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Suppression of post-angioplasty restenosis with an Akt1 siRNA-embedded coronary stent in a rabbit model

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ABSTRACT

Restenosis is the formation of blockages occurring at the site of angioplasty or stent placement. In order to avoid such blockages, the suppression of smooth muscle cells near the implanted stent is required. The Akt1 protein is known to be responsible for cellular proliferation, and specific inhibition of Akt1 gene expression results in the retardation of cell growth. To take advantage of these benefits, we developed a new delivery technique for Akt1 siRNA nanoparticles from a hyaluronic acid (HA)-coated stent surface. For this purpose, the disulfide cross-linked low molecular polyethyleneimine (PEI) (ssPEI) was used as a gene delivery carrier because disulfide bonds are stable in an oxidative extracellular environment but degrade rapidly in reductive intracellular environments. In this study, Akt1 siRNA showed efficient ionic interaction with the ssPEI carrier, which was confirmed by polyacrylamide gel electrophoresis. Akt1 siRNA/ssPEI nanoparticles (ASNs) were immobilized on the HA-coated stent surface and exhibited stable binding and localization, followed by time-dependent sustained release for intracellular uptake. Cellular viability on the nanoparticle-immobilized surface was assessed using A10 vascular smooth muscle cells, and the results revealed that immobilized ASNs exhibited negligible cytotoxicity against the adhering A10 cells. Transfection efficiency was quantified using a luciferase assay; the transgene expression of Akt1 suppression through the delivered Akt1 siRNA was measured using RT-PCR and western blot, demonstrating higher gene silencing efficiency when compared to other carriers. ASN coated on HA stents were deployed in the balloon-injured external iliac artery in rabbits *in vivo*. It was shown that the Akt1 released from the stent suppressed the growth of the smooth muscle at the peri-stent implantation area, resulting in the prevention of restenosis in the post-implantation phase.

1. Introduction

Restenosis remains the most significant consequence of percutaneous transluminal angioplasty, despite efforts to address it both biologically and mechanically [1,2]. Gene therapy has been attempted as a treatment option in terms of controlling gene expression in neighboring cells related to restenosis. Non-viral gene delivery has been considered safer than its viral counterpart. Strategies for enhancing non-viral gene delivery typically involve the complexation of plasmids with cationic polymers or lipids, which can self-assemble with DNA to form particles capable of being endocytosed by cells. Substrate-mediated delivery results in the immobilization of DNA complexed with the carrier onto the substrate. Immobilization on the target surface can enhance gene transfer by maintaining an elevated concentration of DNA within the cellular microenvironment via sustained release and by facilitating subsequent cellular internalization [3].

Polyethyleneimine (PEI) is a commonly used polymeric gene carrier possessing a high density of primary, secondary, and tertiary amino groups that can be protonated at different environmental pH levels. At physiological pH, polycation is very effective in binding to DNA and can mediate the transfection of eukaryotic cells. However, its relatively higher cytotoxicity hinders clinical application [4–6].

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Disulfide cross-linked PEI (ssPEI) is preferred in the design and synthesis of biodegradable polymers for drug delivery. It was previously demonstrated that ssPEI has an efficient transfection property in the A10 vascular smooth muscle cell (VSMC) line derived from the thoracic aorta of embryonic rats. First, disulfide bonds are more hydrolytically stable than ester bonds in the extracellular environment, thus polycation with disulfide bonds can be used to prepare stable complexes with plasmid DNA. Second, disulfide bonds can be cleaved rapidly by glutathione and thioredoxin reductase in the cytoplasm [7–9]. As a result, DNA can be rapidly released from polyplexes in order to mediate efficient gene expression. In addition, cytotoxicity can be reduced by avoiding high charge density and long-term polymer accumulation.

Akt1 siRNA was delivered to the cells with enhanced proliferation for knocking down the Akt1 protein responsible for cellular proliferation at the mRNA level. It has been reported that the inhibition of Akt1 decreases the expression of caspase-8 in endothelial cells. Suppression of downstream Akt1 signaling proteins such as Fas ligand results in the stimulation of caspase activity and apoptosis in VSMCs. It has also been demonstrated that the specific inhibition of Akt1 protein expression results in the retardation of cell growth [10–14].

Hyaluronic acid (HA) is highly compatible with cells and the cellular matrix. It can be efficiently degraded using specific enzymes, such as hyaluronidase; furthermore, its degradation products could induce extracellular matrix production and the neoformation of blood capillaries [15–17]. HA enhances proliferation and migration of endothelial cells at a later stage, whereas Akt1 siRNA delivered from nanoparticles can knock down the initial over-growth of VSMCs near the implanted stent. Here, Akt1 siRNA delivery was evaluated on an HA-coated surface, and the subsequent inhibition of Akt1 protein expression was also determined.

In this study, ssPEI was complexed with Akt1 siRNA (Akt1 siRNA/ssPEI nanoparticles [ASNs]) and immobilized on an HA-coated surface. The binding ability of the ASNs and the release of siRNA from the HA-coated surface were studied. The potential efficacy of ASNs was evaluated with the rat VSMC line. The suppression of the Akt1 protein and its downstream signaling proteins regulating the cellular proliferation after the treatment with ASNs was examined. ASNs were coated on an HA stent deployed in the balloon-injured external iliac artery in a rabbit *in vivo*. Finally, post-angioplasty restenosis was measured by micro-computed tomography (micro-CT) imaging.

2. Materials and methods

2.1. Materials

Branched PEI (bPEI) 25K and linear PEI (lPEI) 25K were purchased from Sigma–Aldrich (St. Louis, USA). Plasmid gWiz-luc (Aldevron, USA) was transformed into the competent cells, *Escherichia coli* DH5α, using the heat shock method. gWiz-luc was then propagated in bacterial cultures grown in Luria-Bertani (LB) media (Becton Dickinson, USA) containing 100 μg/ml of kanamycin (Biosesang Inc., Korea), and extracted and purified using a mini DNA-spin kit (Intron Biotechnol Co., Korea). Akt1 siRNA and scrambled (scr) siRNA were purchased from Genolution Pharmaceuticals (Seoul, Korea). A cassette of oligonucleotides encoding 19-mer hairpin sequences specific to the target Akt1 mRNA was designed. The targeted Akt1 mRNA sequence is GAAGGAAGUCAUCGUGGCCAA; the sense and antisense strands were synthesized. An scr siRNA with the same nucleotide composition as siRNA but lacking significant sequence homology to the genome was also designed. An antibody for Akt1 was purchased from Genolution Pharmaceuticals (Seoul, Korea).

