nude mice using QDots.[27] HA−QDot conjugates with 35 mol % HA modification maintaining enough binding sites for HA receptors were mainly accumulated in the liver, while those with 68 mol % HA modification losing much of HA characteristics were evenly distributed to the tissues in the body. Homeostasis of HA in the body is known to be maintained by the receptor mediated HA degradation in the liver, kidney, lymphatic nodes, and so on. CD44,[22] RHAMM,[7] and LYVE-1[23] have been identified as HA receptors for various biological functions. Therefore, siRNA/PEI−HA complex was thought to be usefully applied as target specific siRNA therapeutics for the treatment of diseases in the tissues with HA receptors.

***In Vivo* Tumor Treatment with siVEGF/PEI−HA Complex.** Recently, inhibition of VEGF production by siRNA was reported as an effective and useful method for antiangiogenic tumor therapy.[43] Accordingly, we decided to assess siVEGF/PEI−HA complex as antiangiogenic therapeutics for

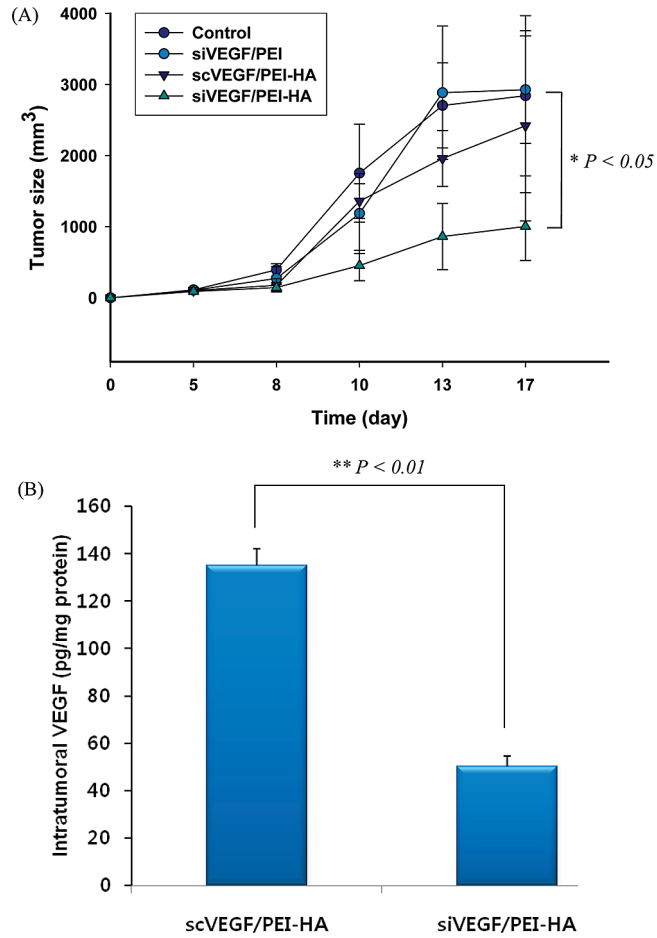

Figure 8. Antitumoral therapeutic effect of anti-VEGF siRNA (siVEGF)/PEI−HA complex in tumor-bearing C57BL/6 mice: (A) Tumor volume change with increasing time after intratumoral injection of a control of 5% glucose solution, siVEGF/PEI complex, scrambled siVEGF (scVEGF)/PEI−HA complex, and siVEGF/PEI−HA complex. The results represent mean ± SD ($n = 3$). * $P < 0.05$ versus control. (B) VEGF levels in tumor tissues in 17 days after treatment with scVEGF/PEI−HA complex and siVEGF/PEI−HA complex. The excised tumors were homogenized in PBS with protease inhibitor. After centrifugation, the amount of VEGF in each supernatant was measured by ELISA. ** $P < 0.01$ versus scVEGF/PEI−HA.

the treatment of tumor. First, B16F1 cells (1×10^6) were injected subcutaneously into the right flank of C57BL/6 mice to prepare tumor models. Tumors were visible at the injection sites in 5 days with a mean size of 75.4 mm^3. Then, four

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kinds of samples, a control of 5 wt % glucose solution, siVEGF/PEI complex, scrambled siVEGF (scVEGF)/PEI−HA complex, and siVEGF/PEI−HA complex, were administered by intratumoral injections. As shown in Figure 8-A, siVEGF/PEI−HA complex significantly suppressed the tumor growth compared to a control, siVEGF/PEI complex, and scVEGF/PEI−HA complex ($P < 0.01$). After anatomization in 17 days, we could confirm the antitumor effect of siVEGF/PEI−HA complex more clearly from the recovered tumor tissues. Furthermore, the amount of VEGF in tumor tissues was quantified by ELISA to assess the therapeutic effect of siVEGF/HA−PEI complex on the inhibition of tumor growth. A lower concentration of VEGF was observed in tumors treated with siVEGF/PEI−HA complex in 17 days compared to that with scVEGF/PEI−HA complex (Figure 8-B). The siVEGF/PEI−HA complex having a slightly negative charge was thought to result in serum stability, low toxicity and enhanced cellular uptake by HA receptor mediated endocytosis contributing for the successful intracellular delivery of siVEGF and in turn effective gene silencing of VEGF producing mRNA.[44,45] In a word, all these results can be summarized as successful synthesis and applications of PEI−HA conjugate as a novel target specific intracellular delivery carrier of siRNA. The novel siVEGF/PEI−HA complex will be investigated further as target specific antiangiogenic therapeutics for the treatment of diseases in the tissues with HA receptors, such as liver cancer and kidney cancer.

Conclusion

According to the bioimaging of HA derivatives using QDots, HA−QDot conjugates with a degree of modification less than 25 mol % appeared to be more efficiently taken up to B16F1 cells by HA receptor mediated endocytosis than QDots alone. On the basis of bioimaging study, PEI−HA conjugate with 24.2 mol % PEI content was developed as a novel target specific intracellular delivery carrier of siRNA. The PGL3-Luc gene silencing efficiency of anti-PGL3-Luc siRNA/PEI−HA complex in B16F1 cells with HA receptors was in the range of 50%−85% depending on the serum concentration up to 50 vol %, which was more significant than that of anti-PGL3-Luc siRNA/PEI complex. While Cy3-siRNA/PEI complex was accumulated mainly in the lung, Cy3-siRNA/PEI−HA complex was observed in the tissues with HA receptors such as liver, kidney, and tumor. Furthermore, the intratumoral injection of anti-VEGF siRNA/PEI−HA complex resulted in an effective inhibition of tumor growth by HA receptor mediated endocytosis to melanoma tumor cells in C57BL/6 mice. HA mostly in the outer layer of siVEGF/PEI−HA complex was thought to play an essential role in target specific delivery of siRNA to the tissues with HA receptors providing serum stability, low toxicity and enhanced cellular uptake by HA receptor mediated endocytosis contributing for the effective gene silencing of VEGF producing mRNA. The novel siVEGF/PEI−HA complex will be investigated further as target specific antiangiogenic therapeutics for the treatment of diseases in the tissues with HA receptors, such as liver cancer and kidney cancer.

Acknowledgment. This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A081026).

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