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*Advances in Brief*

# A Small Interfering RNA Targeting Vascular Endothelial Growth Factor as Cancer Therapeutics

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## Abstract

**Vascular endothelial growth factor (VEGF) plays a critical role during normal embryonic angiogenesis and also in the pathological angiogenesis that occurs in a number of diseases, including cancer. We developed a novel VEGF blockade system using RNA interference. The small interfering RNA (siRNA) targeting human VEGF almost completely inhibited the secretion of VEGF in a human prostate cancer cell line, PC-3, whereas the control scramble siRNA showed no effects. The VEGF siRNA with atelocollagen dramatically suppressed tumor angiogenesis and tumor growth in a PC-3 s.c. xenograft model. Atelocollagen provided a beneficial delivering means by which stabilization and efficient transfection of the siRNA injected into the tumors were achieved.**

## Introduction

Several RNA interference (RNAi) methodologies are rapidly being established and hold promise to specifically inhibit gene expression in mammals (1). RNAi is the sequence-specific, posttranscriptional gene silencing method initiated by double-stranded RNAs, which are homologous to the gene being suppressed. Double-stranded RNAs are processed by Dicer, a cellular RNase III, to generate duplexes of ~21 nt with 3′-overhangs [small interfering RNA (siRNA)], which mediate sequence-specific mRNA degradation (1, 2). RNAi technology, especially chemically synthesized siRNA, is currently being evaluated not only as an extremely powerful instrument for functional genomic analyses but also as a potentially useful method to develop highly specific gene-silencing therapeutics. However, there are only a few studies on the application of siRNA to the organismal level. This study was performed to suppress tumor angiogenesis by siRNA. There is compelling evidence indicating that uncontrolled angiogenesis is a major contributing factor in both tumor growth and metastasis (3–5). A number of growth factors have been identified as potential positive regulators of angiogenesis. Among them, vascular endothelial growth factor (VEGF) is the only growth factor most consistently found in a wide variety of conditions associated with angiogenesis (6). Inhibition of VEGF activity or disabling the function of its receptors has been shown to inhibit both tumor growth and metastasis in a variety of animal tumor models (7–9). We planned to suppress the synthesis of VEGF in the human prostate carcinoma cell line, PC-3, using siRNA and to evaluate its therapeutic significance in a xenograft model. For *in vivo* treatment, we used a new gene transfer method using a biomaterial, atelocollagen, prepared from bovine dermis (10). Atelocollagen is unique in that it is a liquid at 4°C and a gel at 37°C. Therefore, we expected atelocollagen to increase cellular uptake, nuclease resistance, and prolonged release of the siRNA administered into the tumor as has been shown in the case of plasmid DNA (10) and antisense oligodeoxynucleotide modified with a phosphorothioate backbone (11).

## Materials and Methods

**Preparation of siRNAs.** Five siRNAs targeting human VEGF and one scrambled siRNA (used for a positive control) with the following sense and antisense sequences were used: VEGF siRNA no. 1 (bases 131–149), 5′-UGGAUGUCUAUCAGCGCAGdTdT-3′ (sense), 5′-CUGCGCUGAUAGACAUCCAdTdT-3′ (antisense); VEGF siRNA no. 2 (bases 149–167), 5′-GCUACUGCCAUCCAAUCGAdTdT-3′ (sense), 5′-UCGAUUGGAUGGCAGUAGCdTdT-3′ (antisense); VEGF siRNA no. 3 (bases 189–207), 5′-GGAGUACCCUGAUGAGAUCdTdT-3′ (sense), 5′-GAUCUCAUCAGGGUACUCCdTdT-3′ (antisense); VEGF siRNA no. 4 (bases 290–308), 5′-CUGAGGAGUCCAACAUCACdTdT-3′ (sense), 5′-GUGAUGUUGGACUCCUCAGdTdT-3′ (antisense); VEGF siRNA no. 5 (bases 336–354), 5′-CCAAGGCCAGCACAUAGGAdTdT-3′ (sense), 5′-UCCUAUGUGCUGGCCUUGGdTdT-3′ (antisense); and VEGF siRNA no. 3-SCR, 5′-ACGCGUAACGCGGGAAUUUdTdT-3′(sense), 5′-AAAUUCCCGCGUUACGCGUdTdT-3′ (antisense). All siRNAs were designed by B-Bridge International, Inc. (Sunnyvale, CA) and synthesized by Dharmacon, Inc. (Lafayette, CO) using 2′-bis (acetoxyethoxy)-methyl ether-(ACE) protection chemistry. We selected siRNA sequences as reported by Elbashir *et al.* (12). Thus, we (*a*) searched for sequences 5′-AA(N19) or 5′-NA(N19), where N is any nucleotide, in the intended mRNA sequence (within an open reading frame and preferably 50–100 nt downstream of the start codon) and choose those with 47 or 52% G/C content; (*b*) Blast-searched the selected siRNA sequences against expressed sequence tag libraries to ensure that only a single gene is targeted; and (*c*) checked a predicted secondary structure of the intended mRNA to avoid a steric hindrance for its binding. Each freeze-dried siRNA was reconstituted with RNase-free water to prepare a 20 $\mu$M stock solution. The targeting sequence and its location of each siRNA in VEGF cDNA is shown in Fig. 1*A*. To inhibit the synthesis of all five alternative splicing variants of human VEGF, all target sites in this study were selected within exons 3 and 4. We also used fluorescein (FI)- or $^{32}$P-labeled VEGF siRNA no. 3-SCR to monitor the stability of siRNA upon injection into tumors grown in nude mice. To verify the sequence specificity of the RNAi system, VEGF siRNA no. 3 with one or two mismatches was also synthesized (Fig. 1*B*).

