Administration of PLK-1 small interfering RNA with atelocollagen prevents the growth of liver metastases of lung cancer

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Abstract
Liver metastasis is one of the most important prognostic factors in lung cancer patients. However, current therapies are not sufficient. RNA interference provides us a powerful and promising approach for treating human diseases including cancers. Herein, we investigated the in vitro effects of PLK-1 small interfering RNA (siRNA) on human lung cancer cell lines and the in vivo usage of PLK-1 siRNA with atelocollagen as a drug delivery system in a murine liver metastasis model of lung cancer. PLK-1 was overexpressed in cell lines and in cancerous tissues from lung cancer patients. PLK-1 siRNA treatment inhibited growth and induced apoptosis in a concentration-dependent manner. To verify in vivo efficacy, we confirmed that atelocollagen was a useful drug delivery system in our model of implanted luciferase-labeled A549UC cells by detecting reduced bioluminescence after an i.v. injection of luciferase GL3 siRNA/atelocollagen. PLK-1 siRNA/atelocollagen was also successfully transfected into cells and inhibited the progression of metastases. This study shows the efficacy of i.v. administration of PLK-1 siRNA/atelocollagen for liver metastases of lung cancer. We believe siRNA therapy will be a powerful and promising strategy against advanced lung cancer. [Mol Cancer Ther 2008;7(9):2904–12]

Introduction
The discovery of RNA interference (1) led to its wide application as a powerful tool in postgenomic research. Recently, small interfering RNA (siRNA), which induces RNA interference, has been experimentally introduced into cancer therapy. However, the establishment of siRNA therapy depends on the development of suitable delivery systems. Recently, several useful drug delivery systems (DDS) for siRNA have been developed (2–6). Among them, atelocollagen is one of the most unique and attractive DDS. It was developed as a biomaterial carrier for gene delivery and is obtained from type I collagen of calf dermis using pepsin treatment (3, 7).

Polo-like kinases (PLK) belong to the family of serine/threonine kinases and are highly conserved among eukaryotes (8). PLK-1, which is the best characterized of the PLK family, is crucial for cell division. PLK-1 promotes mitotic entry and also controls mitotic progression in mammalian cells (9). Elevated PLK-1 expression is observed in a wide variety of cancers. Moreover, overexpression of PLK-1 has also been reported to positively correlate with the survivals of cancer patients (10–13). Therefore, PLK-1 is considered to be a suitable target for cancer therapy (14). We recently showed that PLK-1 is a prognostic marker of urinary bladder carcinoma and that the intravesical administration of PLK-1 siRNA inhibited the growth of bladder cancer in an orthotopic murine model (15).

Lung cancer is the leading cause of cancer-related deaths. Moreover, liver involvement is a prognostic factor for the patients with lung cancers (16, 17). Despite the development of new chemotherapeutic agents, current therapies are not sufficient to inhibit liver metastasis (18, 19). Herein, we have shown that PLK-1 siRNA induced cell death in lung cancer cells and that the i.v. administration of PLK-1 siRNA/atelocollagen complex inhibited the growth of metastatic liver tumors of lung cancer in a mouse model.

Materials and Methods
Patients and Human Samples
All 79 patients underwent complete resection with lobectomy or pneumonectomy and mediastinal lymph node dissection at the Department of Thoracic Surgery, Kyoto University Hospital, between 1996 and 1998. In...
accordance with the Declaration of Helsinki recommendations, approval was obtained from the institutional review board at Kyoto University Hospital, and informed consent was obtained from all patients. Resected samples were fixed in formalin, embedded in paraffin, and used for microscopic analysis. Clinical and pathologic data were obtained by retrospective chart review.

**Cell Lines, Reagents, and Animals**

The human non-small cell lung cancer cell lines A549 and H1299 and the human small cell lung cancer cell lines SBC-3 and SBC-5 were cultured in RPMI 1640 (Life Technologies) containing 10% heat-inactivated FCS (Invitrogen), L-glutamine (Life Technologies), and 1% penicillin-streptomycin (Life Technologies). The normal human dermal fibroblast (NHDF) cells and Fb normal human fibroblast (NHF) cells were cultured in DMEM (Life Technologies) containing 10% FCS, L-glutamine, and 1% penicillin-streptomycin. All cell lines were maintained at 37°C in a fully humidified atmosphere of 5% CO2 in air. A549 and H1299 lung cell lines were obtained from the American Type Culture Collection. SBC-3 and SBC-5 cell lines were kindly provided by Dr. Nakanishi (Kyusyu University). NHDF cells were purchased from Kurabo and NHF cells were purchased from Cell Systems.