2.2. Stable coating of HA on the stent surface

As reported previously [18], conjugates between HA and dopamine were synthesized using N-Hydroxysuccinimide (NHS) and ethyl(dimethylaminopropyl) carbodiimide (EDC) as an activation agent. Briefly, HA (200 mg), NHS (50 mM; 115 mg), and EDC (100 mM; 383 mg) were dissolved in 2-(N-Morpholino) ethanesulfonic Acid (MES) buffer (50 mM; pH 5.5; 20 ml) to minimize hydrolysis of EDC [19]. The solution was stirred at room temperature for 2 h, and dopamine (15 mM; 60 mg) was then added to the solution. The resulting mixture was stirred at room temperature for an additional 8 h. The resultant solution was transferred to molecular porous dialysis membrane bags and then underwent dialysis for 12 h to remove unreacted coupling reagents. To prevent the self-polymerization of dopamine, 5 mM of HCl was added to the dialysis water. The solution was freeze-dried to obtain a solid-state HA compound. The HA compound was completely dissolved in 10 mM of Tris buffer (pH 8.5), and cobalt–chromium (Co–Cr) alloy stents or circular disks (10 mm in diameter) were immersed in the HA solution. After 10 h incubation, the specimens were removed and sufficiently washed with water.

2.3. Complex formation between ssPEI and Akt1 siRNA

For the complex formation study, siRNA was added to either ssPEI or bPEI solution with varying N/P ratios of 10, 20, 30, and 40. The final volume was adjusted to 20 μL with siRNA concentrations from 100 pmol and was vortexed gently. After 15 min of incubation, complexes were subjected to polyacrylamide gel electrophoresis (PAGE) at 50 V for 4 h. The gel was analyzed on an ultraviolet (UV) illuminator.

2.4. Physico-chemical characterization of Akt1 siRNA/ssPEI nanoparticles (ASNs)

For this study, siRNA (100 pmol) was mixed with ssPEI or bPEI at N/P molar ratios of 10. Complexes were formed in phosphate buffer saline (PBS) and then incubated for 15 min at room temperature. Particle size and zeta potential were measured using Zetasizer Nano Z (Malven Instruments, Malvern, UK).

2.5. ASNs release from the HA-coated stent surface

The YOYO1-labeled plasmid DNA (luciferase) and FITC-labeled siRNA (50 pmol) complexed with either ssPEI or bPEI were prepared at different N/P ratios for immobilization on an HA-coated surface. After 15 min of incubation, complexes were immobilized on the HA-coated surface and incubated for another 24 h. Successful immobilization and release of siRNA from the HA-coated stent surface was checked by observation with fluorescent microscopy and by measuring the absorbance of supernatant samples collected at regular intervals.

2.6. Cell attachment on the surface of an HA-coated stent

For the cell attachment experiment, an HA-coated stent was placed into the 24-well plate and rinsed with 70% ethanol for sterilization. The vascular smooth muscle (A10) cells were seeded on the surface of the HA-coated stent at a density of 5.0×10^4 cells/cm^2 in DMEM medium with supplementation of antibiotics. The attachment of the cells over the stent surface was investigated by scanning electron microscopy (SEM).

2.7. Transgene expression from the luciferase nanoparticle-immobilized HA surface

The A10 cells were cultured at 37 °C with a 5% CO_2 atmosphere in DMEM (Hyclone, USA) and supplemented with 1% sodium pyruvate, 1% penicillin, and 10% heat-inactivated fetal bovine serum (FBS). Cells were seeded at a density of 5×10^4 cells per well in 24 well plates. For reverse transfection, cells were seeded following complex immobilization. Expression levels of luciferase after transfection were measured by the luciferase assay system (Promega, USA). After 24 h transfection, A10 cells were washed with PBS. Cells were lysed with 100 μL cell culture lysis buffer (Promega, USA) and assayed for enzymatic activity. Luciferase activity was normalized to total cellular protein using a bicinchoninic acid (BCA) assay kit (iNtRON, Republic of Korea).

2.8. Cell viability on ASNs-immobilized HA-coated stent surface

The cellular viability of ASNs with A10 cells was analyzed through MTS assay. ASNs were immobilized on the HA-coated surface and incubated at room temperature for 24 h. After incubation, A10 cells were seeded at a density of 5×10^3 cells/well and incubated at 37 °C in a humidified atmosphere of 5% CO_2. The cells were then washed with PBS and 100 μL DMEM containing 10% FBS and 1% penicillin. The viability of the cell population was analyzed using an MTS assay (Promega, USA).

2.9. Measurement of Akt1 expression at the mRNA level using semi-quantitative RT-PCR

For reverse transfection, A10 cells were seeded following complex immobilization on the HA-coated surface. After 24 h, mRNA was extracted from a total of 1×10^6 cells and reverse transcribed into cDNA using Maxime RT PreMix (iNtRon, Korea). Primers used for amplification, as well as internal probes for hybridization, were as follows: Akt1 forward, 5′-ATGAGCGACGTGGCTATTGTGAAG-30, and Akt1 reverse, 5′-GAGGCCGTCAGCCACAGTCTGGATG-3′. The downstream signaling protein mRNA primers for the Akt1 pathway were ordered as follows: mTOR or forward, 5′-CGCTGTCATCCCTTTATCG-3′; reverse, 5′-ATGCTCAAACACCTCCACC-3′; p70S6K forward, 5′-TACTTCGGGTACTTGGTAA-3′; reverse, 5′-GATGAAGGGATGCTTTACT-3′; 4E-BP1 forward, 5′-ACCGGAAATTCCTGATGGAG-3′; reverse, 5′-CCCACTTATCTTCTGGGCTA-3′; GAPDH forward, 5′-GCACCGTCAAGGCTGAGAA-3′; and

reverse, 5′-AGGTCCACCACTGACACGTTG-3′. Quantification of the PCR products was performed after 1% agarose gel electrophoresis.