**Cell Culture Conditions and Transfection of siRNAs.** PC-3 cells (American Type Culture Collection, Manassas, VA) derived from human prostate adenocarcinomas were cultured in Ham's F-12 medium modified by Kaighn (F-12K) with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% $CO_2$. Cells were plated at a density of $2 \times 10^5$ cells/35-mm tissue culture dish. After 20 h and 70–80% confluence, the cells were transfected with siRNAs in serum-free medium using LipofectAMINE PLUS (Invitrogen, Carlsbad, CA). Then, 5 $\mu$l of each siRNA stock solution (20 $\mu$M) and the PLUS reagent (10 $\mu$l) were mixed in Opti-MEM (85 $\mu$l; Invitrogen) in a small sterile tube. After immediate mixing and incubation at room temperature for 15 min, the LipofectAMINE reagent (4 $\mu$l) in Opti-MEM (100 $\mu$l) was added, and the mixture was left at room temperature for 15 min. Then, 0.8 ml of F-12K was added to generate the siRNA-lipid complex. The PC-3 culture medium was removed, and the siRNA-lipid complex (1 ml total volume) was added. After incubation for 4 h at 37°C, 1 ml of F-12K with 10% fetal bovine

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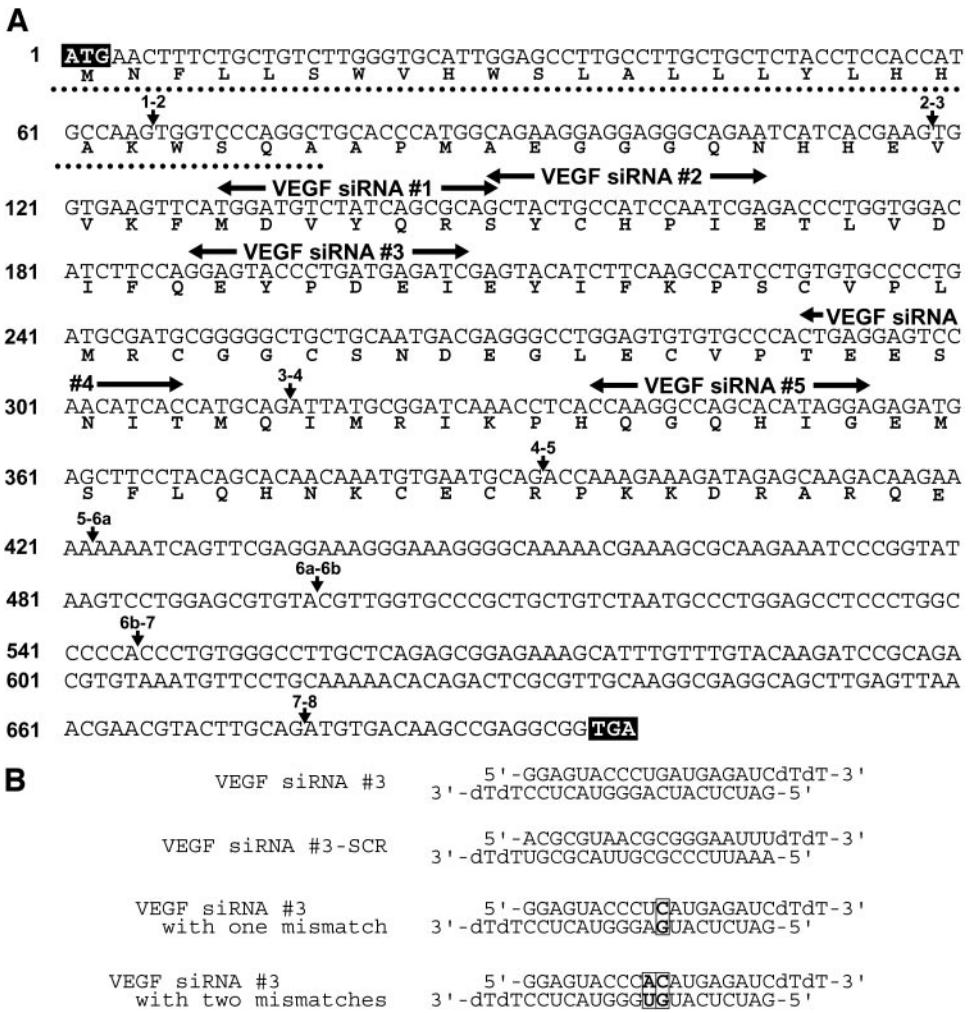


Fig. 1. *A*, design of vascular endothelial growth factor (VEGF) small interfering RNAs (siRNAs). Locations of VEGF siRNA nos. 1, 2, 3, 4, and 5 examined in this study are shown by *horizontal arrows*. ATG (■), the translation initiation site; TGA (■), the translation terminal site; ····, the signal peptide; *vertical arrows*, exon-intron junctions (number indicates exon numbers). *B*, structures of VEGF siRNA no. 3 determined as the most effective siRNA to human VEGF secretion in this study and its scrambled control (VEGF siRNA no. 3-SCR). We also used two siRNAs to verify the sequence specificity of VEGF siRNA no. 3. Structures of VEGF siRNA no. 3 with one mismatch and two mismatches were also shown. Mismatch site(s) was shown in box(es).

serum was added, and incubation was continued for 6 h. The medium was then replaced with fresh F-12K with heparin (20 $\mu$g/ml). After 16 h of incubation, conditioned medium was collected for ELISA.