Four kinds of PLK-1 (GenBank accession no. NM_005030) siRNAs were previously generated and successfully suppressed the PLK-1 expression in bladder cell carcinoma cell lines (15). Of those siRNAs, we used siRNA1412. The sequence of the sense strand was 5′−CCUUAGUAAGAA-GAUCACdTdT-3′ and that of the nonsense siRNA was 5′−UCUCAGAAGUACCGUDdTdT-3′, which was used as the control. The sequence of scramble siRNA, which was used in a modified MTT assay, was 5′−UUGACGCAA-GUGGCAAAATTt-3′. We also synthesized luciferase (Luc) GL3 siRNA using the sequence as reported previously (20). All siRNAs used were chemically synthesized (Takara). LipofectAMINE 2000 (Invitrogen) was used for transfection in the in vitro experiments. Atelocollagen, which was kindly provided by Dainippon Sumitomo Pharmaceutical and Koken, was used for transfection in the in vivo experiments. Specific pathogen-free 6- to 7-week-old BALB/c nu/nu mice (Clea Japan) were used for the in vivo experiments. Approval for these studies was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

**Growth-Inhibitory Effects of PLK-1 siRNA in vitro**

Cell proliferation was determined by the modified MTT assay using the Cell-counting kit-8 (Dojindo Laboratory) as described previously (15). Cells were seeded in a flat-bottomed 96-well plate (Becton Dickinson) at 3 × 103 in 100 μL medium per well and incubated with graded concentrations of siRNA solution of 3, 10, 30, and 100 nmol/L. The mean of four samples was calculated.

**Cell Cycle and Apoptosis Analysis**

Cell cycle analysis using propidium iodide (PI) was done as described previously (21). Apoptosis induced by each siRNA treatment was determined using Annexin V-FITC Apoptosis Detection kit I (BD PharMingen) done as recommended by the manufacturer. Cells were analyzed with FACS Calibur using CellQuest software (BD Bioscience). Caspase-3 activity induced by the siRNA treatment was evaluated using a fluorometric protease assay kit (MBL) according to the manufacturer’s protocol.

**Western Blotting Analysis**

Following the transfection of PLK-1 siRNA into cells, as described above, the medium was aspirated and the cells were washed with ice-cold PBS (-). Ice-cold radioimmuno-precipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 0.25 mol/L NaCl, 5 mmol/L EDTA, 20 mmol/L NaF, 1% NP-40] with freshly added phenylmethylsulfonyl fluoride (1 mmol/L) and protease inhibitor (10 μg/mL) was added to the plate. The cells were then scraped, and the suspension (cells with lysis buffer) was transferred into a centrifuge tube, which was placed on ice for 15 min with an occasional vortex to ensure complete lysis of the cells. The cell suspension was cleared by centrifugation at 14,000 × g for 30 min at 4°C, and the supernatant (total cell lysis) was either used immediately or stored at -80°C. The protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories). Immunoblotting was done as described previously (21). A rabbit polyclonal anti-PLK-1 antibody (Upstate Biotechnology) and a rabbit polyclonal anti-actin antibody (Sigma-Aldrich) were used as the primary antibodies.

**Real-time Quantitative Reverse Transcription-PCR**

We investigated the changes of PLK mRNA transcriptons on A549 cells by PLK-1 siRNA treatment. Total RNA was extracted by using the Micro-to-Midi Total RNA Extraction Kit (Invitrogen) and subjected to reverse transcription (22). The mRNA levels of human PLK-1, PLK-2, and PLK-3 were analyzed by using the LightCycler System (Roche Diagnostics) with FastStart DNA Master SYBER Green I (Roche). Amplicons were validated by their melting curve and electrophoresis. The expression levels of the target mRNAs were normalized with that of the housekeeping gene β-actin. The specific primers for PLK-1 were forward 5′−GCCCTCAAGTCTGCTGTA and reverse 3′−CTGTTTCACTGGAACCCG. The specific primers for PLK-2 were forward 5′−AGGGACTTTGGCAGCTGTA and reverse 3′−GATAATGCGTCTCGAACCCG. The specific primers for PLK-3 were forward 5′−GGCGCTTTGGGTGATACACTGT and reverse 3′−ACTTGAA-GATGCCCCCTTGGA. The specific primers for β-actin were forward 5′−GGACTTCGAGCAAGAGATGG and reverse 3′−GACATGGTTGTGTCAG.