2.10. Measurement of Akt1 expression at protein level by western blot

The A10 cells transfected with Akt1 siRNA and scr siRNA (1 μg) were lysed after 24 h and protein was extracted using PRO-PREP™ (iNtRON, Korea). Total protein was quantified by a BCA protein assay kit (Invitrogen, Korea). For western blot, equal amounts of protein were separated on sodium dodecyl sulfonate (SDS)-PAGE, transferred onto a nitrocellulose membrane, blocked, and incubated for 1 h with Akt1 goat polyclonal antibody. After washing, the membrane was incubated with a horseradish peroxidase-labeled secondary antibody. The bands were analyzed using a luminescent image analyzer (LAS-3000).

2.11. Animal preparation

All the animal experiments were performed according to the Ethics Committee of Chonnam National University Medical School and Chonnam National University Hospital (CNU IACUC-H-2011-5). Experiments were performed on New Zealand white rabbits (3.5 kg average weight; obtained from Damool Science, Daejeon, Korea). To prevent acute thrombosis after stenting, premedication with 20 mg of aspirin per day was given for seven days before the procedure. On the procedure day, rabbits were anesthetized intramuscularly with a mixture of 20 mg/kg ketamine hydrochloride and 2 mg/kg xylazine. They received supplemental oxygen continuously through an oxygen mask.

2.12. Rabbit iliac artery stent implantation

Stent implantation was performed under sterile conditions using portable fluoroscopic imaging (Philips, BV-Pulsera, Eindhoven, Netherlands). Rabbits were anesthetized intramuscularly through the right marginal ear vein with a mixture of xylazine (2.2 mg/kg) and ketamine (22 mg/kg). Body temperature was controlled at 37 °C through the use of a heating pad, and the animals received supplemental oxygen continuously through an oxygen mask. Continuous hemodynamic and surface electrocardiographic monitoring was maintained throughout the procedure. Lidocaine solution (2%) was subcutaneously administered at the cut-down site. An incision was made above the right carotid artery and the vessel dissected free. A Radifocus Introducer with an A 4-fr introducer sheath (Terumo Co., Tokyo, Japan), preloaded with a guide wire, was inserted into the vessel and advanced from the carotid artery into the distal descending aorta. The guide wire was then advanced to the proximal portion of the iliac artery. The stent delivery system was advanced to the distal portion of the iliac artery using the guide wire, and the stent was deployed to a pressure of 12 atm to achieve a stent-to-artery-size ratio range of 1.1–1.2:1.7. Intravenous heparin (100 U/kg) was administered immediately after stent deployment. Successful deployment was verified by angiography and the introducer sheath was removed. The right carotid artery was ligated, and the rabbits were allowed to emerge from anesthesia. Follow up was performed four weeks post implantation to observe sequential alteration; the animals underwent follow-up angiography in the same views as before and were sacrificed with 20 ml of potassium chloride through the left carotid artery injection.

2.13. Measurement of post-angioplasty restenosis by micro-computed tomography (micro-CT) imaging

The retrieved stent was stored in formaldehyde solution. A 1.5-ml Eppendorf tube was filled with clay, and the clay was tuned with a V shape to hold the stent during contrast agent staining. The stents were taken from the solution and placed vertically in the V-shaped opening in the clay. Each stent had to be fixed with the clay such that there was no movement of the stent inside the Eppendorf tube. The contrast agent used was omnihexol. One milliliter of the contrast agent was taken in a 5-ml syringe and injected through the opening at the center of the stent. The stent was incubated with contrast agent overnight and subjected to micro-CT imaging.

3. Results

3.1. Optimization of transfection efficiency with ssPEI

The ssPEI with different N/P ratios was complexed with luciferase pDNA and its transfection efficiency was compared with two positive controls of bPEI 25 kDa and lPEI 25 kDa. After 24 h of transfection, transgene expression was measured by luciferase assay (Fig. 1). Compared to other carriers, ssPEI exhibited higher gene transfection efficiency as well as increased gene expression with an increase in DNA concentration.

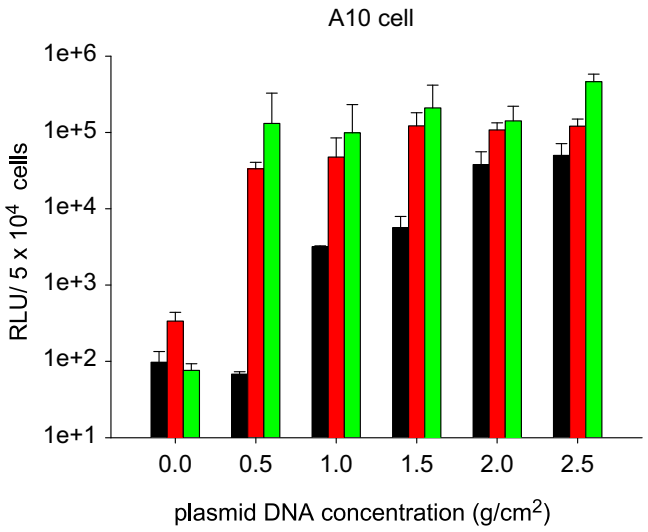

Fig. 1. Transgene expression in rat vascular smooth muscle cells (VSMCs) after the delivery of luciferase plasmid complexed with different gene carriers, such as bPEI 25 kDa (black), lPEI 25 kDa (red), and ssPEI (green), at varying plasmid concentrations (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

