Anticancer activity of RecQL1 helicase siRNA in mouse xenograft models

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Small interfering RNAs (siRNAs) are expected to have a medical application in human therapy as drugs with a high specificity for their molecular target mRNAs. RecQL1 DNA helicase in the human RecQ helicase family participates in DNA repair and recombination pathways in the cell cycle of replication. Silencing the *RecQL1* expression by RecQL1-siRNA induces mitotic death *in vitro* specifically in growing cancer cells. By contrast, the same *RecQL1* silencing does not affect the growth of normal cells, emphasizing that RecQL1 helicase is an ideal molecular target for cancer therapy. In this study, we show that local and systemic administration of RecQL1-siRNA mixed with polyethyleneimine polymer or cationic liposomes prevented cancer cell proliferation *in vivo* in mouse models of cancer without noticeable adverse effects. The results indicate that RecQL1-siRNA in a complex with a cationic polymer is a very promising anticancer drug candidate, and that in particular, RecQL1-siRNA formulated with a cationic liposome has an enormous potential to be used by intravenous injection for therapy specific for liver cancers, including metastasized cancers from the colon and pancreas. (*Cancer Sci* 2008; 99: 1227–1236)

Human RecQL1 DNA helicase (RecQL1), a member of the RecQ helicase family, participates in the maintenance of genomic integrity and is highly up-regulated in rapidly growing cells, including various cancers and transformed cells.[1–5] RecQL1 unwinds specific DNA *in vitro* ATP-dependently and can increase base matching ATP-independently.[6,7] It is assumed to participate in the mismatch repair pathway *in vivo* because it binds to the incision activity of human EXO1 and the mismatch repair recognition complex MSH2/6.[8] Also, RecQL1 acts as an enzyme that resolves Holliday junctions during cell proliferation.[9] Consequently, down-regulation of *RecQL1* expression in HeLa cells by RNA interference (RNAi) increases sister chromatid exchanges resulting from unprocessed Holliday junction structures,[10]. Although *RecQL1*-deficient mice show no apparent phenotypic differences when compared with wild-type mice, embryonic fibroblasts from *RecQL1*-deficient mice are sensitive to ionizing radiation[11] (and our unpublished data Sakamoto *et al*. 1999). All these findings collectively showed that *RecQL1* suppresses chromosomal instability by participating in DNA repair associated with cell proliferation, but its function seems to be nonessential and complemented by other cellular repair system(s).

Silencing *RecQL1* in cancer cells by RNAi with siRNA induces mitotic catastrophe and death in a wide range of cancer cells that have defects in the cell cycle checkpoint system.[12] By contrast, the same silencing does not cause mitotic death in growing normal cells that have a competent checkpoint system, even though their cell cycles slowed down. To test the efficacy of RecQL1-siRNA *in vivo*, we previously studied the multicellular cancer spheroids system, a three dimensional (3-D) model that uses human hepatocarcinoma HuH-7 cell lines to mimic the *in vivo* biology of cancers.[13] In that study,[13] polyethylene glycol-conjugated RecQL1-siRNA showed an appreciable growth inhibition of HuH-7 cells for up to 21 days at IC50 of 6 nM, indicating that silencing *RecQL1* expression indeed inhibits 3-D proliferation of cancer cells.

In this study, we compared the anticancer effects of RecQL1-siRNA in various cancer-bearing mouse models to assess the potential of RecQL1-siRNA as a therapeutic agent and to search for the most effective administration. To this end, a few plausible drug delivery systems were tested that protect siRNA from hydrolysis by nucleases, direct siRNA to specific tissues and organs and facilitate entry into cells. To find the most feasible therapy, several cancer-bearing mouse models were tested by local or systemic administration by intravenous injection. Although siRNA has many excellent profiles as a drug candidate, the highest hurdle to its therapeutic application is finding a way to deliver siRNA molecules safely and specifically to target organs and cells. Our results showed that growth of various human cancers in the mouse xenograft model was prevented *in vivo* without apparent adverse effect by local and systemic injection of RecQL1-siRNA formulated with cationic polymers. We believe this is the first demonstration that confirms anticancer activity *in vivo* for siRNA that silences the DNA helicase participating in genome maintenance associated with cell cycle progression.

Materials and Methods

Cells and cell culture condition. The cell lines used in this study were A549 (lung carcinoma), Hep3B (hepatic carcinoma), AsPC-1 (pancreatic carcinoma), and LS174T (colon carcinoma), all purchased from ATCC (Manassas, VA, USA). The cells were incubated at 37°C in humidified chambers with 5% CO_2 in a medium containing 10% fetal bovine serum and 25 μg/mL gentamicin or following the instructions of ATCC.

RNA interference. The 5′ monophosphorylated form of siRNA was chemically synthesized by Dharmacon or Nippon Shinyaku. All siRNA sequences (21 mers) had overhanging-3′-dTdT. The sequences of siRNAs used in this study were selected and were used as described by Elbashir *et al*.[14] RecQL1 mRNA-specific siRNA (RecQL1-siRNA) contains the sense sequence of 5′-GUUCAGACCACUUCAGCUUdTdT-3′ (corresponding to the position 273–291 in RecQL1 mRNA) that was confirmed not to interact with any known mRNA sequence by cDNA microarray analysis (Affimetrix GeneChip system – Human Genome U133 Plus 2.0 Array) and by homology search by using the Smith and Waterman method. Firefly luciferase gene sequence GL3

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Abbreviations: Small interfering RNA (siRNA), non-silencing (NS), polyethyleneimine (PEI).
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(Promega, Madison, WI, USA), 5′-CUUACGCUGAGUACUUCG-AdTdT-3′ (sense strand) and 5′-UCGAAGUACUCAGCGUA-AGdTdT-3′ (antisense strand) was used as non-silencing siRNA (NS-siRNA) that represented a negative control for the RNA interference reaction.

For *in vitro* transfection 24 h after plating, cells were incubated with 2.5–160 pmol of siRNA duplex using Oligofectamine or Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Because of different transfection efficiencies with various cell lines, pilot experiments were made with individual cell lines to determine the optimal condition by changing the transfection reagent or by modifying the concentration or the ratio of siRNA and transfection reagents or both. For *in vivo* administration to mice, RecQL1-siRNA was mixed with a PEI or a cationic liposome before injection as described below.

Quantitative analysis of RecQL1 mRNA and siRNA by reverse transcription–polymerase chain reaction (RT-PCR). At 30 h after transfection, total RNA was extracted from cultured cells by using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RT-PCR analyses were done to estimate RecQL1 mRNA by using the ABI Prism 7000 Sequence Detection System, Taqman probes, and primers (ABI, Foster, CA, USA). The β-actin mRNA was also analyzed as the internal standard.

To quantitate the amount of RecQL1-siRNA distributed in various tissues, total RNA was extracted by using an miRNeasy Mini Kit (Qiagen) after GL3 siRNA (50 pmole) was added as an internal standard and was incubated at 37°C for 30 min with 1 mM dGTP and terminal deoxynucleotidyl transferase (EC2.7.7.31; TaKaRa Bio, Shiga, Japan). The latter process permits the addition of oligo-dG to the 3′ termini of RecQL1-siRNA consisting of –dTdT. After the 3′ dG-tailing of siRNA, a portion of reaction mixture was diluted with water, and was mixed with an anchor-primer oligonucleotide (36 mer) consisting of 5′ oligodeoxynucleotide primer sequence (20 mer) and 3′ oligo-dC (16 mer) that together function as an anchor to hold the dG-tailed siRNA. The mixture was denatured by heat at 70°C for 10 min to disrupt the duplex structure of dG-tailed siRNA and then was cooled on ice to let the dG-tailed and single-stranded siRNA anneal with the oligo-dC-containing anchor-primer sequence. A mixture of dNTP (where N was A, C, G, or T) and reverse transcription (Toyobo, Osaka, Japan) was added to the mixture, which was incubated for 1 h at 42°C to produce an siRNA-specific cDNA template. To determine the amount of siRNA, quantitative PCR was done with the cDNA template by using a specific primer (19 mer) having the antisense sequence of siRNA but lacking-3′ dTdT, and another primer (19 mer) having the same primer sequence as the primer-anchor oligonucleotide (20 mer), by using the QuantiTect SYBR Green PCR kit (Qiagen). The amount of siRNA was estimated based on the standard curve made with GL3-siRNA. Details of this new procedure to estimate siRNA will be described elsewhere.