**VEGF ELISA.** Secretion of VEGF into the cell culture supernatant and tumor contents of VEGF in the PC-3 xenografts were determined using a Quantikine human VEGF Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

**Quantifying mRNA of VEGF.** The concentrations of human VEGF mRNA were determined with a Quantikine mRNA colorimetric quantitation kit (R&D Systems) according to the manufacturer's instructions. Total RNA samples (5 $\mu$g) from PC-3 cells transfected with various siRNAs were assayed using human VEGF gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes. The concentration of human VEGF mRNA was calculated by interpolation of a standard calibration curve.

**Semiquantitative Reverse Transcription-PCR Analysis for VEGF Family Gene Expression.** Reverse transcription-PCR was performed with the isolated total RNA (1 $\mu$g) using Omniscript RT kit and HotStarTaq PCR kit (Qiagen) according to the manufacturer's instructions. Two primer set to detect VEGF-B (13), VEGF-C (14), VEGF-D (14), and placenta growth factor (13) was used, respectively. Details in conditions for PCR reaction were reported previously (13, 14).

**Preparation of $^{32}$P-Labeled siRNA.** VEGF siRNA no. 3-SCR were labeled at the 5′-end by T4 polynucleotide kinase (Takara Shuzo Co. Ltd.) and [$\gamma$-$^{32}$P]ATP (Amersham Bioscience) in 50 $\mu$l of reaction mixture. Thus, 5 $\mu$l of VEGF siRNA no. 3-SCR stock solution (20 $\mu$M), RNase-free water (30 $\mu$l), 10× kinase buffer (5 $\mu$l; Takara), 5 $\mu$l of T4 polynucleotide kinase (10 units/$\mu$l), and 5 $\mu$l of [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) were incubated at 37°C for 2 h. The reaction mixture was placed in the upper chamber of Microcon YM3 (cutoff value $M_r$ 3000; Millipore Corp.). After adding 200 $\mu$l of RNase-free water, it was centrifuged (4000 × *g*), and the procedure was repeated four times. Material remaining in the upper chamber was regarded as $^{32}$P-labeled VEGF siRNA (200 $\mu$l). Analysis of $^{32}$P-labeled VEGF siRNA by PAGE (15% gel) followed by autoradiography gave a single band.

**Cellular Uptake of $^{32}$P-Labeled siRNA.** PC-3 cells were transfected with $^{32}$P-labeled VEGF siRNA (100 nM; 213,965 cpm/dish) using LipofectAMINE PLUS as described above. On the next day, the cells were vigorously washed with PBS to remove incorporated $^{32}$P-labeled siRNA on the cell surface and were lysed with CelLytic-MT Mammalian Tissue Lysis/Extraction Reagent (Sigma Chemical Co.). After centrifugation, radioactivity in the supernatant was determined by a liquid scintillation counter (Beckman). Then, the efficiency for transfection was calculated.

**Tumor Therapy.** A total of 3.0 × 10$^6$ PC-3 cells were s.c. inoculated in 0.3 ml of serum-free F-12K medium through a 24-gauge needle into the lower flank of 8-week-old athymic nude mice obtained from SLC (Tokyo, Japan) as described previously (11, 15). After 3 weeks when the tumors had reached an average volume of ~50–60 mm$^3$, the tumor-bearing nude mice were treated with VEGF siRNA no. 3 with atelocollagen (Koken Co. Ltd., Tokyo, Japan). The final concentration of atelocollagen was 1.75% and that of the siRNA is described in Fig. 3*A*. siRNAs were used after dilution with PBS. As positive controls, PBS mixed with atelocollagen was injected. Each therapeutic reagent was injected into the tumors every 10 days after the first injection as indicated in Fig. 3*A*. Tumor diameters were measured at regular intervals with digital calipers, and the tumor volume in mm$^3$ was calculated by the formula: volume = (width)$^2$ × length/2 (16). Data are presented as mean ± SE. Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine.

**Intratumor Microvessel Density Assessment.** Immunohistochemical staining for CD31 and microvessel counting of CD31-positive vessels were performed as described previously (11).