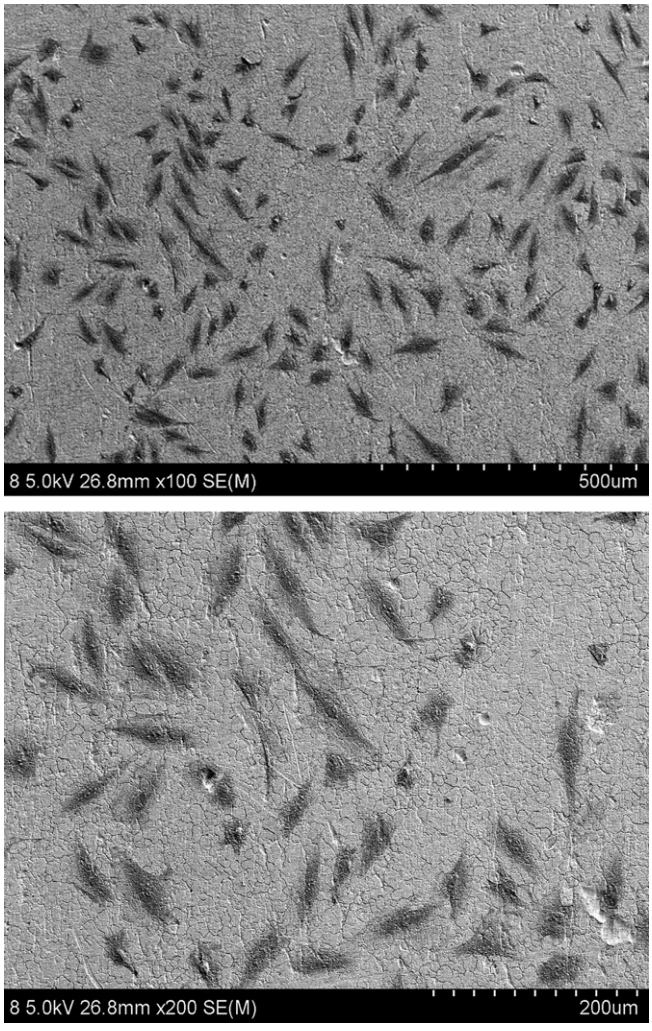

Fig. 2. SEM images of VSMCs adhering on the HA-coated surface after overnight culture.

3.2. Cell attachment studies on HA-coated stents

A10 VSMC lines were used to study cell attachment on HA-coated stents. It was found that a larger number of cells were well attached onto the surface of the stent, indicating a better binding of the cells on the stent surface, as illustrated in Fig. 2. Furthermore, the cells were spread over the stent uniformly, helping in the uptake of the immobilized material of ASNs. It is believed that the successful immobilization and delivery of ASNs to the cells attached on the stent would stop the migration and over-growth properties of the VSMCs happening as a side effect post-angioplasty.

3.3. Gel retardation assay

For an efficient siRNA delivery, ssPEI must form stable complexes with siRNA to prevent leaching and must condense it into compact nanoparticles. PAGE was performed with ASNs and Akt1 siRNA/bPEI 25 kDa nanoparticles (ABNs). siRNA was complexed efficiently with ssPEI or bPEI, and its movement was retarded in the gel, which was observed as the disappearance of thick bands in the gel due to the retarded migration at the loading well, for an N/P ratio of 10:40 (Fig. 3B). Compacted nanoparticles usually produce diminished fluorescence signals due to fluorescence quenching.

3.4. Size and surface charge of ASNs or ABNs

The ssPEI was complexed with Akt1 siRNA, and their physicochemical properties were analyzed using dynamic light scattering. The average particle size of ASNs was around 112 nm, which was similar to that of control ABNs. The surface charge of ASNs was found to be +35 mV. The sizes of the particles are important for the facilitated uptake, whereas surface charge plays a role in cellular interaction (Fig. 3A).