**Immunohistochemical Staining**

Paraffin-embedded tumor sections were immunolabeled with an anti-human PLK-1 monoclonal antibody (Upstate Biotechnology). The primary antibody at 1:100 dilution was visualized by the conventional avidin-biotin-peroxidase complex method (Vectorstain Elite ABC kit; Vector Laboratories) as described previously (22). Sections were counterstained with hematoxylin and mounted. Normal mouse IgG was used as a negative control. Two investigators independently calculated the number of PLK-1-positive cells in 1,000 tumor cells in a minimum of 15 fields.
Tailvein. Photons were detected 24 h after the treatment. siRNA or 25 A either siRNA/atelocollagen complex containing 25 A photons were counted using the (150 mg/kg; Promega) by i.v. injections. Five minutes later, siRNA solution and mixed by rotating overnight at 4 C. For transmission electron microscopic analysis, 3 x 10^7 to 5 x 10^7 A549 cells were transfected with PLK-1 or nonsense siRNA at 100 nmol/L for 48 h. Then, transfected cells were spun down into a pellet and fixed in 2% glutaraldehyde in a 0.1 mol/L phosphate buffer at 4°C for 2 h. After washing in an isotonic PBS-sucrose, the cells were refixed in PBS-1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in Luveak 812 (Nakalai). Sections were cut 70 to 90 nm thick with a diamond knife on a Sorvall MT-5000 ultramicrotome (DuPont), stained with uranyl acetate and lead citrate, and observed with a Hitachi H-7000 electron microscope (Hitachi).

**Morphologic Evaluation by Light and Electron Microscopy**

Forty-eight hours after the transfection with PLK-1 or nonsense siRNA at 100 nmol/L, 2 x 10^4 to 3 x 10^5 A549 cells were spun down onto slides using Cytospin-3 (ThermoShandon), stained with Wright-Giemsa staining solutions, and subsequently examined by light microscopy. For transmission electron microscopic analysis, 3 x 10^7 to 5 x 10^7 A549 cells were transfected with PLK-1 or nonsense siRNA at 100 nmol/L for 48 h. Then, transfected cells were spun down into a pellet and fixed in 2% glutaraldehyde in a 0.1 mol/L phosphate buffer at 4°C for 2 h. After washing in an isotonic PBS-sucrose, the cells were refixed in PBS-1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in Luveak 812 (Nakalai). Sections were cut 70 to 90 nm thick with a diamond knife on a Sorvall MT-5000 ultramicrotome (DuPont), stained with uranyl acetate and lead citrate, and observed with a Hitachi H-7000 electron microscope (Hitachi).

**Generation of a Stable Luc-Expressing Cancer Cell Line and a Mouse Model**

A lung cancer cell line stably transfected with the Luc gene was generated. The pGL3 control vector (Promega) was cotransfected with pSV2Neo vector (American Type Culture Collection) into an A549 cell line using Lipofect-AMINE 2000 as described previously (24).

We established a model of liver metastasis as described previously (2). BALB/c nu/nu mice were anesthetized with pentobarbital, and the spleens were exposed to allow direct intrasplenic injections of 1 x 10^6 Luc-labeled A549 (A549Luc) cells in 50 μL PBS on day 0. Ten minutes after the injection of tumor cells, the spleens were removed. For in vivo imaging, the mice were administered the d-luciferin (150 mg/kg; Promega) by i.v. injections. Five minutes later, photons were counted using the in vivo imaging system (IVIS; Xenogen). Approval for these studies was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

**In vivo Effects of PLK-1 siRNA Using a Murine Model**

We prepared the siRNA/atelocollagen complex as reported previously (25). Briefly, equal volumes of atelocollagen (0.1% in PBS at pH 7.4) were combined with siRNA solution and mixed by rotating overnight at 4°C. The final concentration of atelocollagen was 0.05%. We first verified that atelocollagen was a useful carrier for i.v. delivery of siRNA in this mouse model. After confirmation of liver metastasis by an IVIS, we i.v. injected 200 μL of either siRNA/atelocollagen complex containing 25 μg GL3 siRNA or 25 μg GL3 siRNA solution alone into mice via the tail vein. Photons were detected 24 h after the treatment.