Cell proliferation assays. Cell proliferation was measured by colorimetic assays of cell viability based on the cleavage of tetrazolium salt WST-8 by mitochondrial dehydrogenase (Nacalai Tesque) 96 h after transfection. The 450 nm absorbance of the formazan dye formed was measured at 3 h after adding the reagent.

Flow cytometric analysis. Trypsin-treated cells were washed with phosphate-buffered saline (PBS) and were fixed in ice-cold methanol for 2 h. The cells were treated with pancreatic RNase A (Nippon Gene, Toyama, Japan), stained with propidium iodide (Sigma, St. Louis, MO) for 30 min, and then analyzed by using a flow cytometer. Fluorescence was measured by using EPICS XL (Beckman Coulter, Tokyo, Japan). For each sample, 7000 events were analyzed.

Preparation of RecQL1-siRNA/PEI and RecQL1-siRNA/LIC-101 complexes. PEI has a linear configuration and a molecular weight of 10 000 Da. *In vivo*-jetPEI (PolyPlus, Illkirch, France) and RecQL1-siRNA were mixed in their solutions. The ratio of PEI to siRNA was N/P = 4. Cationic liposome LIC-101 consists of 2-O-(2-diethylaminoethyl)-carbamoyl-1, 3-O-dioleylglycerol, and egg phosphatidylcholine. The RecQL1-siRNA/LIC-101 complex was formulated in the laboratory of Nippon Shinyaku (Kyoto, Japan) as described by Hirabayashi *et al*.[15] and Yano *et al*.[16] The ratio of siRNA to LIC-101 liposome was 1:16 (w/w).

Mouse models of cancers. Animal procedures were approved by the committee for the institutional care and use of animals at GeneCare Research Institute in accordance with the guidelines for animal experimentation prepared by the Japanese Association for Laboratory Animal Science.

Lung and liver cancers inoculated under mouse dorsal skin. Male BALB/c nu+/+mice (4 weeks old) were purchased from CLEA Japan (Tokyo, Japan). For a mouse model of lung and liver cancers, mice were inoculated with 5×10^6 A549 human lung carcinoma cells and Hep3B human liver carcinoma cells, respectively, in 100 μL of PBS under the dorsal skin on day 0. The siRNA treatment started on days 8–14 after cancer nodules of approximately 100 mm^3 were confirmed. The volumes of cancer nodule were estimated by two-dimensional caliper measurement; the formula for estimating the ellipsoid cancer volume was $L \times W^2/2$, where L is the major axis and W is the width of the cancers. The results were assessed for statistical significance by using Multiple Comparison Procedures (Holm–Sidak method).

Liver metastasis model with colorectal and pancreatic cancers. The liver metastasis model of mice was made according to the method of Hirabayashi *et al*.,[15]. Briefly, mice (BALB/c nu+/+) were anesthetized with pentobarbital, and the spleen was exposed to allow direct intrasplenic injection of 1.5×10^6 LS174T human colorectal cancer cells or 2.0×10^6 AsPC-1 human pancreatic cancer cells in 50 μL of PBS on day 0. Ten minutes after injection, the spleen was removed. Mice with liver metastasis were administrated with an intravenous injection of the RecQL1-siRNA/LIC-101 complex into the tail vein from day 4.

Local administration of RecQL1-siRNA/PEI and RecQL1-siRNA/LIC-101 complexes

RecQL1-siRNA/PEI complex. RecQL1-siRNA (25 μg) was mixed with 5 μg PEI (MW 10 000) in 50 μL saline. The mixture was administrated on days 8, 11, 14, 17, 20, 23, 27, and 32 directly onto cancer nodules made by inoculation of A549 lung carcinoma cells.

RecQL1-siRNA/LIC-101 complex. The RecQL1-siRNA/LIC-101 complex containing 50 μg siRNA was suspended in 50 μL 10% maltose solution (w/v) and was directly injected on days 14, 16, 18, 21, 23, 25, 27, 32, and 36 onto cancer nodules made by inoculation of Hep3B hepatic carcinoma cells.

Intravenous injection of RecQL1-siRNA/LIC-101 complex. In the mouse model of AsPC-1 liver metastasis, the RecQL1-siRNA/LIC-101 complex (2 mg siRNA/kg mouse body weight, equivalent to about 50 μg siRNA/mouse) in 100 μL of 10% (w/v) maltose solution was given intravenously on days 4, 6, 8, 11, 13, 15, 18, 20, 22, and 25 until a total of 10 injections were administered. In addition, mouse model with LS174T liver metastasis, the RecQL1-siRNA/LIC-101 complex (1 mg siRNA/kg mouse body weight, equivalent to about 25 μg siRNA/mouse) in 100 μL of 10% (w/v) maltose solution was administrated intravenously on days 4, 6, 7, and 8 and days 11, 12, 13, 14, and 15 into two groups of mice, each group consisting of six to 10 mice; a third group was a sham-operated group consisting of four mice. The mice were euthanized after completion of the injection schedule, and the anticancer effects were evaluated from various aspects. The average liver weights were analyzed by using Dunnett's test.

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Distribution and toxicity of siRNA administrated by intravenous injection. The distribution of siRNA in various organs was monitored after systemic administration of the RecQL1-siRNA/LIC-101 complex (50 μg/mouse equivalent to 2 mg/kg) into the tail vein of BALB/c mice. To monitor the distribution of siRNA in organs, the mice were sacrificed after 15 min, and the RecQL1-siRNA from the excised organs was analyzed quantitatively as described above. To visualize the distribution, RecQL1-siRNA was labeled by Alexa Fluor 546 or FAM (5′-carboxyfluorescein) fluorescences, and the labeled-siRNA/LIC-101 complex (25 μg/mouse) was injected intravenously into the tail vein. After 24 h, the distribution of labeled-siRNA was visualized by using a fluorescence image analyzer FM-BIO II (Hitachi Software Engineering, Yokohama, Japan). Distribution of siRNA within the liver was observed for sliced section by a confocal laser scanning microscope (Fluoview; Olympus, Tokyo, Japan). Here, endothelial cells consisting blood vein were stained by anti-PECAM1 (BD Pharmingen, San Diego, NJ, USA), and the non-cancer cells were distinguished from cancer cells by selective staining with Hoechst33342. For toxicological analysis, a group of mice ($n = 5$) were given 2 or 10 mg/kg of RecQL1-siRNA/LIC-101 complex by intravenous injection into the tail vein every other day for 4 weeks.

Immunohistochemical staining. The hepatic tissue array consisting of 60 hepatocellular carcinomas, which was confirmed by the standard immunohistochemical staining of proliferating cell nuclear antigen, was purchased from Cybrdi (Frederick, MA, USA; hepatocellular carcinoma, grade I–III with normal tissue control tissue array). Cells in the tissue arrays were stained by incubation with anti-RecQL1 polyclonal antibodies,[17] after removal of a wax covering and reactivation of protein antigens. Immunohistochemical staining was done as described before,[17] to monitor the expression levels of RecQL1 helicase protein in cancer nodules by staining with rabbit anti-RecQL1 polyclonal antibody.

TdT-mediated dUTP nick end-labeling (TUNEL) assays. Cancer nodules were fixed with 10% (w/v) formaldehyde and thin slices were prepared for TUNEL assay. Apoptotic-like cells were detected by TUNEL assay by using the *in situ* Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol.

Quantitation of cancer markers in the blood of cancer-bearing mice. The levels of cancer markers carcinoembryonic antigen (CEA) and cytokeratin fragment (CYFRA 21-1) in the circulating blood of cancer-bearing mice were evaluated by using ELISA kits supplied by DRG instruments (Marburg, Germany). Quantitation was made by using the protocols of the manufacturer.

Quantitation of interferon-alpha (IFN-α) in the blood of normal mouse. Excretion of IFN-α in the blood of mice treated with the RecQL1-siRNA/LIC-101 complex was monitored by using a mouse IFN-α ELISA kit (PBL Biomedical Laboratory, Piscataway, NJ, USA). The RecQL1-siRNA/LIC-101 complex (50 μg siRNA/mouse) was injected intravenously into three Jcl-Imperial Cancer Research mice (CLEA). After 3 h, the concentrations of mouse IFN-α excreted into the blood were determined by using a PBL ELISA kit using the manufacturer's protocol. As a positive control that can induce IFN-α, Poly (I:C) (Amersham, Piscataway, NJ, USA) was injected after mixing with LIC-101 liposome.