**Analyses of Tumors Administered with FI-Labeled siRNA.** FI-labeled VEGF siRNA no. 3-SCR (VEGF siRNA-FI; 55.2 $\mu$g/tumor) with atelocollagen was directly injected into PC-3 tumor (tumor volume: ~100 $mm^3$) in the same manner as described in the above section. After 1 and 8 days, tumors were removed and snap frozen in liquid nitrogen and then 2-$\mu$m thick sections were cut with cryostat and observed under a confocal microscope system (MRC 1024; Bio-Rad). Various amounts of the VEGF siRNA-FI (10, 5, and 1 $\mu$M) with atelocollagen were also injected. After 1 and 8 days, excised tumors were homogenized in ice-cold CelLytic-MT Mammalian Tissue Lysis/Extraction Reagent (1 ml) with protease inhibitor mixture (Sigma Chemical Co., St. Louis, MO) and then centrifuged. The fluorescence intensity in each supernatant was measured by a Shimadzu Spectrofluorophotometer (Model RF-5000). The wavelengths for excitation and emission were 490 and 530 nm, respectively. Each fluorescence intensity value was corrected by a protein concentration.

**PAGE Analysis of $^{32}$P-Labeled siRNA Injected into the Tumors.** $^{32}$P-labeled siRNA with atelocollagen ($^{32}$P-labeled siRNA, 10 $\mu$M; atelocollagen, 1.75%) was injected into PC-3 tumors. On the next day and 2 and 7 days after the injection, each tumor was excised and homogenized in 0.5 ml of ice-cold 10 mM Tris-HCl (pH 8.0) with 0.1% Triton X-100, 50 mM NaCl, and RNase inhibitor (1 units/$\mu$l, Roche) and then centrifuged. Proteinase K (1 mg; Roche) was directly added to the supernatant and incubated for 6 h at 37°C and centrifuged. The supernatant was analyzed by PAGE (15% gel) followed by autoradiography.

**Statistical Analysis.** The data were analyzed using the Mann-Whitney *U* test, and *P*s $< 0.05$ were considered to indicate significant differences.

## Results

**Effects of VEGF siRNAs on the Expression of VEGF from PC-3 Cells.** We examined five VEGF siRNAs to target human VEGF as described in Fig. 1*A*. Of the five siRNAs synthesized, VEGF siRNA no. 3 potently suppressed the synthesis and secretion of VEGF in human prostate carcinoma cells, PC-3, after transfection with LipofectAMINE-PLUS (Fig. 2*A*). Scrambled siRNA (VEGF siRNA no. 3-SCR) showed no effects (Fig. 2*B*). ELISA for VEGF revealed that VEGF siRNA no. 3 (100 nM) suppressed VEGF production to 1.3–1.6% of that in the control cultures as shown in Fig. 2, *A* and *B*. On the basis of these results, we selected VEGF siRNA no. 3 as the most highly functional. To investigate the specificity of the RNAi system, we made mutant siRNAs containing either one or two central mismatches (Fig. 1*B*). We found that the single and double mutants lost their RNAi activity (Fig. 2*B*). VEGF siRNA no. 3 decreased the VEGF mRNA level compared with VEGF siRNA no. 3-SCR or untreated cultures (Fig. 2*C*), whereas the expression level of VEGF-B, VEGF-C, VEGF-D, and placenta growth factor did not change upon transfection of VEGF siRNA no. 3 (Fig. 2*D*). We also investigated cellular uptake of siRNA after transfection. After uptake of VEGF siRNA-FI by PC-3 cells, a characteristic spotty distribution around the nuclei was observed (Fig. 2*E*). The efficiency for transfection (percentage) to the cells using $^{32}$P-labeled siRNA was estimated as 85.2 ± 1.2% ($n = 3$ dishes).

**Treatment of the Established PC-3 Xenograft by VEGF siRNA.** PC-3 cells ($3.0 \times 10^6$) were injected s.c. into the flank of nude mice. By 3 weeks, visible tumors had developed at the injection sites (mean tumor volume, 54.2 $mm^3$; $n = 30$). To determine the therapeutic effectiveness of VEGF siRNA, intratumoral treatment with VEGF siRNAs with atelocollagen or atelocollagen alone was started and repeated every 10 days for a total of four times. As shown in Fig. 3, *A* and *B*, VEGF siRNA no. 3 markedly suppressed tumor growth compared with VEGF siRNA no. 3-SCR or atelocollagen alone ($P < 0.001$). These growth inhibitory effects were dependent on the dose of the VEGF siRNA no. 3 (Fig. 3*A*). Furthermore, we also found