Next, we investigated the in vivo effects of PLK-1 siRNA. After the injection of A549Luc cells and detection of photons by IVIS, 200 μL PLK-1 siRNA/atelocollagen complex (25 μg siRNA), nonsense siRNA/atelocollagen complex (25 μg siRNA), or PBS/atelocollagen complex were administered by i.v. injection for 10 consecutive days from day 1 of transplantation. Tumor growth was investigated by IVIS every week. On day 70, mice were euthanized and their livers were removed for macroscopic evaluation. In some experiments, we evaluated the PLK-1 expression in the liver metastatic tumors after the consecutive 10 days of PLK-1 treatment by immunohistochemical examinations.

We determined the body weights and measured hematologic and biochemical variables of the mice to investigate the feasibility of the systemic administration of the siRNA/atelocollagen complex. We collected peripheral blood and then measured the WBC counts, hematocrit, and serum levels of aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, creatinine, total protein, albumin, and blood glucose.

**IFN Induction in Mice Treated with siRNA/Atelocollagen Complex**

We investigated whether the administration of siRNA/atelocollagen complex induced the production of interferon in BALB/c nu/nu mice. The mice were i.v. injected with either PLK-1 siRNA/atelocollagen complex (25 μg siRNA) or nonsense siRNA/atelocollagen complex (25 μg siRNA) via the tail vein. Poly(I:C) (Sigma-Aldrich) was i.p. injected into mice as a positive control of IFN induction. Blood was harvested from mice 2 h after injection and serum levels of IFN-β were measured using a mouse IFN-β ELISA Kit (PBL Biomedical Laboratories) according to the manufacturer's instructions.

**Statistical Analysis**

The effects of siRNA on the in vivo experiments were analyzed by the Student's t test. P values < 0.05 were considered to be statistically significant.

**Results**

**Relationship between Expression of PLK-1 and Clinicopathologic Features of Patients**

First, we examined the immunohistochemical levels of PLK-1 expression in lung cancer tissues (Table 1). PLK-1 was overexpressed in lung cancer tissues but not in normal pulmonary epithelial cells (Fig. 1). Among 37 patients at progressed stages of cancer (postsurgical stage ≥II), 12 had low levels of PLK-1 expression and 25 had high levels. In contrast, 27 patients had low levels at postsurgical stage I, an early stage of cancer, and 15 had high levels of PLK-1 expression. Moreover, according to the differentiation grade of cancer, 25 patients had low and 11 had high PLK-1 expression among the 36 patients with well-differentiated lung cancers, whereas 5 had lower and 12 had higher PLK-1 expression among 17 patients with poorly differentiated cancers.

**Inhibition of Lung Cancer Cell Growth by PLK-1 siRNA Treatment In vitro**

Next, we investigated the inhibitory effects of PLK-1 siRNA on lung cancer cells in vitro. We examined the expression of PLK-1 on four lung cancer cell lines: A549,
H1299, SBC-3, and SBC-5 cell lines. All cell lines examined expressed a higher level of PLK-1 than noncancerous cells (Fig. 2A). We then examined the ability of PLK-1 siRNA to knock down the endogenous level of PLK-1 protein in lung cancer cell lines. Western blot analysis showed that the transfection of PLK-1 siRNA (100 nmol/L) but not of nonsense siRNA (100 nmol/L) led to a marked loss of PLK-1 expression in cancer cells (Fig. 2B). Then, we examined the growth-inhibitory effects of PLK-1 siRNA on lung tumor cells using a modified MTT assay. As shown in Fig. 2C, PLK-1 siRNA transfection inhibited cell growth in a concentration-dependent manner. However, no significant inhibitory effects were detected on NHDF or NHF cells (Fig. 2D). We also examined the modified MTT assay using a scramble siRNA and there were no inhibitory effects on all four lung cancer cell lines or NHF cells (data not shown). Wolf et al. showed that PLK-1 was overexpressed in 107 of 111 lung cancer tissues obtained from patients, indicating that PLK-1 expression is elevated in almost of lung cancer cells (10). Taken together, our observations suggest that PLK-1 siRNA transfection specifically inhibits the growth of lung cancer cells.

