the most resistant to mouse serum. Thus, we succeeded in developing a serum-resistant vector system.

Discussion

We succeeded in enhancing the transfection efficiency of HVJ-E by combining it with cationic polymers. For cultured cells *in vitro*, the most efficient transfection was obtained by combining HVJ-E with both cationized gelatin and protamine sulfate. However, for *in vivo* transfection, CG-HVJ-E without protamine sulfate resulted in the highest gene expression. These findings are consistent with our previous report indicating that the particle size of cationic liposomes may affect gene transfection efficiency [36]. By adding both protamine sulfate [37] and cationized gelatin to HVJ-E, the size and charge of the resulting complex may have been the most suitable for *in vitro* transfection. Protamine sulfate and cationized gelatin affected gene transfection efficiency in a variety of cell lines as well as in primary cells, although the efficiency was varied among cell types. The ratio of protamine sulfate and cationized gelatin used for these experiments was determined by gene transfection experiments with CT26 cells. Thus, gene expression in the other cell types may be enhanced when the conditions are optimized for each cell type.

We determined that cell fusion is the mechanism responsible for a PS-CG-HVJ-E-mediated gene transfer system. Although endocytosis appeared to be involved in gene transfection based on the wortmannin experiments, transfection was completely inhibited by antibody against the fusion protein of HVJ. Since the fusion activity of HVJ is pH-independent [31], HVJ can fuse with the cell membrane both on the cell surface and in endocytotic vesicles. Even for the HVJ-E complex with protamine sulfate and cationized gelatin, the F protein of HVJ appeared to associate with the cell membrane, and fusion activity appeared to be necessary for gene transfection.

As shown in Figure 5, HVJ-E complexed with cationized gelatin targeted the liver. With protamine sulfate, gene expression in the liver after intravenous injection was lower than with CG-HVJ-E. We speculate that larger particles with positive charge are less mobile when intravenously administered. Comparison with PS-HVJ-E and PS-CG-HVJ-E suggests that CG-HVJ-E may have the appropriate size and potential for targeting the liver after intravenous injection.

Numerous biocompatible polymers have been developed to enhance gene delivery systems [38–45]. Pullulan complexed with naked DNA targets the liver [46,47]. However, pullulan–HVJ-E complexes failed to transfect tissues, including the liver. Dextran–HVJ-E was also not an efficient complex for gene transfer. Only low molecular weight cationized gelatin has formed an effective complex with HVJ-E that enhances transfection efficiency both *in vitro* and *in vivo*, although the precise mechanism is still unknown.

Our results suggest that the CG-HVJ-E vector may be effective and practical for the treatment of liver diseases, such as liver cirrhosis and hepatitis, when therapeutic genes encoding secreted proteins, such as HGF, soluble TGF-β receptor and decorin, are employed. Moreover, long-term gene expression in the liver can be achieved with Epstein-Barr virus replicon plasmid [33] and the Sleeping Beauty transposon system [48]. CG-HVJ-E may be clinically tested in the near future because it does not require a large volume of solution to be injected (as used in the hydrodynamic method) [48,49]. An adverse effect of this treatment is that coagulation function is transiently decreased by CG-HVJ-E in mice, although it recovered in 1 day (H. Mima and Y. Kaneda, unpubl. obs.). This adverse effect is probably caused by HVJ hemagglutinating protein, which is necessary for binding with sialic acid, a virus receptor [32]. When HVJ-E is complexed with cationized gelatin, cationized gelatin may perform the function of hemagglutinating protein and enhance the association with cell membranes. If HVJ-E without hemagglutinating protein is combined with cationized gelatin, the complex may reduce adverse effects to a much lower level.

An additional advantage of cationized gelatin is that it protects HVJ-E from degradation in fresh mouse serum. Although the *in vitro* transfection efficiency of HVJ-E was not inhibited by culture medium containing 10% FBS [32], the activity of HVJ-E was rapidly lost in the presence of fresh mouse serum (Figure 6). However, CG-HVJ-E was significantly stable in 50% fresh mouse serum. The high transfection activity of CG-HVJ-E after intravenous injection appears to be mediated by the stability of the vector in fresh serum. Retrovirus [50] and HIV [51] are degraded in human serum due to complement lysis. Liposomes composed of hydrogenated egg phosphatidylcholine and cholesterol activate the complement system in rats by interacting with IgG and IgM [52]. Although it is unproven that HVJ is degraded by complement lysis in mouse serum, the interaction of serum proteins with HVJ-E may be involved in the loss of transfection activity of HVJ-E. Conjugation to cationized gelatin appears to protect the surface molecules of HVJ-E from the detrimental effects of serum proteins.