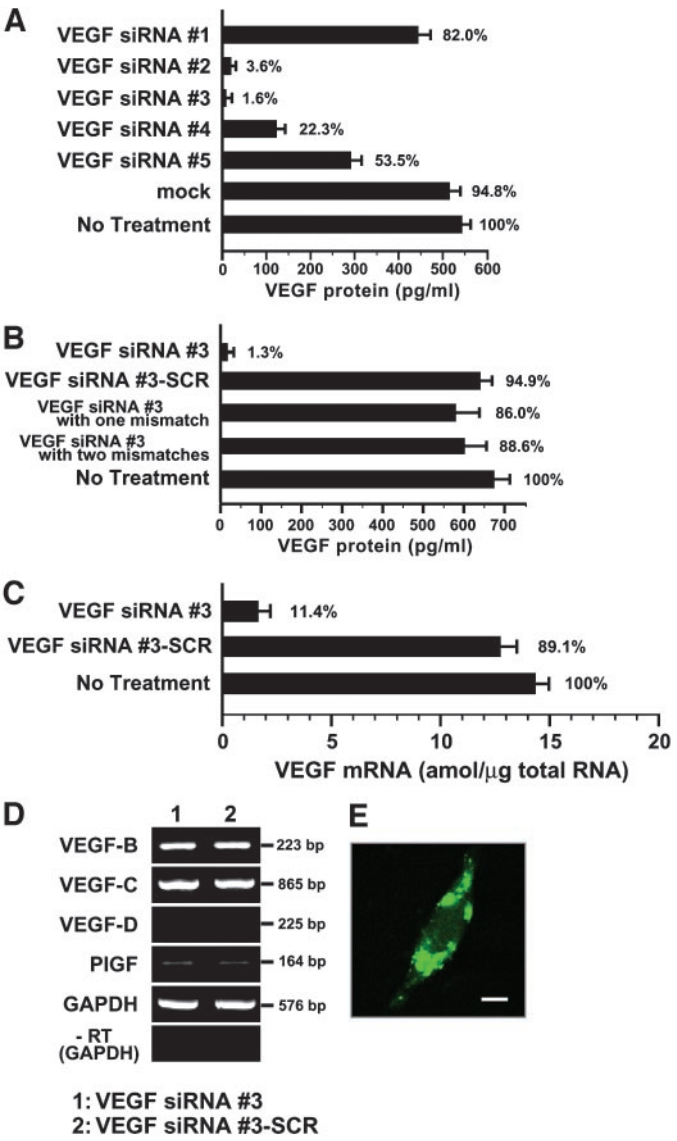


Fig. 2. Decrease in secreted vascular endothelial growth factor (VEGF) and the mRNA levels in PC-3 cells transfected with various small interfering RNAs (siRNAs). *A*, cells were transfected with VEGF siRNA nos. 1, 2, 3, 4, and 5, LipofectAMINE-PLUS alone (mock), or no siRNA, respectively. *B*, cells were transfected with VEGF siRNA no. 3, VEGF siRNA no. 3-SCR, VEGF siRNA no. 3 with one mismatch, VEGF siRNA no. 3 with two mismatches, or no siRNA, respectively. In both *A* and *B*, VEGF concentration in the conditioned media was determined by ELISA for human VEGF. Each *bar* represents the mean ± SD ($n = 4$ dishes). *C*, decreased VEGF mRNA levels of PC-3 cells transfected with various siRNAs. VEGF mRNA levels were determined with a Quantikine mRNA colorimetric quantification kit. Each *bar* represents the mean ± SD ($n = 3$ dishes). *D*, the expression of VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) mRNA in the cells transfected with VEGF siRNA no. 3 or VEGF siRNA no. 3-SCR. Total RNA was extracted from the cells and processed for reverse transcription-PCR (RT-PCR). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a control. *Lane 1*, VEGF siRNA no. 3; *Lane 2*, VEGF siRNA no. 3-SCR. Predicted size of each PCR product was shown in the figure. −RT, no reverse transcriptase. *E*, cellular uptake of VEGF siRNA-FI. PC-3 cells were transfected with VEGF siRNA-fluorescein for 4 h, washed with PBS, fixed in 4% paraformaldehyde, mounted with a ProLong Antifade kit, and subjected to confocal microscopy. *Bar*, 10 $\mu$m.

that the decrease of the VEGF contents in tumors treated with VEGF siRNA no. 3 was correlated with the injected doses of VEGF siRNA no. 3 (Fig. 3*C*). No gross adverse effects, *i.e.*, the loss of body weight, were observed during the experimental periods (data not shown).

**Tumor Vascularity *in Vivo*.** To assess the relationship between the therapeutic effects by VEGF siRNA and tumor-associated neovascularization, we stained intratumoral vessels with CD31/PECAM-