Next, we investigated the changes of other PLK transcript on A549 cell after PLK-1 siRNA transfection. The mRNA of PLK-1 was decreased 24 h after PLK-1 siRNA transfection, whereas PLK-2 and PLK-3 transcript were increased 48 and 72 h after PLK-1 depletion (Supplementary Fig. S1).

Next, we assessed the morphologic changes of A549 lung cancer cells after siRNA transfection by light and electron microscopy. PLK-1 is known to play important roles in mitosis (9) and the knockdown of PLK-1 induces M-phase arrest and abnormalities of cell division. Using light microscopy, we observed cells with fragmented nuclei after PLK-1 siRNA transfection. Some of the PLK-1 siRNA-transfected cells had dumbbell-like and misaligned nuclei (Fig. 3A). These observations showed that PLK-1 siRNA transfection induces abnormalities of cell division during M phase. Transmission electron microscopic evaluation also clarified that PLK-1 siRNA transfection induces abnormalities of cell division during M phase. Transmission electron microscopic evaluation also clarified that PLK-1 siRNA-transfected cells had fragmented and condensed nuclei, which are typical of apoptotic cells, and some cells had missegregated their chromosomes (Fig. 3B).

**Mechanisms of Cell Death Induced by PLK-1 Knockdown**

We investigated the mechanisms of cell death caused by PLK-1 siRNA transfection. In cell cycle analysis, the G2-M arrest occurred 24 h after PLK-1 siRNA transfection.
(100 nmol/L); thereafter, the sub-G1 fraction was increased through 48 and 72 h after transfection (Fig. 4A). Early apoptotic cells (Annexin V+/PI- fraction), and late apoptotic cells and necrotic cells (Annexin V+/PI+ fraction) increased 48 and 72 h after PLK-1 siRNA transfection, respectively (Fig. 4B). A fluorometric protease assay also showed that caspase-3 activity was augmented up to 11.3-fold 48 h after PLK-1 siRNA transfection (Fig. 4C). Thus, PLK-1 siRNA transfection induced apoptosis by activating caspase-3.

**Inhibition of Liver Metastatic Tumor Growth by PLK-1 siRNA In vivo**

We established a liver metastatic mouse model by injecting A549Luc lung cancer cell lines into mice. First, we evaluated the efficacy of atelocollagen as a carrier of siRNA by i.v. delivery in our mouse model. After the confirmation of liver metastasis by IVIS, we then administered Luc GL3 siRNA alone or GL3 siRNA/atelocollagen complex by i.v. injection via the tail vein. In mice receiving the Luc GL3 siRNA/atelocollagen complex, bioluminescence was inhibited by 90% compared with mice receiving GL3 siRNA alone or before treatment (Supplementary Fig. S2). Thus, these observations indicate that atelocollagen is a useful DDS for the i.v. administration of siRNA in a liver metastatic mouse models.

Next, we evaluated the growth inhibition of liver metastases by PLK-1 siRNA. After the detection of bioluminescence in the livers of mice on day 1 of transplantation, we administered PLK-1 siRNA/atelocollagen, nonsense siRNA/atelocollagen, or PBS/atelocollagen complexes by i.v. injection via the tail vein for 10 consecutive days. The expression of PLK-1 in the mice treated with PLK-1 siRNA/atelocollagen complex was diminished (Fig. 5A). The progression of liver metastasis was noninvasively monitored by detecting bioluminescence with IVIS every week. On day 35, mice treated with the nonsense siRNA/atelocollagen complex or the PBS/atelocollagen complex showed extensive metastases in the liver compared with the mice treated with PLK-1 siRNA/atelocollagen complex (Fig. 5B and C). As shown in Fig. 5C, the luminescence in PLK-1 siRNA/atelocollagen-treated mice was significantly suppressed compared with that in nonsense siRNA/atelocollagen-treated or PBS/atelocollagen-treated mice ($P < 0.05$). We also evaluated the inhibition of tumor growth macroscopically. On day 70 after inoculation of the tumor cells, mice were euthanized...
and their livers were removed. We have evaluated the weights of livers of mice on day 70 after treatment. The weights of livers of mice treated with the nonsense siRNA/atelocollagen complex, PBS/atelocollagen complex, and PLK-1 siRNA/atelocollagen complex were 2.68 ± 1.02, 2.54 ± 1.0, and 1.79 ± 0.09 g, respectively. There is a tendency that the liver weights of mice treated with PLK-1 siRNA/atelocollagen complex were lighter than those of mice treated with nonsense siRNA/atelocollagen complex ($P = 0.08$). Besides, livers of mice treated with nonsense siRNA/atelocollagen or PBS/atelocollagen complex had numerous large tumor nodules, whereas the livers of mice treated with PLK-1 siRNA/atelocollagen complex were lighter than those of mice treated with nonsense siRNA/atelocollagen complex ($P = 0.08$). There is a tendency that the liver weights of mice treated with PLK-1 siRNA/atelocollagen complex were lighter than those of mice treated with nonsense siRNA/atelocollagen complex ($P = 0.08$). Besides, livers of mice treated with nonsense siRNA/atelocollagen or PBS/atelocollagen complex had a much lower number of smaller nodules (Fig. 5D). We found no statistical differences in the body weights or in the measured hematologic and biochemical parameters of mice between the groups (data not shown). These observations indicate that PLK-1 siRNA/atelocollagen complex is a novel and potent therapeutic tool for liver metastasis of lung cancer without severe adverse effects.