The results of this study suggest that low molecular weight cationized gelatin may be appropriate for complex formation with various envelope viruses, such as retrovirus, herpes virus and HIV, and that the cationized gelatin–envelope virus vector may enhance transfection efficiency both *in vitro* and *in vivo*. This technology may lead to the achievement of an ideal vector system with high efficiency and minimal toxicity.

References

1. Marshall E. Gene therapy's growing pains. *Science* 1995; 269: 1052–1055.
2. Mulligan RC. The basic science of gene therapy. *Science* 1993; 260: 926–932.

3. Li S, Huang L. Non-viral gene therapy; promises and challenges. *Gene Ther* 2000; **7**: 31–34.
4. Hwang SJ, Davis ME. Cationic polymers for gene delivery; designs for overcoming barriers to systemic administration. *Curr Opin Mol Ther* 2001; **3**: 183–191.
5. Pannier AK, Shea LD. Controlled release systems for DNA delivery. *Mol Ther* 2004; **10**: 19–26.
6. Han S, Mahato RI, Sung YK, Kim SW. Development of biomaterials for gene therapy. *Mol Ther* 2000; **2**: 302–317.
7. Qiang B, Segev A, Beliard I, Nili N, Strauss BH, Sefton MV. Poly(methylidene malonate 2.1.2) nanoparticles: a biocompatible polymer that enhances peri-adventitial adenoviral gene delivery. *J Control Release* 2004; **98**: 447–455.
8. Han S, Mahato RI, Kim SW. Water-soluble lipopolymer for gene delivery. *Bioconjugate Chem* 2001; **12**: 337–345.
9. Lim YB, Kim SM, Suh H, Park JS. Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and nontoxic gene delivery carrier. *Bioconjugate Chem* 2002; **13**: 952–957.
10. Le Doux JM, Landazuri N, Yarmush ML, Morgan JR. Complexation of retrovirus with cationic and anionic polymers increases the efficiency of gene transfer. *Hum Gene Ther* 2001; **12**: 1611–1621.
11. Koping-Hoggard M, Tubulekas I, Guan H, *et al.* Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Gene Ther* 2001; **8**: 1108–1121.
12. Wang J, Zhang PC, Mao HQ, Leong KW. Enhanced gene expression in mouse muscle by sustained release of plasmid DNA using PPE-EA as a carrier. *Gene Ther* 2002; **9**: 1254–1261.
13. Sano A, Maeda M, Nagahara S, *et al.* Atelocollagen for protein and gene delivery. *Adv Drug Deliv Rev* 2003; **55**: 1651–1677.
14. Honma K, Ochiya T, Nagahara S, *et al.* Atelocollagen-based gene transfer in cells allows high-throughput screening of gene functions. *Biochem Biophys Res Commun* 2001; **289**: 1075–1081.
15. Nguyen JB, Sanchez-Pernaute R, Cunningham J, Bankiewicz KS. Convection-enhanced delivery of AAV-2 combined with heparin increases TK gene transfer in the rat brain. *Neuroreport* 2001; **12**: 1961–1964.
16. Kichler A. Gene transfer with modified polyethylenimines. *J Gene Med* 2004; **6**: S3–10.
17. Fischer D, Bieber T, Li Y, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm Res* 1999; **16**: 1273–1279.
18. Sweeney P, Karashima T, Ishikura H, *et al.* Efficient therapeutic gene delivery after systemic administration of a novel polyethylenimine/DNA vector in an orthotopic bladder cancer model. *Cancer Res* 2003; **63**: 4017–4020.
19. Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther* 1999; **6**: 595–605.
20. Hosseinkhani H, Aoyama T, Ogawa O, Tabata Y. Ultrasound enhancement of in vitro transfection of plasmid DNA by a cationized gelatin. *J Drug Target* 2002; **10**: 193–204.
21. Hosseinkhani H, Tabata Y. In vitro gene expression by cationized derivatives of an artificial protein with repeated RGD sequences, pronectin. *J Control Release* 2003; **86**: 169–182.
22. Fukunaka Y, Iwanaga K, Morimoto K, Kakemi M, Tabata Y. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. *J Control Release* 2002; **80**: 333–343.
23. Wagner E. Strategies to improve DNA polyplexes for in vivo gene transfer: will "artificial viruses" be the answer? *Pharm Res* 2004; **21**: 8–14.
24. Mahato RI, Anwer K, Tagliaferri F, *et al.* Biodistribution and gene expression of lipid/plasmid complexes after systemic administration. *Hum Gene Ther* 1998; **9**: 2083–2099.
25. Pun SH, Davis ME. Development of a nonviral gene delivery vehicle for systemic application. *Bioconjugate Chem* 2002; **13**: 630–639.