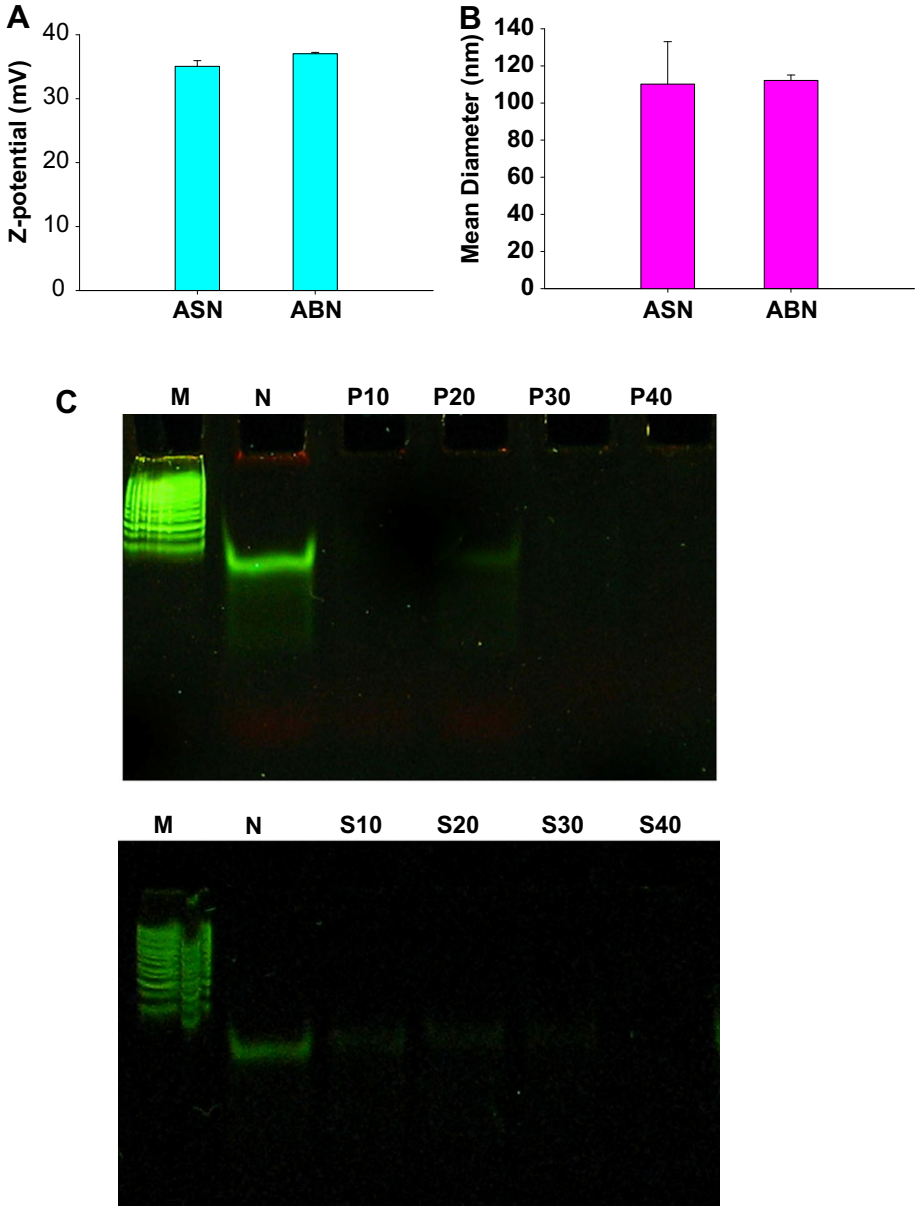

Fig. 3. Physico-chemical characteristics of ASNs. (A) Hydrodynamic size of ASNs and ABNs at a fixed N/P molar ratio of 10. (B) Zeta potential of ASNs and ABNs at an N/P molar ratio of 10. (C) A fixed amount of Akt1 siRNA was incubated with ssPEI (S) or bPEI (P) according to the different N/P molar ratios and was loaded onto polyacrylamide gel for electrophoresis. The complexes formed between Akt1 siRNA and ssPEI (or bPEI) were completely retarded from an N/P of 10.

3.5. Immobilization study of ASNs on HA-coated stent surface

In order to visualize the immobilization of the nanoparticles over the HA-coated stent surface, the pDNA was labeled with YOYO1 fluorescent dye. The YOYO1-labeled pDNA was incubated with ssPEI to form the complex. The complex was then coated over the HA-coated surface. After 24 h incubation, the complex was immobilized efficiently on the HA-coated stent surface and it was confirmed by observation with fluorescent microscopy, as shown in Fig. 4. The complexes were distributed uniformly over the stent, which is evident from the green color signal from the YOYO1-labelled DNA complexed with ssPEI. The HA coating over the stent had a negative charge, which helps in ionic interaction with the positively charged ssPEI/pDNA complex.

The FITC-labeled Akt1 siRNA was used to quantitate the percentage of immobilization over the stent. The FITC-labeled Akt1 siRNA was incubated with ssPEI at various N/P ratios to form the fluorescent ASNs. After complexation, they were immobilized over the HA-coated stent surface and placed in a plate with PBS buffer. After 24 h incubation, the solution was collected and the absorbance was measured. The supernatant solution was transferred to UV-transparent cuvettes and the absorbance was measured at 490 nm using a UV spectrophotometer. More than 80% of the ASNs were bound over the HA-coated surface and the binding efficiency of the ASNs on the HA-coated surface was not much affected, irrespective of the different mixing molar ratios of ssPEI and Akt1 siRNA (N/P 10 and N/P 20) (Fig. 5).

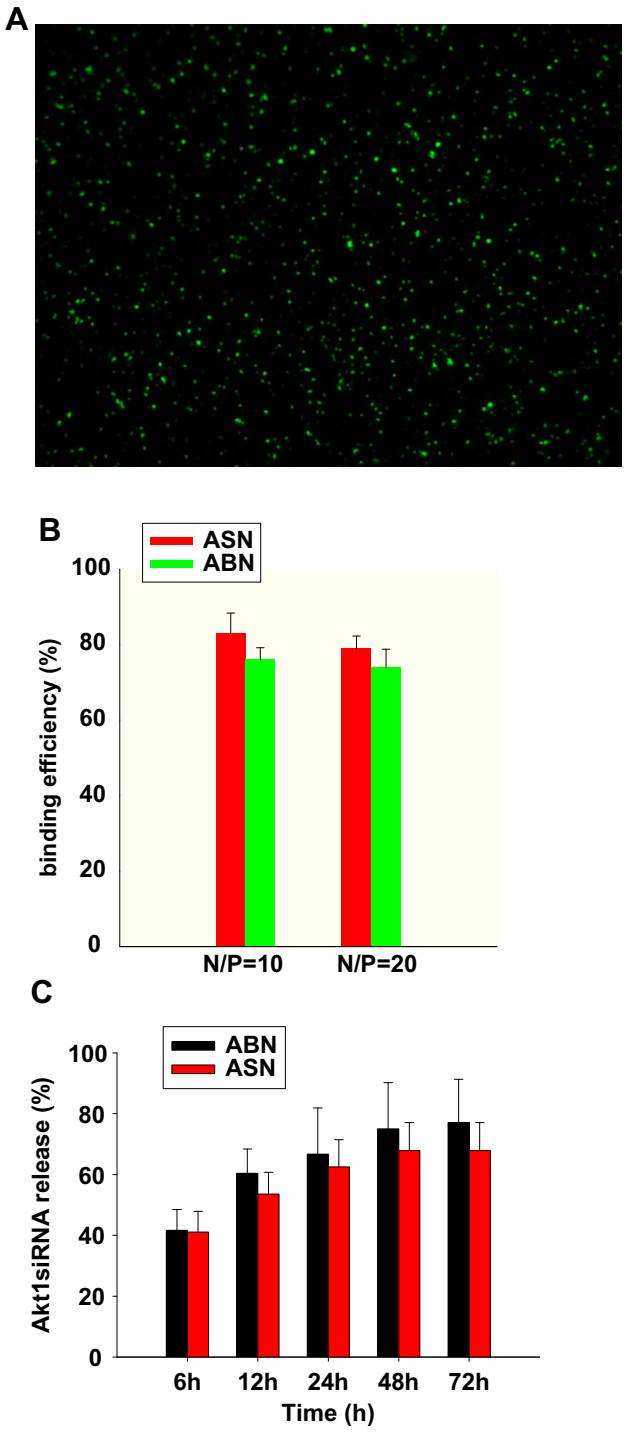

Fig. 4. (A) Fluorescent microscopic image of YOYO1-labeled ASNs immobilized on the HA-coated stent surfaces. (B) The binding efficiency of FITC-Akt1 siRNA/ssPEI nanoparticles on an HA-coated stent surface. FITC-Akt1 siRNA was complexed with ssPEI at N/P ratios of 10 and 20 and was then immobilized on the HA-coated stent surface. The unbound siRNA was measured by a UV spectrophotometer. (C) The release kinetics of Akt1 siRNA from either ASNs or ABNs immobilized on the HA-coated stent surface were measured at various time intervals, from 6 h to 72 h, using a UV spectrophotometer.