An important consideration for siRNA treatment is the activation of innate immunity (26, 27). We investigated the serum levels of IFN-β after the administration of siRNA/atelocollagen complex to examine whether the siRNA/atelocollagen complex activated innate immunity in mice. The siRNA/atelocollagen complex did not induce IFN-β response, whereas poly(IC) induced a strong response (data not shown). This result indicated that it is possible to administer siRNA/atelocollagen complexes without inducing the expression of nonspecific genes, such as those that lead to an immune response.

Discussion

PLK-1 is the best characterized member of the PLK family and participates in several steps of mitosis in proliferating cells: mitotic entry through CDK1 activation, bipolar spindle formation, chromosome alignment, segregation of chromosomes, and cytokinesis (9, 28). Overexpression of PLK-1 is observed in various cancer types and is shown to be an indicator of a poor prognostic outcome (10–13, 15). In this study, we showed that PLK-1 is overexpressed in lung cancer tissues. Therefore, we selected PLK-1 as a new target of siRNA therapy for lung cancer. Depletion of PLK-1 resulted in inhibition of lung cancer cell proliferation. The morphologic evaluation of cells treated with PLK-1 siRNA showed that the chromosomes were dumbbell-like, fragmented, misaligned, or missegregated. Electron microscopic evaluation confirmed that the PLK-1-depleted cells had fragmented and condensed nuclei, suggesting that PLK-1 siRNA treatment induced apoptosis by activating caspase-3.

The kinases of PLK family cooperatively act in mitosis. Quantitative real-time reverse transcription-PCR data showed that PLK-2 and PLK-3 transcripts were increased after PLK-1 siRNA treatment. Unlike PLK-1, PLK-2 and PLK-3 play inhibitory roles. PLK-2 is regulated by p53 and PLK-3 is activated by the DNA damage checkpoint (29). These observations suggest that PLK-1 depletion induced mitotic catastrophe and activation of spindle checkpoint and DNA damage checkpoint, resulting in increased transcription of PLK-2 and PLK-3. Consequently, these PLK family kinases cooperatively prevented G2-M transition and induction of apoptosis. Although PLK-1 is important for cell mitosis, PLK-1 depletion did not inhibit the growth of NHDF or NHF cells as the previous report (30). The expression of PLK-1 is much lower in normal cells compared with that in cancer cells (Fig. 2A). It is speculated that some other kinases compensate loss of PLK-1 function during mitosis in normal cells.

Numerous methods to transfet siRNA into cells have been developed, and viral vectors are the most efficient...
among them (31, 32). However, the feasibility of viral vectors is limited because they can disrupt important genes and induce mutagenesis and the development of cancers (33, 34). Several nonviral carriers have also been developed (2–6). Among them, atelocollagen is one of the most attractive nonviral DDS currently available (25, 35–37). It is obtained from calf dermis after the removal of immunogenic telopeptides located at the NH2 and COOH terminals of the collagen molecules. It provides a clinically safe and readily available biomaterial (7). The size of the complex can be changed by altering the ratio of siRNA to atelocollagen (7). A high concentration of atelocollagen persists locally for a long time, whereas a low concentration of atelocollagen is suitable for systemic delivery. As i.v. administration of atelocollagen at a low concentration results in its accumulation in the livers of mice (25), atelocollagen could be used as a liver-directed DDS.