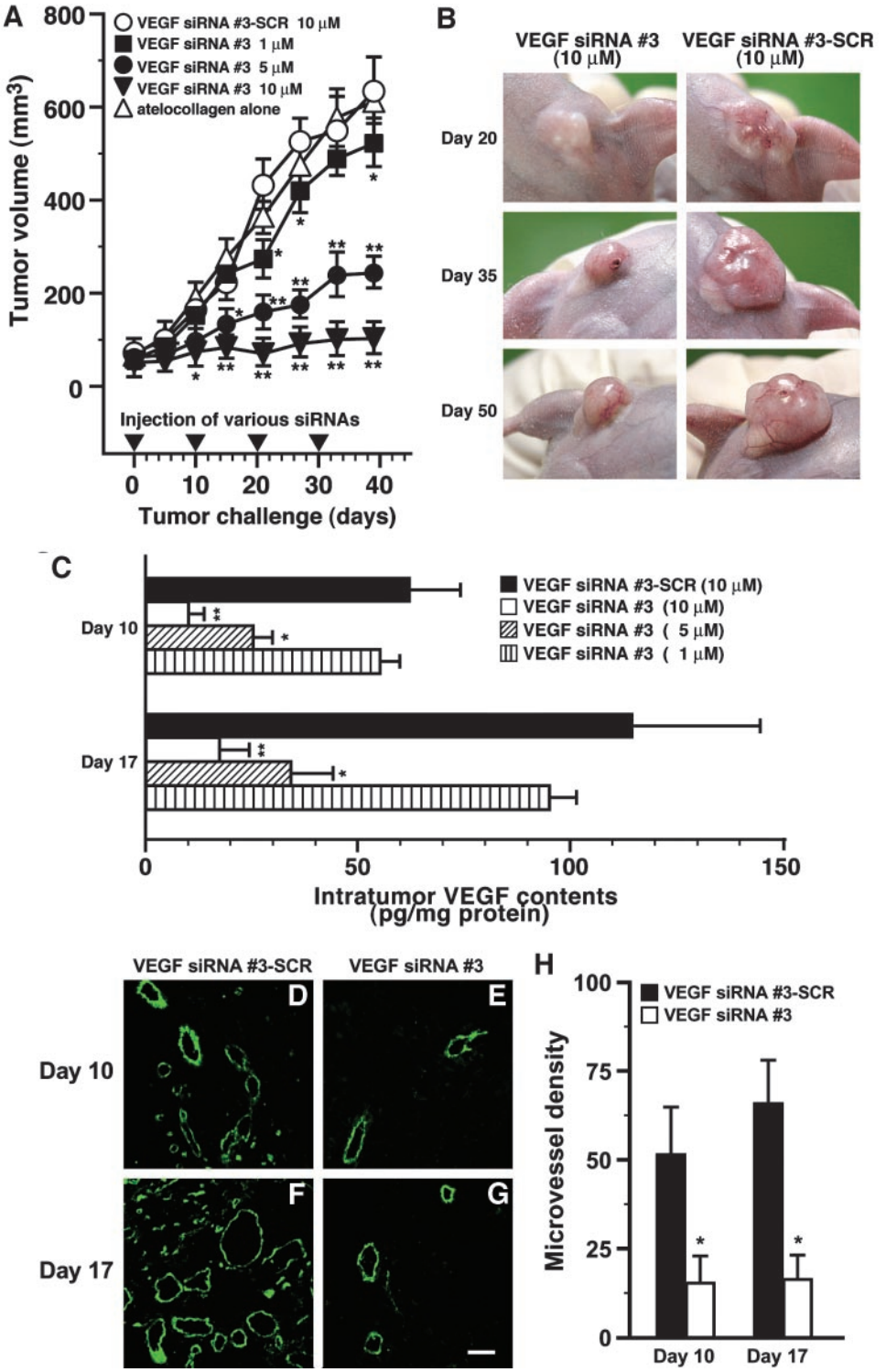


Fig. 3. Antitumor effect of vascular endothelial growth factor (VEGF) small interfering RNA (siRNA) in the PC-3 xenograft. *A*, tumor growth curves. On days 0, 10, 20, and 30, VEGF siRNA no. 3 (■, 1 μM; ●, 5 μM; ▲, 10 μM), VEGF siRNA no. 3-SCR (○, 10 μM), or PBS (△) was mixed with atelocollagen, and 50 μl of each mixture were injected into the tumor region, as indicated in the figure. Day 0 corresponds to 3 weeks after inoculation of cells when the tumor volume was ~50–60 $mm^3$. Tumor diameters were measured at a regular interval for up to 40 days with calipers, and the tumor volume was calculated. Results represent the means ± SE ($n = 6$ tumors). *, $P < 0.05$; **, $P < 0.001$ *versus* VEGF siRNA no. 3-SCR 10 μM. *B*, photos of PC-3 xenografts. During VEGF siRNA treatment (concentration of siRNA: 10 μM), PC-3 tumors were photographed. *C*, VEGF contents in tumors. Experiments were performed as in *A*. The excised tumors (on days 10 and 17) were homogenized in ice-cold CelLytic-MT Mammalian Tissue Lysis/Extraction Reagent with protease inhibitor mixture (Sigma Chemical Co.) and then centrifuged. The amount of VEGF in each supernatant was measured by ELISA. **, $P < 0.001$; *, $P < 0.01$ *versus* VEGF siRNA no. 3-SCR (10 μM). Results represent the mean ± SD ($n = 4$ tumors). *D–H*, decreased vessel density in tumors treated with VEGF siRNA. Histological sections from tumors injected with VEGF siRNA no. 3 or VEGF siRNA no. 3-SCR mixed with atelocollagen were immunostained for endothelial cells with anti-CD31 antibodies. We examined four excised tumors on both day 10 and 17 after injection and the intratumoral microvessel density (vessels/$mm^2$) was determined. *D* and *F*, VEGF siRNA no. 3-SCR; *E* and *G*, VEGF siRNA no. 3. *Bar*, 50 μm. *H*, decreased microvessel density in tumors treated with VEGF siRNA no. 3. Results represent the mean ± SD ($n = 4$ tumors). *, $P < 0.001$ *versus* VEGF siRNA no. 3-SCR.

1-specific antibody. Representative results of this staining are shown in Fig. 3*D–G*, and quantitative results of the microvessel density analysis are given in Fig. 3*H*. We observed a dramatically lower microvessel density in tumors treated with VEGF siRNA no. 3 at both 10 and 17 days after the first injection, whereas the tumors treated with VEGF siRNA no. 3-SCR showed a higher microvessel density (Fig. 3*D–H*).