3.6. ASNs release from the HA-coated stent

The release of ASNs from the HA-coated stents surface in PBS buffer was analyzed by measuring the absorbance of the supernatants after each sampling interval; the buffer solution was removed and replaced with fresh PBS buffer (pH 7.4, 137 mM NaCl) and incubated. The solution was transferred into UV-transparent cuvettes and the absorbance at 490 nm was measured. From the absorbance value, the percentage of ASNs released from the HA-coated surface was calculated. Initially, at 6 h post-incubation, about 40% of the ASNs were released, and as time proceeded, gradual release of the ASNs was observed up to 72 h incubation. At 72 h, 60% of the complex was released, which was regarded as a controlled release in comparison with ABNs control (Fig. 4C).

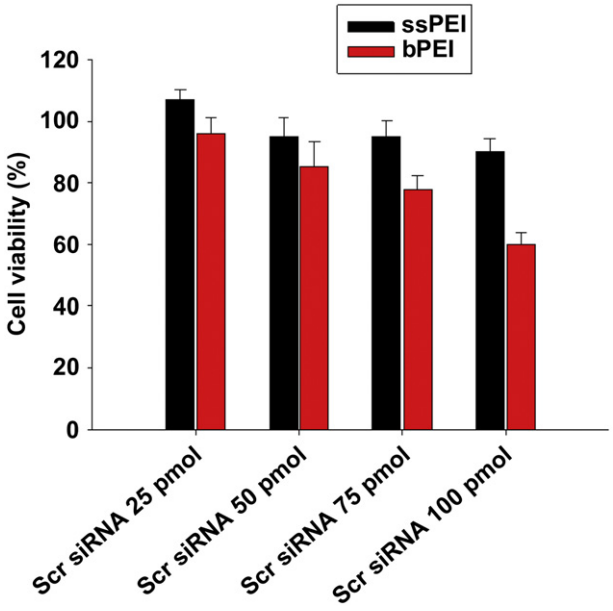

Fig. 5. The cell viability of VSMCs cultured on a siRNA/ssPEI nanoparticle-immobilized, HA-coated surface. Different amounts of Akt1 siRNA were complexed with ssPEI at a fixed N/P ratio and immobilized on the HA-coated surface. The cellular viability of VSMCs on the surface was measured using MTS assay.

3.7. Cell viability

The scr siRNA was complexed with ssPEI and bPEI to test their cytotoxicity against A10 cells. The scr siRNA was used to avoid the functional growth inhibitory effects of Akt1 siRNA. To analyze the cytotoxicity of ssPEI, an MTS assay was performed and the carrier bPEI/scr siRNA nanoparticles were used as a positive control. Varying concentrations of scr siRNA, 25 pmol, 50 pmol, 75 pmol, and 100 pmol, were tested for viability. The ssPEI/scr siRNA nanoparticles showed above 90% cell viability, even at the 100 pmol concentration of siRNA, when compared with the control. The viability of bPEI/scr siRNA nanoparticles was only 70% at 100 pmol concentration of siRNA (Fig. 5).

3.8. Akt1 suppression in VSMCs mediated by ASNs

A10 VSMCs were seeded on the ASNs-immobilized and HA-coated stent surface. After 24 h of incubation, the cells were collected from the stent surface and further processed for RT-PCR and western blotting. The total RNA and protein was isolated to check the suppression of the Akt1 protein at the mRNA and protein level, respectively. Compared to the control carrier, ABN-treated A10 cells, Akt1 mRNA was reduced in ASNs-treated cells, which is evident from the RT-PCR analysis data shown in Fig. 6A. The Akt1 siRNA delivered from ssPEI suppressed the Akt1 protein expression in A10 cells in an enhanced manner when compared to control carrier ABN treated cells (Fig. 6B). Akt1 protein suppression also