After the development of metastatic liver tumors, we evaluated the efficacy of atelocollagen as a carrier of delivery for siRNA. We administered the Luc GL3 siRNA/atelocollagen complex (final concentration of atelocollagen was 0.05%) into mice by i.v. injection via tail vein, which inhibited bioluminescence by 90%. The successful transfection of GL3 siRNA in our liver metastasis mouse model confirmed that atelocollagen at low concentrations was effective as a systemic DDS as reported previously (25). Next, we investigated the efficacy of PLK-1/atelocollagen complex as an anticancer agent. After confirming the engraftment of Luc-transfected lung cancer cells in murine livers by the IVIS, we i.v. administered PLK-1 siRNA/0.1% atelocollagen complex (final concentration, 0.05%) for 10 days. IVIS evaluation on day 35 revealed that the growth of the liver metastases of PLK-1 siRNA/atelocollagen-treated mice were significantly suppressed compared with that of nonsense siRNA/atelocollagen-treated or PBS/atelocollagen-treated mice ($P < 0.05$). Even at day 70 after the engraftment, which was 60 days after finishing the treatment, the growth of the liver tumors was markedly inhibited by PLK-1 siRNA treatment. In contrast, expanded liver tumors were observed in PBS-treated and nonsense siRNA-treated mice. This long-lasting silencing effects using atelocollagen were observed in the previous reports. Moreover, the siRNA/atelocollagen complex is also resistant to nucleases and is efficiently transduced (35). When

![Figure 4. Induction of apoptosis in A549 lung cancer cell line by PLK-1 siRNA treatment (100 nmol/L). A, cell cycle analyses of siRNA-treated A549 cells. G2-M arrest was observed in the PLK-1 siRNA-treated cells and an increased in the sub-G fraction was observed in a time-dependent manner. B, frequencies of apoptotic cells by siRNA treatment (white columns, no treatment; solid columns, nonsense siRNA treatment; striped columns, PLK-1 siRNA treatment). Apoptosis induction was identified by Annexin V/PI staining using a flow cytometer. Early apoptotic cells (Annexin V+ /PI− fraction; left) and late apoptotic cells and necrotic cells (Annexin V+/PI+ fraction; right) increased 48 and 72 h after PLK-1 siRNA treatment, respectively. Representative of three independent experiments. C, caspase-3 activity in siRNA-treated cells (white columns, no treatment; solid columns, nonsense siRNA treatment; striped columns, PLK-1 siRNA treatment). Caspase-3 activity increased 48 h after PLK-1 siRNA transfection. Representative of three independent experiments.](mct.aacrjournals.org/article-figures/2910_Figure-4.jpg)
anti-intercellular adhesion molecule-1 antisense oligonucleotide with atelocollagen was administered i.v. 3 days before the induction of allergic dermatitis by 2,4-dinitrofluorobenzene, the induction of inflammation was prevented (38). Takeshita et al. showed that siRNA delivered with atelocollagen existed intact for at least 3 days in tumor tissues (25). Taken together with our study, the PLK-1 depletion by PLK-1 siRNA delivered with atelocollagen can yield a long-lasting inhibition of cancer growth. Moreover, the administration of the siRNA/atelocollagen complex did not enhance the innate immunity of the treated mice, indicating that the i.v. administration of PLK-1 siRNA/atelocollagen complex inhibited the growth of lung cancer by interfering with specific RNA transcription.

We used a model of liver metastases by lung cancer cells in this study. Liver metastases are important prognostic factors also in patients with other cancers including colon cancer. We confirmed that PLK-1 is overexpressed in colon cancer cells and the transfection of PLK-1 siRNA induced apoptosis in colon cancer cells (data not shown) as reported previously (39, 40). As atelocollagen is liver-directed DDS, atelocollagen-mediated PLK-1 siRNA delivery could also be a useful strategy for liver metastases.

In conclusion, we showed that PLK-1 siRNA induced the cell death of lung cancer cells and that the systemic administration of PLK-1 siRNA/atelocollagen complex inhibited the growth of lung cancer in a liver metastatic murine model. These observations show that systemic siRNA/atelocollagen complex therapy may be an attractive strategy for lung cancer patients even with advanced liver metastases. The feasibility of this strategy should be verified by investigational clinical trials for these patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dainippon Sumitomo Pharmaceutical and Koken for providing atelocollagen.
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Molecular Cancer Therapeutics

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