Results

Correlation of RecQL1 helicase expression levels with progression of hepatocarcinoma cell malignancy. Mitotic cell death in cancer cells induced by *RecQL1* silencing depends primarily on the progression of the cell cycle, i.e. proliferation of cells, and secondarily on the highly required function of RecQL1 in cancer cells that appears to be correlated with the expression level of *RecQL1*.[12] A third essential point is failure of cancer

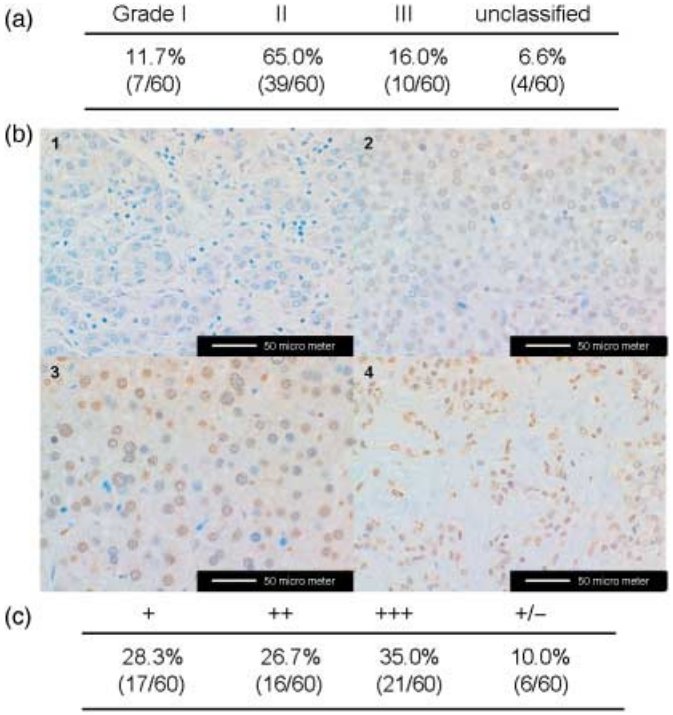

Fig. 1. Expression of RecQL1 helicase in the liver cancer tissue arrays classified from grade I to III based on progression of cancer. A total of 60 human liver cancer specimens classified from grade I to III (provided by Cybrdi, Frederick, MA, USA) were stained immunohistochemically by an antibody specific to RecQL1.[17] The level of staining was compared between the grades and with normal liver tissues ($n = 3$) that were not stained under the same conditions. (a) Progression of cancers classified by supplier of the liver cancer array; (b_1) normal liver tissue –; (b_2) high degree of staining +; (b_3) higher degree of staining ++; (b_4) highest degree of staining +++; (c) immunohistochemical staining of RecQL1 classified in this study.

cells to arrest the cell cycle due to a defect in the G2 checkpoint that is shown in most cancer cells by mutations in p53.[18,19] In this context, the expression of *RecQL1* in growing cancer cells in the culture was extremely high, as reported previously.[2,12] To test if the RecQL1 is also highly expressed *in vivo* in cancer tissues, we analyzed the level of RecQL1 expression in human hepatocarcinoma tissues of various progression grades. The liver cancer tissue array consisting of 60 hepatocarcinoma samples of different cancer grades and three non-cancer tissues were stained with RecQL1-specific antibody.

Immunohistochemical staining data showed that hepatocarcinoma tissues classified as advanced Grade II and III contained increased levels of RecQL1 helicase that correlated with the progression of cancer, and that the less advanced grade of hepatocarcinoma tissue (Grade I) showed a dispersed staining profile (Fig. 1b_{2-4}). By contrast, the non-cancer tissues showed no clear staining, suggesting a low level of expression of RecQL1 in normal cells (Fig. 1b_1).

Therapeutic effect of local administration of the RecQL1-siRNA/PEI complex on subcutaneously inoculated A549 human lung cancer cells. Human lung cancer A549 cells express RecQL1 helicase highly and undergo mitotic catastrophe *in vitro* by *RecQL1* silencing with RecQL1-siRNA transfection.[12] They grew efficiently in subcutaneously inoculated nude mice, and solid cancers formed by A549 cells were visible at ~8 days after inoculation. RecQL1-siRNA mixed with PEI in PBS was injected onto the top of cancer nodules. A total of eight injections were made in the relatively early stage of cancer proliferation (Fig. 2a). Control mice that were injected with NS siRNA developed a large nodule of about 1600 mm^3 whose

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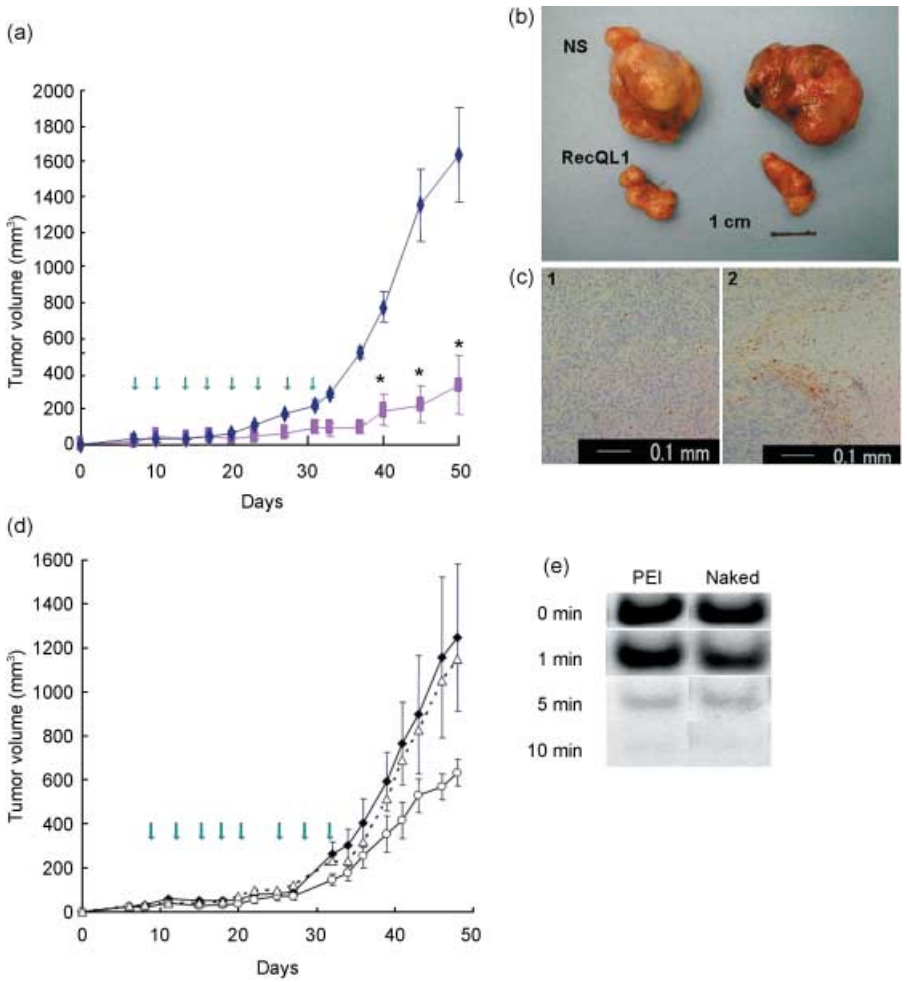

Fig. 2. Anticancer effect of RecQL1-siRNA/PEI complex on subcutaneously inoculated lung cancer cells in mouse model by local and systemic administration. (a) A549 human lung cancer cells were inoculated under the dorsal skin of mice as described in the 'Materials and Methods', and cancer nodules formed after 8 days were injected subcutaneously with RecQL1-siRNA (25 μg) mixed with polyethylenimine carrier (5 μg) in 50 μL saline. NS-siRNA was similarly injected as a negative control. Injections were made on days 8, 11, 14, 17, 20, 23, 27, and 32 (arrow heads). Six mice formed a group and their average size of cancer nodule was plotted. The blue diamonds show NS-siRNA/PEI complex and the pink squares show RecQL1-siRNA/PEI complex. *indicates the statistical significance at $P < 0.05$. (b) Comparison of the size of A549 lung cancer nodules formed at 50 days after cancer cell inoculation. NS-siRNA/PEI complex-treated cancer nodules (upper); RecQL1-siRNA/PEI complex-treated cancer nodules (lower). (c) Detection of TdT-mediated dUTP nick end-labeling (TUNEL) staining-positive cells in cancer nodules. Sliced sections of A549 cancer nodules were stained with hematoxylin–eosin. Cells undergoing apoptotic-like death were detected by TUNEL staining. (c_1) NS-siRNA/PEI complex-treated cancer nodule; (c_2) RecQL1-siRNA/PEI complex-treated cancer nodule. (d) Effect of RecQL1-siRNA/PEI complex on subcutaneously inoculated lung cancer A549 cells in a mouse model by systemic administration. RecQL1-siRNA/PEI complex (containing 50 μg RecQL1-siRNA) was injected intravenously into the tail vein. Injections were made on days 8, 11, 14, 17, 20, 23, 27, and 32 (arrow heads). Six mice formed one group, and average cancer nodule volumes are shown. Open triangles, solid squares, and open circles indicate mice treated with saline, NS-siRNA/PEI, and RecQL1-siRNA/PEI complex, respectively. (e) Retention of the RecQL1-siRNA/PEI complex in the circulation blood of mice. RecQL1-siRNA fluorescently labeled with tetramethylrhodamine (TAMRA) at the 5′-terminus of the sense strand was mixed with PEI, and the retention time in the circulating blood was investigated according to the method described by Sato *et al*.[31] The labeled RecQL1-siRNA/PEI complex was injected into the tail vein and was extracted from the eye vein at 1, 5, and 10 min after the injection. Parts of the extracted blood samples were extracted with phenol-chloroform and were analyzed by using 10% polyacrylamide gel electrophoresis. As a control, fluorescent RecQL1-siRNA without PEI was also similarly analyzed. RecQL1-siRNA mixed with PEI (PEI). RecQL1-siRNA without PEI (naked).