26. Takakura Y, Nishikawa M, Yamashita F, Hashida M. Development of gene drug delivery systems based on pharmacokinetic studies. *Eur J Pharm Sci* 2001; **13**: 71–76.
27. Bragonzi A, Boletta A, Biffi A, *et al.* Comparison between cationic polymers and lipids in mediating systemic gene delivery to the lungs. *Gene Ther* 1999; **6**: 1995–2004.
28. Liu F, Qi H, Huang L, Liu D. Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration. *Gene Ther* 1997; **4**: 517–523.
29. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum Gene Ther* 2001; **12**: 861–870.
30. Tabata Y, Nagano A, Ikada Y. Biodegradation of hydrogel carrier incorporating fibroblast growth factor. *Tissue Eng* 1995; **5**: 127–138.
31. Okada Y. Sendai-virus induced cell fusion. *Methods Enzymol* 1993; **221**: 18–41.
32. Kaneda Y, Nakajima T, Nishikawa T, *et al.* Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. *Mol Ther* 2002; **6**: 219–226.
33. Saeki Y, Wataya-Kaneda M, Tanaka K, Kaneda Y. Sustained transgene expression in vitro and in vivo using an Epstein-Barr virus replicon vector system combined with HVJ liposomes. *Gene Ther* 1998; **5**: 1031–1037.
34. Shpetner H, Joly M, Hartley D, Corvera S. Potential sites of PI-3 kinase function in the endocytic pathway revealed by the PI-3 kinase inhibitor, wortmannin. *J Cell Biol* 1996; **132**: 595–605.
35. Chen X, Wang Z. Regulation of intracellular trafficking of the EGF receptor by Rab5 in the absence of phosphatidylinositol 3-kinase activity. *EMBO Rep* 2001; **2**: 68–74.
36. Saeki Y, Matsumoto N, Nakano Y, Mori M, Awai K, Kaneda Y. Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for in vitro and in vivo gene transfer. *Hum Gene Ther* 1997; **8**: 2133–2141.
37. Yang YW, Hsieh YC. Protamine sulfate enhances the transduction efficiency of recombinant adeno-associated virus-mediated gene delivery. *Pharm Res* 2001; **18**: 922–927.
38. Merdan T, Kopecek J, Kissel T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Adv Drug Deliv Rev* 2002; **54**: 715–758.
39. Maruyama K, Iwasaki F, Takizawa T, *et al.* Novel receptor-mediated gene delivery system comprising plasmid/protamine/sugar-containing polyanion ternary complex. *Biomaterials* 2004; **25**: 3267–3273.
40. Putnam D, Gentry CA, Pack DW, Langer R. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proc Natl Acad Sci U S A* 2001; **98**: 1200–1205.
41. Hofland HE, Masson C, Iginla S, *et al.* Folate-targeted gene transfer in vivo. *Mol Ther* 2002; **5**: 739–744.
42. Su J, Kim CJ, Ciftci K. Characterization of poly((N-trimethylammonium)ethyl methacrylate)-based gene delivery systems. *Gene Ther* 2002; **9**: 1031–1036.
43. Schakowski F, Gorschluter M, Junghans C, *et al.* A novel minimal-size vector (MIDGE) improves transgene expression in colon carcinoma cells and avoids transfection of undesired DNA. *Mol Ther* 2001; **3**: 793–800.
44. Wang J, Zhang PC, Lu HF, *et al.* New polyphosphoramidate with a spermidine side chain as a gene carrier. *J Control Release* 2002; **83**: 157–168.
45. Yun YH, Goetz DJ, Yellen P, Chen W. Hyaluronan microspheres for sustained gene delivery and site-specific targeting. *Biomaterials* 2004; **25**: 147–157.
46. Kaneo Y, Tanaka T, Nakano T, Yamaguchi Y. Evidence for receptor-mediated hepatic uptake of pullulan in rats. *J Control Release* 2001; **70**: 365–373.
47. Hosseinkhani H, Aoyama T, Ogawa O, Tabata Y. Liver targeting of plasmid DNA by pullulan conjugation based on metal coordination. *J Control Release* 2002; **83**: 287–302.
48. Mikkelsen JG, Yant SR, Meuse L, Huang Z, Xu H, Kay MA. Helper-independent *Sleeping Beauty* transposon-transposase vectors for efficient nonviral gene delivery and persistent gene expression *in vivo*. *Mol Ther* 2003; **8**: 654–665.
49. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther* 1999; **10**: 1735–1737.
50. Fujita F, Yamashita-Futsuki I, Eguchi S, *et al.* Inactivation of porcine endogenous retrovirus by human serum as a function of complement activated through the classical pathway. *Hepatol Res* 2003; **26**: 106–113.