**Effects of Atelocollagen on Stability of Injected siRNA.** To verify the capability of atelocollagen to stabilize the injected siRNA in tumors, we injected FI-labeled VEGF siRNA no. 3-SCR (VEGF siRNA-FI) mixed with atelocollagen directly into PC-3 tumors. Representative images of the cryostat sections were shown in Fig. 4*A*. We observed a strong green fluorescence in tumors upon injection of VEGF siRNA-FI with atelocollagen at least up to 8 days after injection while only a weak fluorescence in VEGF siRNA-FI alone. Furthermore, fluorescence intensity in tumors treated with VEGF siRNA-FI with atelocollagen was quite higher than those of VEGF siRNA-FI alone (Fig. 4*C*). Intensities of fluorescence remained in the tumors correlated with the injected amounts of VEGF siRNA-FI mixed with atelocollagen (Fig. 4*C*). These observations suggested that

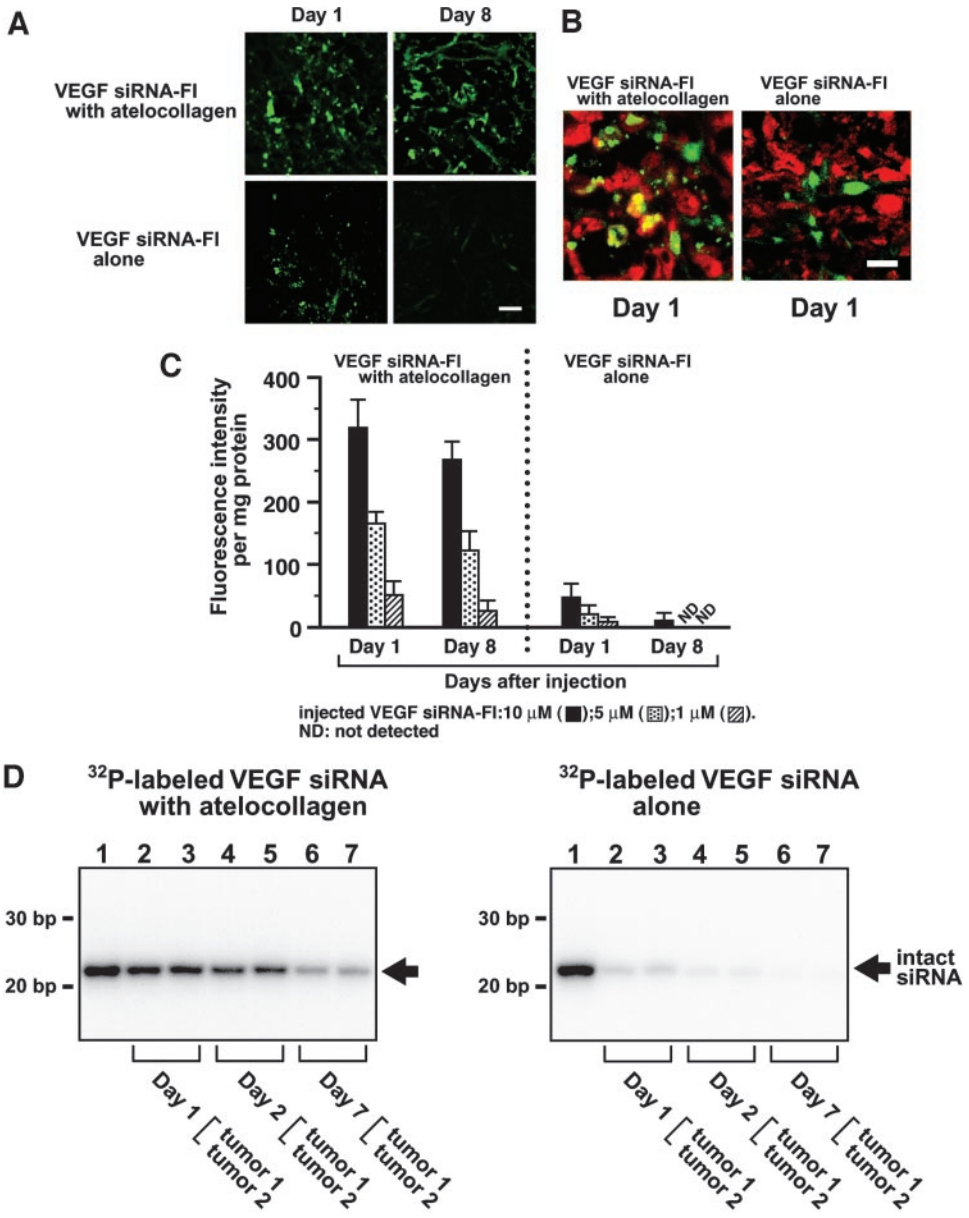


Fig. 4. Role of atelocollagen as a delivery reagent of siRNA *in vivo*. *A*, vascular endothelial growth factor (VEGF) small interfering RNA-fluorescein (siRNA-FI; 55.2 $\mu$g) with or without atelocollagen were directly injected in PC-3 tumors ($\sim$100 mm$^3$). On the next day and 8 days after injection, tumors were excised and sections were cut. Fluorescence was observed under a confocal microscope. *Bar*, 100 $\mu$m. *B*, nuclei were stained with propidium iodide (0.3 $\mu$g/ml), and the merged images with higher magnifications using oil lens were obtained. *Bar*, 20 $\mu$m. *C*, fluorescence intensity in homogenized supernatant from each tumor was measured. Each concentration of VEGF siRNA-FI (10, 5, and 1 $\mu$M) with or without atelocollagen were injected in tumors as in *A*. On the next day and 8 days after injection, tumors were excised and homogenized. ND, not detected. Results represent the mean $\pm$ SD ($n$ = 3 tumors). *D*, autoradiograms of $^{32}$P-labeled VEGF siRNA injected into tumors. $^{32}$P-labeled VEGF siRNA with or without atelocollagen was injected into PC-3 tumors. On the next day and 2 and 7 days after injection, tumors were excised, homogenized, and digested with proteinase K. Supernatant after centrifugation was analyzed with PAGE (15% gel), and autoradiogram was obtained. *Left panel*, $^{32}$P-labeled VEGF siRNA with atelocollagen; *right panel*, $^{32}$P-labeled VEGF siRNA alone. *Lane 1*, intact $^{32}$P-labeled VEGF siRNA; *Lanes 2* and *3*, $^{32}$P-labeled VEGF siRNA from tumors on day 1; *Lanes 4* and *5*, $^{32}$P-labeled VEGF siRNA on day 2; *Lanes 6* and *7*, $^{32}$P-labeled VEGF siRNA on day 7. *Arrows*, intact $^{32}$P-labeled VEGF siRNA (21-mer) before injection.

atelocollagen contributed to the increased stability of VEGF siRNA injected in tumors and that the injection interval (every 10 days) of VEGF siRNA upon tumor treatments (Fig. 3*A*) was appropriate. Moreover, VEGF siRNA-FI was visible inside tumor cells in the case of transfection with atelocollagen *in vivo* (yellow color) but not in the case of transfection without atelocollagen (Fig. 4*B*).

Finally, using $^{32}$P-labeled siRNA, we investigated whether the siRNA injected in tumors remained intact and, if intact, how long it persisted. $^{32}$P-labeled siRNA mixed with atelocollagen existed at least 7 days in tumors and remained intact (Fig. 4*D*). On the other hand, $^{32}$P-labeled siRNA alone was rapidly degraded and vanished from the tumors (Fig. 4*D*).