volume rapidly increased from around day 30, but mice injected with RecQL1-siRNA developed only small cancers of about 200 mm^3. After 50 days, the size difference between NS- and RecQL1-siRNA-treated cancers was marked when the cancers were removed from the mice and were compared (Fig. 2b). As expected, the RecQL1-siRNA-treated cancers showed TUNEL-positive staining that provided evidence of apoptosis-like cell death (Fig. $2c_2$). These results clearly showed that the RecQL1-siRNA/PEI complex inhibited growth of human lung cancer under the dorsal skin of nude mice. However, free RecQL1-siRNA injected similarly in a separate experiment showed no inhibitory effect (data not shown). The results also indicated that PEI that has a slightly weak positive charge and a molecular weight of 10 000 Da was efficacious when siRNA is administrated locally. We speculated three roles of PEI: (1) stabilization of siRNA by protection from endonuclease in body fluids; (2) slow release of siRNA molecules; and (3) facilitation of siRNA entry into cells across the cell membrane.

Limited therapeutic effect of the RecQL1-siRNA/PEI complex by systemic administration on subcutaneously inoculated cancer cells. Intravenous injection into the tail vein of mice was tested to examine if the systemic administration of RecQL1-siRNA/PEI complex is effective to subcutaneously inoculated A549 cancer cells under the dorsal skin. Figure 2d shows that therapeutic

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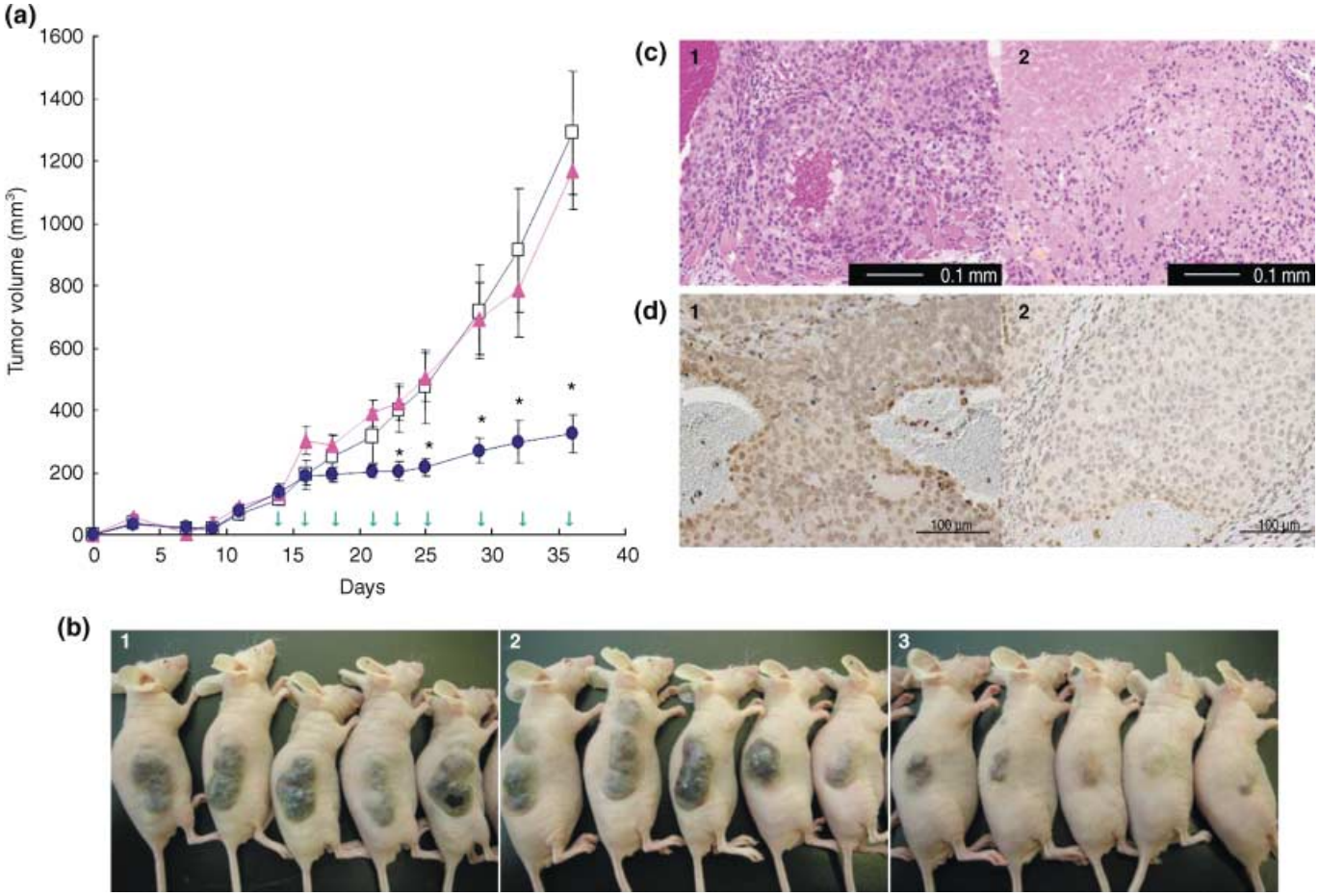

Fig. 3. Anticancer effect of the RecQL1-siRNA/LIC-101 complex on liver cancer in a mouse model by local administration. (a) Time course for measuring the inhibitory effects of RecQL1-siRNA/LIC-101 complex on the growth of Hep3B cancer nodule. Hep3B cells were inoculated under the dorsal skin of mice, and the RecQL1-siRNA/LIC-101 complex was injected subcutaneously as described in the Figure 1 legend. The injections were made on days 14, 16, 18, 21, 23, 25, 27, 32, and 36 (shown by arrow heads). Seven mice formed one group and average sizes of cancers are shown. Open squares, closed triangles, and closed circles indicate mice treated with 10% maltose, NS-siRNA/LIC-101, and RecQL1-siRNA/LIC-101 complex, respectively. *indicates the statistic significance at $P < 0.05$. (b_1) Mice treated with 10% maltose (w/v) solution (42 days after inoculation), (b_2) Mice treated with NS-siRNA/LIC-101 complex (42 days); (b_3) Mice treated with RecQL1-siRNA/LIC-101 complex (42 days). (c) Tissue stained by hematoxylin–eosin. (c_1) NS-siRNA/LIC-101 complex-treated cancer nodule, (c_2) RecQL1-siRNA/LIC-101 complex-treated cancer nodule. (d) Immunohistochemical staining with RecQL1-specific antibody. (d_1): NS-siRNA/LIC-101 complex-treated cancer nodule, (d_2) RecQL1-siRNA/LIC-101 complex-treated cancer nodule.

effect of the systemically administrated RecQL1-siRNA/PEI complex was inferior to the local injection shown in Figure 2a. The reduced therapeutic potential by systemic administration by intravenous injection may be due to low concentrations available in the areas of cancers. Consistently, when the amount of RecQL1-siRNA in the circulating blood was measured by using the fluorescence-labeled RecQL1-siRNA/PEI complex, RecQL1-siRNA disappeared from the circulation blood in the short time of ~ 5 min (Fig. 2e; PEI), similar to the free fluorescence-labeled RecQL1-siRNA tested as a control (Fig. 2e; naked). When approximate retention time in the circulating blood in mice was calculated, both free siRNA and PEI-complexed siRNA showed short half-times of 2 min and 3.5 min, respectively. These results indicated that PEI is not an adequate vector for systemic delivery to skin.