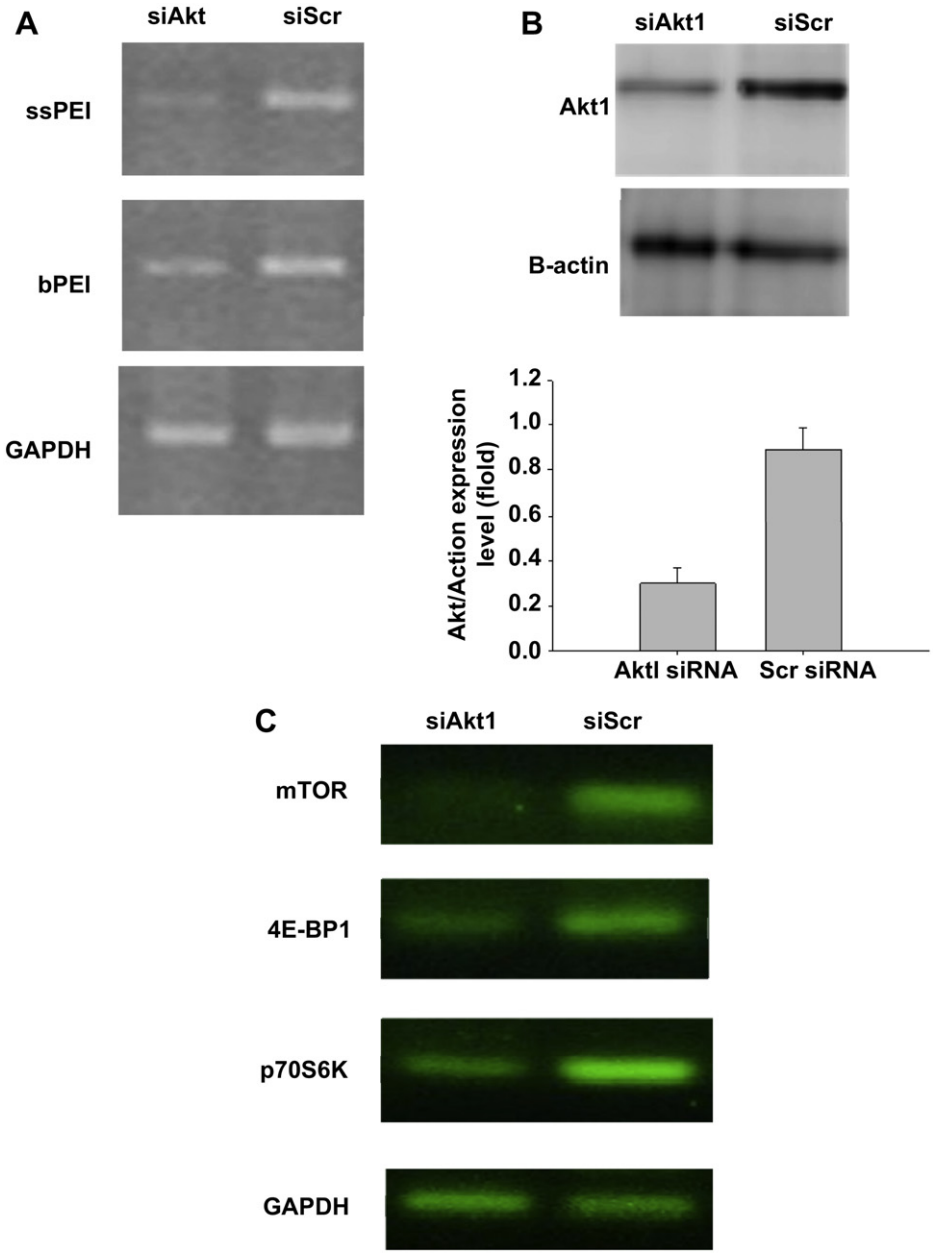

Fig. 6. Confirmation of Akt1 expression silencing after Akt1 siRNA treatments using an ssPEI carrier: (A) Semi-quantitative RT-PCR analysis for the Akt1 mRNA level and (B) western blot analysis for the Akt1 protein level (graph plotted after normalizing with housekeeping protein concentration, actin). (C) Suppression of Akt1 and downstream signaling proteins after the delivery of Akt1 siRNA to mRNA; the mRNA level was measured using RT-PCR.

affected its downstream signaling proteins, which was involved in cell survival. mTOR, 4E-BP1, and p70S6K were the downstream signaling proteins involved in the Akt1 pathway (Fig. 6C). These proteins were also down-regulated once the Akt1 is suppressed, which was confirmed by RT-PCR. The amount of mTOR was significantly reduced compared to the scramble siRNA-treated cells. In a similar fashion, 4E-BP1 and p70S6K protein expression was also reduced. This suggests that the Akt1 protein was successfully suppressed by the delivered Akt1 siRNA.

3.9. Micro-CT imaging of restenosis after angioplasty

After removing the implanted stents, they were subject to micro-CT imaging for calculating the percentage of tissue deposition over them. A stent without HA coating was used as the control in comparison with the HA-coated stent and ASNs-immobilized HA-coated stents. The in-stent restenosis (ISR) area over the stent surface was calculated according to the formula given in Fig. 7. The ISR area over the bare metal stent was aggravated compared to the

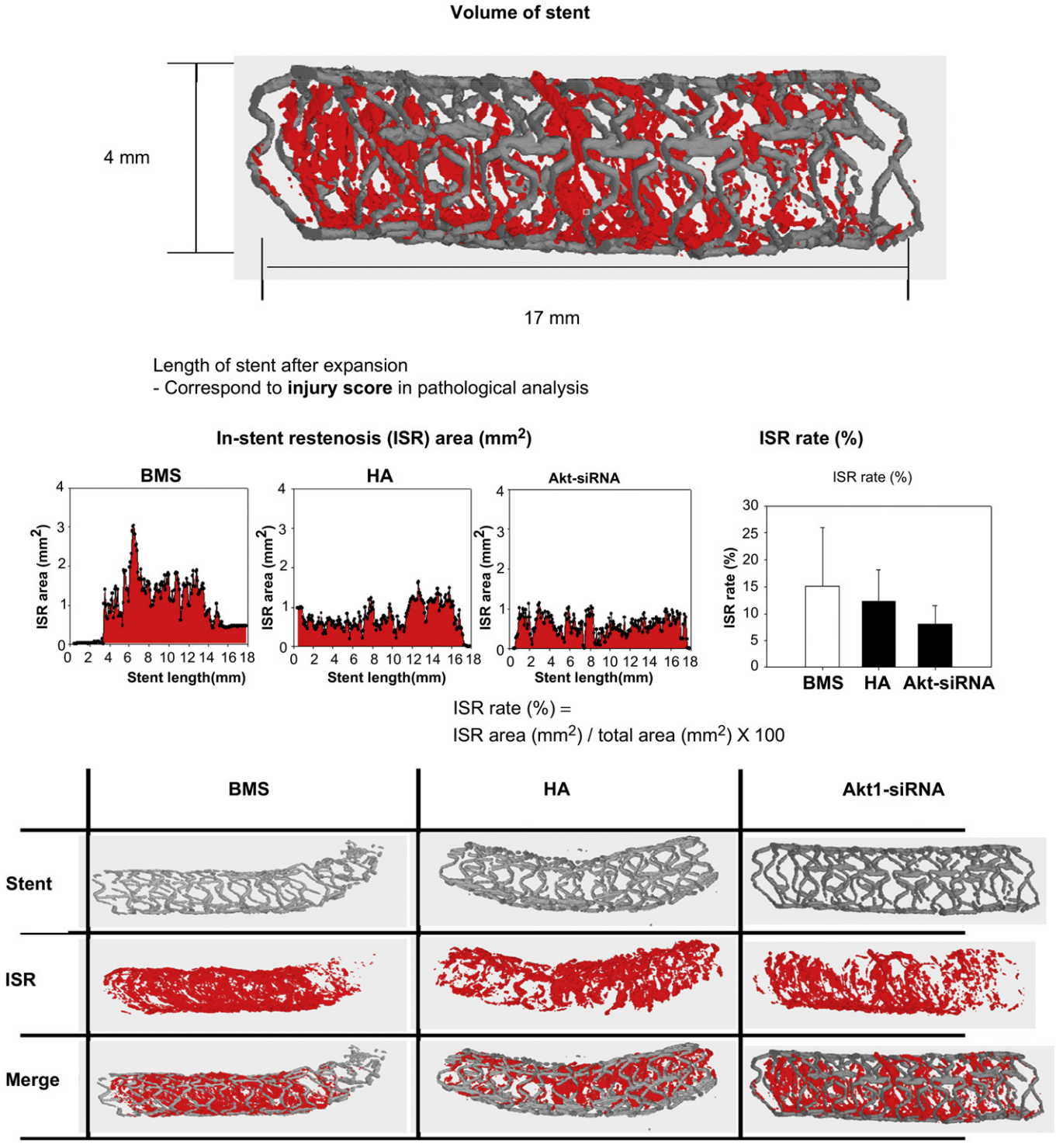

Fig. 7. Micro-CT images of restenosis with a bare metal stent, HA-coated stent, and ASNs/HA-coated stents at four-week angioplasty in the rabbit stent model.