## Discussion

A siRNA to VEGF successfully inhibited the secretion and expression of VEGF in PC-3, human prostate carcinoma cells, leading to the potent suppression of tumor growth in its xenograft model. These results clearly demonstrated that a novel VEGF blockade system by RNAi is valid as a therapeutic. It is indispensable to use a suitable siRNA delivery system to obtain the maximal therapeutic effects. In this study, atelocollagen, a new gene delivery system, contributed significantly to the antitumoral therapeutic effect of VEGF siRNA. Atelocollagen, which was developed by Ochiya *et al.* (10), is soluble at a lower temperature ($<$10°C) but solidifies to refibrillation upon injection into an animal body (37°C). They have developed a technology in which plasmid DNA is embedded in atelocollagen by which the quantity and period of the gene expression are well controlled *in vivo* (10). Atelocollagen can protect plasmid DNA, antisense oligodeoxynucleotide, and adenoviral vectors from degradation by several nucleases, proteases, and antibodies, thereby prolonging the half-life of embedded biomaterials (10, 17). In the present study, we successfully demonstrated, by using FI- and radiolabeled siRNAs, that atelocollagen actually stabilized the siRNA injected in the tumors for at least a week (Fig. 4). Atelocollagen was, thus, crucial to extend a half-life of the siRNA dramatically and to keep it intact when embedded in the animal body. We also observed that atelocollagen was effective on the transfection of siRNAs *in vivo* (Fig. 4*B*). Furthermore, atelocollagen shows neither antigenicity nor toxicity in animals be-

cause antigenic telopeptides attached to both ends of collagen are eliminated by pepsin digestion (10). Consistently, atelocollagen caused no obvious hepatocellular or renal damage upon local injection into the tumor in nude mice (Y. Takei, unpublished data). Taken together, we conclude that atelocollagen is a superior delivery reagent of siRNAs *in vivo*.

Bergers *et al.* (18) have shown that the makeup of the tumor vasculature varies at different stages of tumor development, so the inhibitor efficacy might depend on its application during a specific phase of tumorigenesis. In fact, they observed that the VEGF receptor inhibitor SU5416 blocks the angiogenic switch and prevents the growth of premalignant tumors but does not induce regression of late-stage, well-vascularized tumors (18). This reveals the importance of VEGF signaling during the angiogenic switch and initial tumor growth but not in large tumors with an established vasculature. In light of these observations, we began the treatment with VEGF siRNA when the tumors inoculated in nude mice were still small (initial tumor volume = 50–60 $mm^3$) before the vasculature was well established in the tumors.

In conclusion, we demonstrated potent growth inhibitory effects of VEGF siRNA on prostate carcinoma *in vivo*, potentially applicable to the treatment of cancers as an antiangiogenic therapeutic.

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