Effect of local administration of the RecQL1-siRNA/LIC-101 complex on subcutaneously inoculated Hep3B human liver cancer cells. We tested the effect of RecQL1-siRNA that was formulated with LIC-101 cationic liposome, as described by Hirabayashi *et al*. and Yano *et al*.[15,16] on the Hep3B hepatocarcinoma cell line originally derived from a young boy chronically infected with B-type hepatitis virus (HBV). Hep3B cells contain part of the HBV genome.[20] RT-PCR showed that the expression level of RecQL1 mRNA was as high as that of A549 cells (data not shown). Hep3B liver cancer cells inoculated subcutaneously under the dorsal skin of nude mice ($n = 7$) grew much faster than A549 lung cancer cells and formed solid cancer nodules of size 1200 mm^3 at 36 days after inoculation as estimated by two-dimensional caliper measurement. As controls, the NS-siRNA/LIC-101 complex or 10% maltose solution which suspended siRNA-liposome complex were similarly analyzed. Local injection of 50 μg of the RecQL1-siRNA/LIC-101 complex into growing Hep3B cancer nodules clearly prevented further growth (Fig. 3a). Figure $3b_{1–3}$ shows mice injected with the 10% (w/v) maltose solution, NS-siRNA/LIC-101 complex, and RecQL1-siRNA/LIC-101 complex, respectively.

Cytological analysis was done for slices of cancer nodules excised from mice at 42 days after inoculation. Staining with hematoxylin–eosin (HE) showed that the population of intact cancer cells was greatly diminished, even scarce, in cancer slices obtained from RecQL1-siRNA/LIC-101 complex-treated mice (Fig. $3c_2$), but cancer cells in the NS-siRNA/LIC-101 complex-treated mice were tightly packed and the cell density remained high (Fig. $3c_1$). When slices of cancer nodules were

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further analyzed by immunohistochemical staining with RecQL1-specific antibody, cancer cells from RecQL1-siRNA/LIC-101 complex-treated mice were only weakly stained (Fig. $3d_2$), contrasting with cells from NS-siRNA/LIC-101 complex-treated mice that were all stained strongly (Fig. $3d_1$). These cytological data clearly showed that *RecQL1* silencing correlated *in vivo* with inhibition of cell growth within the cancer nodules. The results also indicate that local administration of RecQL1-siRNA combined with cationic liposome LIC-101 permits delivery *in vivo* to Hep3B cells in cancer nodules resulting in prevention of cell proliferation.

Effect of systemic administration of the RecQL1-siRNA/LIC-101 complex on AsPC-1 pancreatic cancer cells metastasized to liver. We investigated the therapeutic efficacy of RecQL1-siRNA against AsPC-1 pancreatic cancer cells metastasized to liver by using cationic liposome LIC-101 that carries siRNA to the cytosol of liver cells, as well as to cancer cells metastasized to liver.[16] The preliminary *in vitro* cell experiments showed that growth of AsPC-1 cells was inhibited to 50% by *RecQL1* silencing (to about 10%) with RecQL1-siRNA transfection (Fig. 4a). Flow cytometric analysis indicated that cells underwent mitotic death that is characteristic of mitotic catastrophe with increased cell populations in sub-G1 and G2/M fractions (Fig. 4b; NS and RecQL1).

In the *in vivo* experiments, the RecQL1-siRNA/LIC-101 complex was administrated at dose 2 mg/kg (three times weekly, for a total of 10 injections, as described in 'Materials and Methods') by intravenous bolus injection into mice having AsPC-1 liver metastasis. Figure $4c_{1-3}$ shows the spread and growth of AsPC-1 pancreatic cancer cells in metastasized liver, and the anticancer effect of the RecQL1-siRNA/LIC-101 complex. The results indicate multiple cancers consisting of metastasized AsPC-1 cells all over the liver of mice injected with 10% maltose solution (Fig. $4c_1$) or with NS-siRNA/LIC-101 complex (Fig. $4c_2$). In contrast, only a limited number of cancer nodules were seen on the liver of mice injected with the RecQL1-siRNA/LIC-101 complex (Fig. $4c_3$), indicating that RecQL1-siRNA effectively prevented growth of metastasized pancreatic cancer cells. A clear statistical difference ($P < 0.05$) in liver weight was observed between mice injected with maltose and mice injected with the RecQL1-siRNA/LIC-101 complex, although the NS-siRNA/LIC-101 complex prevented liver weight increase slightly (Fig. 4d). Measurement of cancer marker proteins CEA and CYFRA 21-1, which human AsPC-1 cells excrete into the circulating blood of cancer-bearing mice, indicated a maximum 70% inhibition of cancer marker production (Fig. 4e,f); statistical significances of CEA ($P = 0.0029$) and CYFRA 21-1 ($P = 0.0016$) were observed between mice injected with maltose and RecQL1-siRNA. These results showed that the RecQL1-siRNA/LIC-101 complex can prevent effectively the growth of pancreatic cancer metastasized into liver by the systemic administration. To confirm the down-regulation of *RecQL1* expression in AsPC-1 cancer cells in metastasized liver, the amounts of RecQL1 mRNA and RecQL1 protein in the RecQL1-siRNA/LIC-101 complex-treated liver were measured by using RT-PCR (Fig. 4g) and Western blot analysis (Fig. 4h) using livers from mice injected with 10% maltose solution or NS-siRNA/LIC-101 complex as controls. The data showed that *RecQL1* expression was down-regulated in the liver of mice injected with the RecQL1-siRNA/LIC-101 complex with respect to the RecQL1 mRNA as well as protein levels.

Effect of the RecQL1-siRNA/LIC-101 complex on LS174T colorectal cancer cells metastasized to the liver. To further evaluate the efficacy against other cancer types, we tested the RecQL1-siRNA/LIC-101 complex in mice having liver cancer metastasis of human LS174T colorectal cancer cells. Intravenous bolus injection of the RecQL1-siRNA/LIC-101 complex (1 mg/kg; a total of 10 injections on days 4–8, 11–15) into the tail vein of mice markedly prevented the spread and growth of LS174T colorectal cancers in the metastasized liver (Fig. $5a_4$), but which spread during 37 days with sham mice and in control mice injected with 10% maltose or the NS-siRNA/LIC-101 complex (Fig. $5a_{2,3}$). There was a significant difference ($P < 0.05$) in the ratio of liver weight to body weight between mice treated with maltose and mice treated with the RecQL1-siRNA/LIC-101 complex (Fig. 5b). Cancer marker CYFRA 21-1 protein, which LS174T cells excrete into the blood of mice, was reduced by a maximum of 70% inhibition ($P = 0.008$) in mice treated with the RecQL1-siRNA/LIC-101 complex, indicating that the RecQL1-siRNA/LIC-101 complex is therapeutically effective in a mouse model of liver-metastasized colorectal cancers (Fig. 5c). Under similar conditions, but in a separate experiment, when the effect of the RecQL1-siRNA/LIC-101 complex on the survival time of cancer-bearing mice ($n = 15$) was preliminarily assessed, 10% maltose, the NS-siRNA/LIC-101 complex, and the RecQL1-siRNA/LIC-101 complex administered intravenously 10 times markedly extended the survival time, based on the average 50% survival times of 84.4 days, 89.1 days, and 123.2 days, respectively, after beginning treatment, despite being given only at an early stage of treatment, from day 6 to day 17 (data not shown). These results suggest that the RecQL1-siRNA/LIC-101 complex can effectively prevent growth of malignant colorectal cancer cells in mouse liver and provide a greater survival time in the liver metastasis model of colorectal cancer.