HA-coated and ASNs-immobilized HA-coated stents. In particular, the ISR area over the ASNs-immobilized HA-coated stent was greatly reduced due to the suppression of the over-growth of VSMCs adhering to the stent surface, which was mediated by RNA interference with Akt1 siRNA. When the cells attached over the ASNs-immobilized HA-coated stent, the ASNs was internalized in cells efficiently and the Akt1 protein expression in VSMCs was suppressed, leading to cell death (Fig. 6).

4. Discussion

Substrate-mediated delivery enhances gene transfer by increasing the concentration of DNA in the cellular microenvironment. Immobilized complexes in a substrate-mediated fashion deliver therapeutic genes to primary cells with improved cellular viability and transfection efficiency, in comparison with bolus delivery. Maximal transfection from this approach requires the affinity of the DNA complex to the substrate that is sufficient for immobilization, but not so excessive that it limits cellular internalization [4–6].

In terms of carriers for gene delivery, although viral gene carriers possess high transfection efficiency, they have a major disadvantage due to their cellular mechanism, which results in toxicity. To overcome this, we focused on non-viral carriers. A cationic polymer PEI was used in this study, which forms a complex with a nucleic acid and is delivered intracellularly, increasing the transfection efficiency to match that of viral carriers. However, cytotoxicity was observed due to the carrier's non-degrading nature and higher charge density. Considering degradability, a disulfide bond was introduced to the PEI, which was stable in the oxidative extracellular environment, but degraded in the intracellular reductive environment. The ssPEI formed a complex with Akt1 siRNA, which was confirmed by PAGE analysis showing thick bands due to complex formation. The immobilization of ASNs over the HA-coated stent surface was efficient and their release was controlled from the surface. Animal and clinical studies have consistently shown that mural thrombosis and arterial VSMC proliferation occur predominantly near stent struts, and hence, a relatively small amount of stent-immobilized gene vectors strategically placed at the interface between the tissue and implant might be sufficient to produce a clinically significant therapeutic transduction of regional cells [20]. The spread of the vector to the non-target can be eliminated by attaching the complex on the surface of the stent, thereby protecting it from the shearing effect of the blood flow.

In our study, dopamine-conjugated HA was coated on the stent surface stably followed by the loading of ASNs on the HA surface of the stent. It is thought that steric hindrance of the surface-coated HA and the masking of HA with ASNs may delay the enzymatic degradation of HA-coated on coronary stent in the blood stream. It was also reported that polydopamine modified Polystyrene grafted with HA retains bioactivity in the long term without getting degraded in the presence of serum [21]. This is due to the fact that HA-linked dopamine forms a strong bond with various inorganic/organic surfaces [22].

Results from our study showed a better attachment of ASNs onto the HA-coated stent surface, and controlled release from them was also demonstrated. ASNs adhere electrostatically on the HA surface and the release of ASNs occurs as the ionic blood components and chemical ions weaken the ionic interaction of ASN with HA. In our study, it is believed that the enzymatic degradation of HA in the blood stream happens slowly after a faster release of ASNs to neighboring SMCs. The mechanistic and functional studies of an ASN-coated HA stent are currently underway in a cardiac infarction model and will be reported elsewhere.

The cells attached over the stent surface internalized the ASNs complex. The siRNA released inside the cytoplasm of the cells resulted in the suppression of Akt1 mRNA and protein, which was also confirmed through RT-PCR and western blotting analysis.

In vivo stent implantation studies also reflected *in vitro* results: the ISR area over the ASNs-immobilized HA-coated stent was reduced compared to the HA-coated stent and bare stent, demonstrating the suppression of restenosis.

The adhesion and proliferation of SMCs on the HA surface was already demonstrated in our study. It was also reported previously that HA suppressed SMC growth [23]. Based on micro-CT results (Fig. 7) and histological analysis (data not shown), it is believed that HA may have a role in the suppression of SMC growth. This suppressive effect of HA on SMC growth might be partially due to the slow degradation of the HA component from the stent surface.

In this work, we have demonstrated the successful coating of a biodegradable ssPEI carrier with Akt1 siRNA on the surface of HA-coated stents. Binding, transfection, and knock-down studies were successfully done with the developed carrier, and the results revealed that the effective knock-down of pro-proliferative Akt1 protein by Akt1 siRNA could retard the undesired growth of VSMCs near the stent, thereby preventing restenosis.

5. Conclusion

VSMC proliferation over the stent was suppressed by the delivery of Akt1 siRNA complexed with an ssPEI carrier on an HA-coated stent surface. The surface-mediated delivery resulted in higher protein expression compared to the bolus-mediated delivery, which is a benefit of stent-mediated delivery. The ssPEI showed reduced toxicity and higher gene expression compared to its non-degradable counterparts. The Akt1 siRNA was successfully delivered, and Akt1 suppression at the mRNA and protein level was confirmed with RT-PCR and western blotting. The downstream signaling proteins of Akt1, such as mTOR, 4E-BP1, and p70S6K, were down-regulated, which was determined by RT-PCR analysis. The Akt1 siRNA-coated stents were implanted in rabbits and the rates of tissue deposition over the stents were measured using micro-CT imaging. The results confirmed that the rate of restenosis was less pronounced when stents were coated with ssPEI complexed with Akt1 siRNA.

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