Preferential delivery of siRNA/LIC-101 complex to the liver. The bcl-2-siRNA/LIC101 complex intravenously administrated can be delivered effectively to cancer cells in the mouse model of liver metastasis as shown by Yano *et al*.[16] We confirmed that the RecQL1-siRNA/LIC-101 complex administrated intravenously into the tail vein is investigated the retention in the blood of the intravenously injected RecQL1-siRNA more quantitatively. Our preliminary pharmacokinetical data obtained by the newly developed siRNA detection procedure (described in 'Materials and Methods') showed that 80% of the intravenously injected RecQL1-siRNA disappeared from the circulating blood within 5 min and almost no siRNA was detected after 6 h after injection (Fig. 6a). After 15 min when the distribution of RecQL1-siRNA in the organs was determined by extracting RecQL1-siRNA from several specified organs, more than 50% of the total siRNA injected was found in liver (Fig. 6b). Then, we also confirmed that the RecQL1-siRNA/LIC-101 complex administrated intravenously into the tail vein is also preferentially delivered to the liver by injecting the fluorescence-labeled siRNA/LIC-101 complex and by analyzing the distribution of fluorescent siRNA at 24 h after injection (Fig. 6c). Some low levels of siRNA fluorescence were noted to be delivered to the kidney. The results showed that LIC-101 liposome preferentially delivered the intravenously injected RecQL1-siRNA to the liver. Consistent with the study by Hirabayashi *et al*.,[21] the fluorescence-labeled RecQL1-siRNA incorporated into the liver was in both the cancerous and non-cancerous regions of the mouse liver (Fig. $6d_{1,2}$).

No apparent toxicity or IFN induction is associated with RecQL1-siRNA/LIC-101 complex treatment. During this study, we saw no noticeable adverse effects, such as weight loss or sudden death, by mice treated with the RecQL1-siRNA/LIC-101 complex, and no hair loss in BALB/c normal mice after treatment with much higher doses (up to 10 mg/kg) of human RecQL1-siRNA formulated with cationic LIC-101 liposome. When the liver and kidney functions were investigated in BALB/c normal mice after administration of a high dose of the RecQL1-siRNA/LIC-101 complex (2 or 10 mg/kg) every other day for 4 weeks, the aspartate transaminase and alanine transaminase values representing the liver function, and the blood urea nitrogen and creatinine values representing kidney functions, remained similar to those markers in mice administrated with 10%

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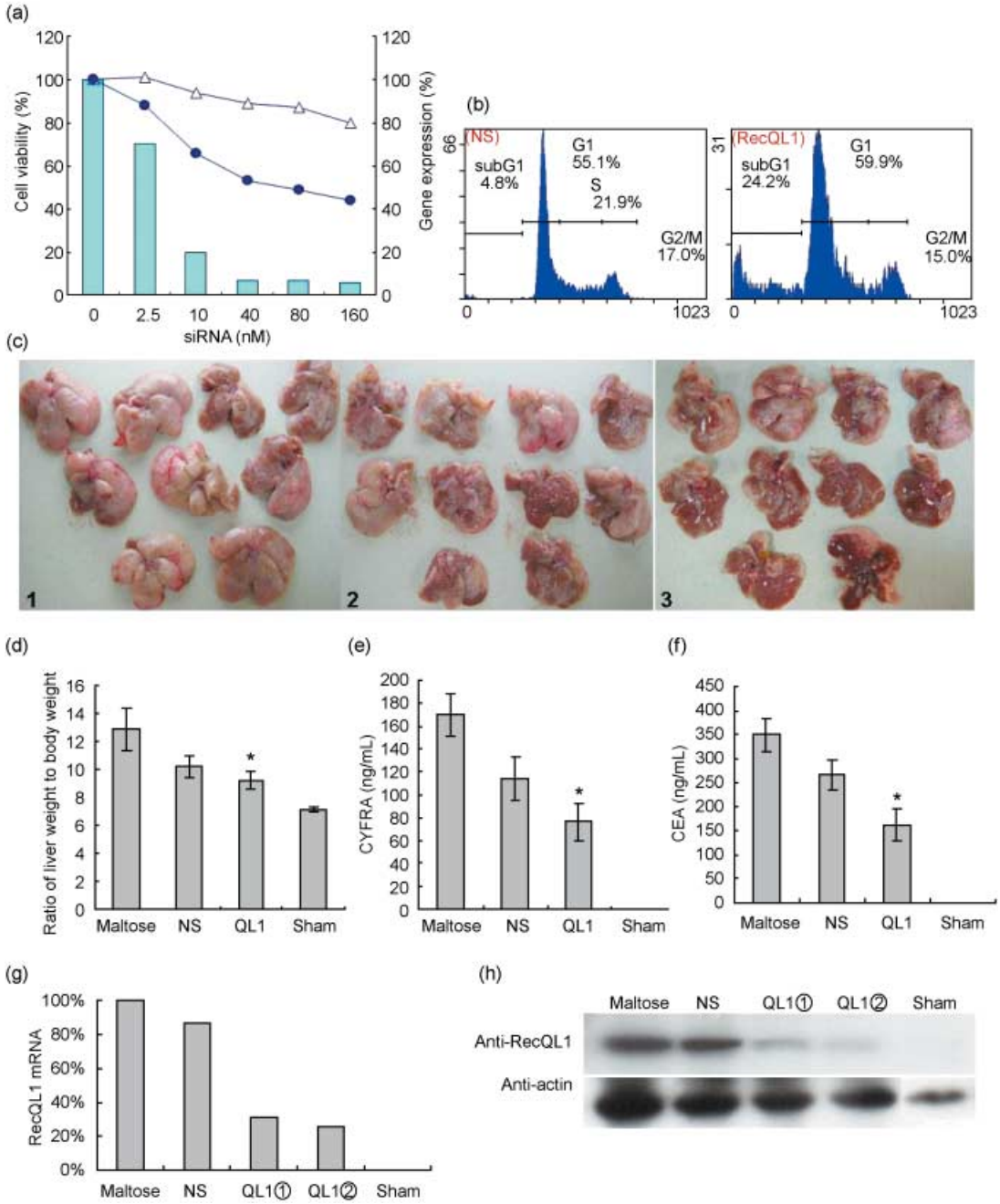

Fig. 4. Inhibiton of growth of AsPC-1 pancreatic cancer cells by systemic administration of the RecQL1-siRNA/LIC-101 complex in a mouse model of metastasized liver cancer. (a) Inhibition of AsPC-1 cell proliferation *in vitro* by *RecQL1* silencing. AsPC-1 cells were transfected *in vitro* by RecQL1-siRNAs with Lipofectamine 2000, and the inhibitory effect on proliferation was investigated. The viability of cells was measured by colorimetric assay as described in the 'Materials and Methods'. Open triangles show NS-siRNA–treated cells, and closed circles show RecQL1-siRNA-treated cells. The histograms show levels of RecQL1 mRNA in the *RecQL1*-silenced cells that are represented as a proportion (%) of NS-treated cells. (b) AsPC-1 cells with 40 nM RecQL1-siRNA in the presence of Lipofectamine 2000 were analyzed by using flow cytometry after 72 h of culture. NS, Non-silencing siRNA-treated cells; RecQL1, RecQL1-siRNA-treated cells. (c) Photographs of mouse livers treated with the NS-siRNA/LIC-101 complex and RecQL1-siRNA/LIC-101 complex at 29 days after intrasplenic inoculation with AsPC-1 cells. (c_1) 10% maltose, (c_2) NS-siRNA/LIC-101 complex, (c_3) RecQL1-siRNA/LIC-101 complex-treated liver. (d) Anticancer effect of the RecQL1-siRNA/LIC-101 complex evaluated by liver weight change in mouse liver metastasis model. AsPC-1 cells were inoculated into mouse liver intrasplenically on day 0. The RecQL1-siRNA/LIC-101 complex was administrated as described in 'Materials and Methods'. Liver weights were measured on day 29. *Mean SE (n = 10) is the significant difference from the maltose by Dunnett's test; $P < 0.05$. NS; NS-siRNA/LIC-101 complex, QL1; RecQL1-siRNA/LIC-101 complex-treated, sham; sham-operation. (e,f) Cancer markers CYFRA 21-1 and CEA indicating the anticancer effect of RecQL1-siRNA/LIC-101 complex intravenously injected into mice having pancreatic cancer cells metastasized to liver. *Mean SE (n = 10) is the significant difference from maltose by Dunnett's test; $P = 0.0016$ (CYFRA 21-1) and 0.0029 (CEA). (g) Expression of RecQL1 mRNA in human AsPC-1 cancer cells metastasized in liver determined on day 29 by reverse transcription–polymerase chain reaction (RT-PCR). This PCR analysis strictly precludes the spill-over from mouse RecQL1 mRNA. (h) Expression of RecQL1 protein in human AsPC-1 cancer cells metastasized in the liver determined on day 29 by Western blotting. The AsPC-1 cells metastasized in the mouse liver were homogenized, and the portion (containing 50 μg protein) was analyzed for human RecQL1 protein expression by using antibodies specific for human RecQL1 and actin. The antibody to human actin, though weakly, cross reacted with mouse actin.

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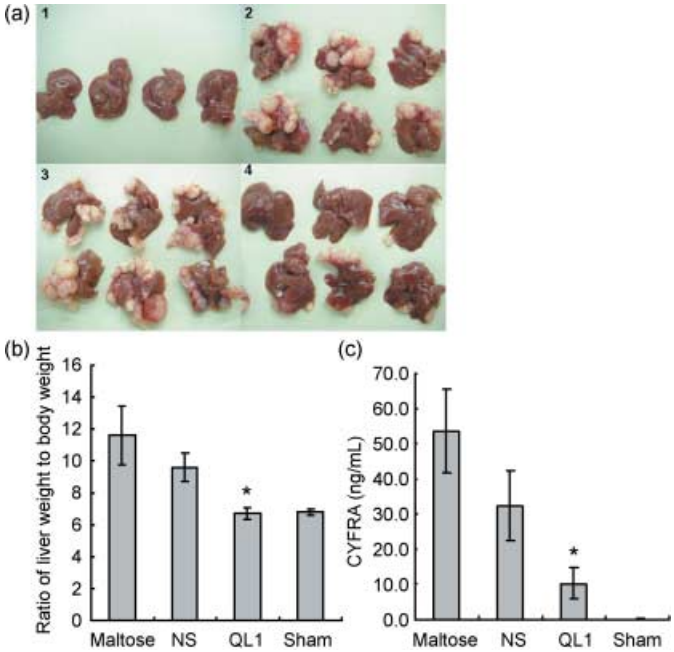

Fig. 5. Inhibition of LS174T colon cancer cell growth by intravenous injection of the RecQL1-siRNA/LIC-101 complex in a mouse model of metastatic liver cancer. (a) Livers treated with the NS-siRNA/LIC-101 complex and RecQL1-siRNA/LIC-101 complex at 37 days after intrasplenic inoculation with LS174T. (a_1) sham operation; (a_2) 10% maltose; (a_3) NS-siRNA/LIC-101 complex; (a_4) RecQL1-siRNA/LIC-101 complex. (b) LS174T was intrasplenically inoculated into nude mice on day 0. The RecQL1-siRNA/LIC-101 complex was administrated as described in 'Materials and Methods'. Liver weights were measured on day 37. *Mean SD ($n = 7$) is the significant difference from the maltose by Dunnett's test; $P < 0.05$. (c) Cancer marker CYFRA 21-1 indicating the anticancer effect of RecQL1-siRNA/LIC-101 complex intravenously injected into mice having liver metastasis cancer. *Mean SE ($n = 7$) is the significant difference from the maltose by Dunnett's test; $P = 0.008$.

maltose solution (Fig. 6e). HE staining of the liver slices showed no aberrant phenotype in mice administrated with an intravenous injection of a high dose of the RecQL1-siRNA/LIC-101 complex (Fig. $6f_2$).

Interferon induction that often accompanies *in vivo* injection of large amounts of double-stranded RNA was not seen with administration of the RecQL1-siRNA/LIC-101 complex under the same conditions of 2 mg/kg that we used in most of the study (Fig. 6g). These data indicated that systemic administration of the RecQL1-siRNA/LIC-101 complex by bolas intravenous injection is free from significant liver toxicity and physiological damage, despite RecQL1-siRNA/LIC-101 complex being preferentially delivered to the liver.

Discussion

DNA helicases have important roles in cellular processes, including DNA replication, recombination, repair, and transcription, by unwinding the duplex genome strands. Among many kinds of DNA helicases in living cells, the RecQ helicase family has unique properties that participate in maintaining genomic integrity, and is conserved in all organisms participating in various repair functions required to maintain genomic integrity. Human cells have five helicases belonging to the RecQ helicase family: RecQL1 (also known as RecQL or RecQ1), BLM, WRN, RTS, and RecQ5. We and others have shown that mutated BLM, WRN, and RTS helicases cause Bloom syndrome, Werner syndrome, and a subset of Rothmund–Thomson syndrome, respectively, all of which are recessive genetic disorders that show genomic instability and a high risk of cancer.[3–5] We believe association of mutations in RecQL1 and RecQ5 helicases with human disorders has not been shown.

We previously reported that silencing the expression of RecQL1 DNA helicase induces specific mitotic catastrophe and cell death in various human cancer cells.[12] This effect by RecQL1-siRNA is dependent on: (i) progression of the cell cycle in cell proliferation, because DNA damage forms endogenously in association with DNA replication resulting in long-term arrest in the M-phase and mitotic cell death after activation of the spindle checkpoint system; (ii) the defective nature of cancer cells in which the checkpoint system loosens due to mutations, such as in the *p53* gene; and (iii) most malignant cancer cell lines having a high level of expression of RecQL1 helicase that participates in DNA repair and which appears to be a prerequisite condition for efficient mitotic cell death by *RecQL1* silencing by siRNA. The results obtained from *in vitro* cell biological studies using RecQL1-siRNA are indeed consistent with the above mechanism underlying the selective mitotic catastrophe in cancer cells. It is intriguing to refer to the idea raised by Weinstein,[22] that cancer cells are often 'addicted to' (that is, physiologically dependent on) the continued activity of specifically activated or over-expressed oncogenes to maintain their malignant phenotype; these kinds of genes and gene products have been pursued as an ideal chemotherapeutic target for anticancer agents with few adverse effects.[23] RecQL1 has never been assumed to be essentially an oncogene-related product, but the possibility exists that cancer cells may also attract greater copy numbers of DNA repair enzymes, such as RecQL1, so that DNA damage is resolved in a short time, even if ample time of cell cycle arrests is unavailable due to defects in the checkpoint activity. To our knowledge this study may be the first to show that a nonessential DNA repair enzyme is a promising molecular target of cancer therapy, based on the mitotic catastrophe theory.

To apply this excellent property of RecQL1-siRNA for anticancer drug development, we tested cationic drug delivery systems PEI and LIC-101 liposome in several mouse cancer models by using different administration passages of local injection and systemic intravenous injection. Delivery of siRNA to target cells, tissues or organs is the major issue to be solved to use siRNA in human therapy. Our results with local injection showed that PEI promoted delivery of RecQL1-siRNA to A549 lung cancer and efficiently prevented the growth of cancers (Fig. 2a). In contrast, the RecQL1-siRNA/PEI complex was not effective by systemic administration to prevent A549 cancers under the skin (Fig. 2d), suggesting that PEI is not an adequate vector for systemic delivery of siRNA. The pharmacokinetics experiment in this study that used fluorescence-labeled RecQL1-siRNA showed that the retention time of RecQL1-siRNA in the blood was extremely short with PEI (Fig. 2e), mainly due to most of the RecQL1-siRNA complex with PEI of short linear configuration being discharged into the urine (our unpublished results). Further studies with various types of drug delivery system should help to overcome the hurdles confronting the medical application of siRNA.

Cationic vector LIC-101 liposome was found to be very effective for both local administration (shown in Fig. 3) and systemic administration by intravenous injection (Figs 4–6). LIC-101 delivers siRNA molecules to the cytosol, not to the nuclei of liver cells, having the advantage of avoiding toxicity in the nucleus.[16] Our study showed that IFN-α was not induced by intravenous bolus injection (Fig. 6g) and showed no sign of apparent adverse effects on normal cells, including the body weight of mice. Because of its liver-prone distribution after intravenous injection, LIC-101 liposome seems to be the most appropriate system of choice to deliver siRNA to the liver because it has been shown to be effective in delivering much

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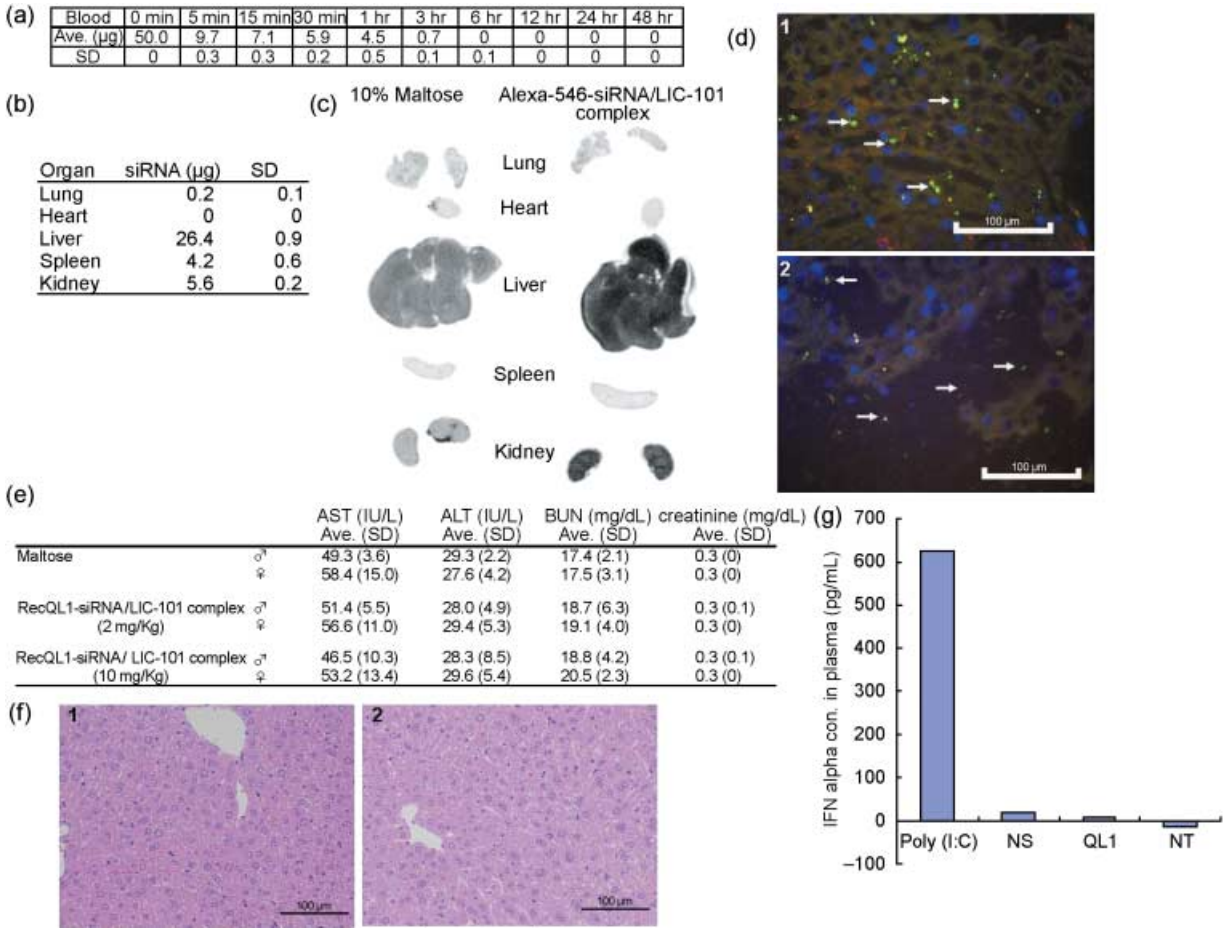

Fig. 6. Distribution of siRNA/LIC-101 complex and toxicological studies. (a) Kinetics of retention of siRNA/LIC-101 complex in circulating blood: after the intravenous injection, RecQL1-siRNA was extracted from the blood and was quantitated by using the method described in the 'Materials and Methods'. (b) Distribution of siRNA in organs: After the intravenous injection, RecQL1-siRNA was extracted from various organs 15 min after administration and was quantitated by using the method described in the 'Materials and Methods'. (c) Distribution of siRNA in organs: Alexa-546 labeled-siRNA/LIC-101 complex (1 mg/kg) was administrated into BALB/c mice by intravenous injection into the tail vein. Distribution of siRNA in organs was visualized by the fluorescence after 24 h. (d) Distribution of intravenously injected FAM-labeled-siRNA/LIC-101 complex in the liver after 24 h. (d_1) non-cancer cell region; (d_2) cancer cell region. Photographic pictures of the stained sliced liver are shown. Arrow heads show the FAM-labeled-siRNA, and blue dots show the nuclei of the non-cancer cells selectively stained by Hoechst33342. (e) Toxicological studies with liver and kidney: 2 or 10 mg/kg of RecQL1-siRNA/LIC-101 complex was given to BALB/c mice every other day for 4 weeks. The serum was investigated for liver and kidney function markers. Data represent the mean ± SD (n = 5). (f) Pathological studies on mice injected with the RecQL1-siRNA/LIC-101 complex (intravenous administration of 10 mg/kg every other day for 4 weeks). Liver slices were stained by hematoxylin–eosin. (f_1) 10% maltose, (f_2) RecQL1-siRNA/LIC-101 complex. (g) Absence of interferon-alpha (IFN-α) induction by RecQL1-siRNA/LIC-101 liposome. BALB/c mice were administrated with RecQL1-siRNA (50 μg) together with LIC-101 liposome in 10% maltose by intravenous injection into the tail vein. The positive control group was injected with poly (I:C). Serum was collected 3 h after injection; the IFN-α level was determined by ELISA.

larger molecules of Poly (I:C) to the human liver in clinical studies.[15,21] The relevant phase 1 clinical study also showed that LIC-101 liposome was non-toxic to humans when used alone. An apoptosis-attenuating Bcl-2 siRNA is effective in a complex with LIC-101 liposome to prevent growth of human cancers in mouse models, such as in A549 liver-metastasized lung cancer, by systemic intravenous injection of 10 mg/kg siRNA or in PC3 prostate cancer by local subcutaneous injection of 0.1 mg/kg siRNA.[16] Local administration of the siRNA/LIC-101 complex, which silences the M phase-modulator *PLK* gene, is effective in inhibiting the growth of UM-UC-3 bladder cancer.[24] Bovine atelocollagen, a fraction of collagen lacking N- and C-terminal terminal peptides, is another delivery system to deliver VEGF siRNA locally to prostate cancers.[25] Atelocollagen has also been used for systemic delivery of siRNA by intravenous injection; EZH2 and p110-alpha siRNAs mixed with atelocollagen prevents growth of cancers in animal models of bone-metastasized cancer.[26]

We believe that the RecQL1-siRNA/LIC-101 complex can prevent proliferation of hepatocarcinoma cells in the liver because most hepatocarcinoma cells, particularly cells in advanced liver cancers, express *RecQL1* and are likely to be sensitive to treatment with RecQL1-siRNA (Fig. 1), although no appropriate mouse xenograft models of endogenous human liver cancer are available to test the possibility now. Instead, in this study the RecQL1-siRNA/LIC-101 complex contributed to therapy for metastasized liver cancers, such as pancreatic cancers as shown for AsPC-1 (Fig. 4) and colorectal cancer as shown by LS174T (Fig. 5), both of which very often take place clinically, in addition to spontaneous and endogenous liver cancers caused by chronic HBV or hepatitis C virus infection or both. Encouragement in this direction is that the RecQL1-siRNA/LIC-101 complex showed promising results in the prevention of expansion of metastasized liver cancers (Figs 4,5), and increased significantly the survival time of malignant pancreatic cancer LS174T.

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Throughout this study we used GL3-siRNA which silences the firefly luciferase gene *GL3* as a negative control NS-siRNA, because it shows no homology to human genes and because it has been used by several studies previously for similar purposes. We noted, however, that this non-human NS-siRNA surprisingly shows, although faint, an inhibitory effect on the growth of human cancer cells when compared with another negative control consisting of plain 10% maltose (Figs 4,5). A similar weak inhibitory effect by the NS (GL3)-siRNA/LIC-101 complex was also obtained previously by Yano *et al*.[16] in their *in vivo* studies with liver-metastasized A549 cells,[16] and also in our *in vitro* HeLa cell studies with lipofectamine.[12] Why GL3-siRNA shows toxicity, although weak, for cancer cells should be investigated in the strict sense because it is widely used as an important standard sequence.

In the therapeutic application of siRNA for oncology, a combination therapy of siRNA with an existing drug is considered to increase the activity of the drug or to reduce the dose of drug or both, that is, to have equal clinical efficacy resulting in a lower adverse effect often associated with anticancer genotoxic drugs. For example, silencing the gene coding for the subunit polypeptide of ribonucleotide reductase RRM2 and survivine by cognate siRNAs increases the sensitivity of cancer cells to gemcitabine[27] and adriamycin.[28] In this context, siRNA which down-regulates the expression of WRN helicase, lowers the effective dose of camptothecin, a well-known genotoxic anticancer agent used for colorectal cancer treatment,[29,30] indicating that such combination therapy should permit lowering the adverse effects of camptothecin. An analogy is the combination therapy of the RecQL1-siRNA/LIC-101 complex, which is expected to be free from adverse effects, with other drugs that is expected to produce a synergistic therapeutic effect to help improve the quality of life of patients, and at the same time keeping (or raising) the high therapeutic capability of both drugs.

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