51. Okada H, Wu X, Okada N. Complement-mediated cytolysis and azidothymidine are synergistic in HIV-1 suppression. *Int Immunol* 1998; **10**: 91–95.
52. Ishida T, Yasukawa K, Kojima H, Harashima H, Kiwada H. Effect of cholesterol content in activation of the classical versus the alternative pathway of rat complement system induced by hydrogenated egg phosphatidylcholine-based liposomes. *Int J Pharm* 2001; **224**: 69–79.

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Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin

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Abstract

Background Every cancer therapy appears to be transiently effective for cancer regression, but cancers gradually transform to be resistant to the therapy. Cancers also develop machineries to resist chemotherapy. Short interfering RNA (siRNA) has been evaluated as an attractive and effective tool for suppressing a target protein by specifically digesting its mRNA. Suppression of the machineries using siRNA may enhance the sensitivity to chemotherapy in cancers when combined with an effective delivery system.

Methods To enhance the anti-cancer effect of chemotherapy, we transferred siRNA against Rad51 into various human cancer cells using the HVJ (hemagglutinating virus of Japan, Sendai virus) envelope vector in the presence or absence of cis-diamminedichloroplatinum(II) (CDDP, cisplatin). The inhibition of cell growth was assessed by a modified MTT assay, counting cell number, or fluorescence-activated cell sorting (FACS) analysis after Annexin V labeling. The synthetic Rad51 siRNA was also introduced into subcutaneous tumor masses of HeLa cells in SCID mice with or without intraperitoneal injection of CDDP, and tumor growth was monitored.

Results When synthetic Rad51 siRNA was delivered into HeLa cells using the HVJ envelope vector, no Rad51 transcripts were detected on day 2, and Rad51 protein completely disappeared for 4 days after siRNA transfer. When HeLa cells were incubated with 0.02 μg/ml CDDP for 3 h after siRNA transfer, the number of colonies decreased to approximately 10% of that with scrambled siRNA. The sensitivity to CDDP was enhanced in various human cancer cells, but not in normal human fibroblasts. When Rad51 siRNA was delivered into tumors using the HVJ envelope vector, the Rad51 transcript level was reduced to approximately 25%. Rad51 siRNA combined with CDDP significantly inhibited tumor growth when compared to siRNA or CDDP alone.

Conclusions Rad51 siRNA could enhance the sensitivity to CDDP in cancer cells both *in vitro* and *in vivo*. Our results suggest that the combination of CDDP and Rad51 siRNA will be an effective anti-cancer protocol.

Keywords chemotherapy; siRNA; Rad51; non-viral vector; drug delivery; cancer therapy

Introduction

Although many different therapeutic strategies or regimens have been developed, there is no definitive treatment for cancer. Every cancer therapy appears to be transiently effective for cancer regression, but cancers gradually

transform to be resistant to the therapy. Although strategies have been developed to reverse the resistance, cancer cells develop mechanisms to escape the immune system and anti-neoplastic treatments [1–3]. cis-Diamminedichloroplatinum(II) (CDDP) is one of the most widely used anti-cancer drugs [4–6]. CDDP inhibits cellular growth by inducing DNA double-strand breaks [7–9]. However, cells can use DNA repair machinery to respond to the DNA damage. The levels of DNA repair proteins correlate with resistance to anti-cancer drugs, especially alkylating agents, in human cancer cell lines [10]. Two pathways, homologous recombination and non-homologous end joining, are used to repair DNA double-strand breaks [11,12]. BRCA 1 and 2 in a complex with Rad51 are involved in homologous recombination [11–13]. Non-homologous repair is performed by the complex of NBS1, MRE11, and Rad50 with the aid of Ku 70, Ku 80, the DNA-dependent protein kinase catalytic subunit, DNA ligase IV, and XRCC4 [11,14]. Different studies have drawn conflicting conclusions regarding the pathway used to repair CDDP-induced DNA double-strand breaks in mammalian cells. Initially, non-homologous end joining was believed to responsible for the repair of CDDP-induced DNA damage [15–17]. However, CDDP sensitivity was not affected by the level of the Ku70, which is needed for non-homologous end joining repair [18]. However, sensitivity to other DNA-damaging agents, such as bleomycin and methyl methanesulfonate, was elevated by suppression of Ku70 [18]. These findings suggest that non-homologous end joining is not used to repair DNA damage induced by CDDP. Recent evidence suggests that homologous recombination is involved in the repair of DNA double-strand breaks generated by CDDP [19–21]. Cancer cells may become resistant to CDDP by increasing the activity of homologous recombination repair machinery. Indeed, a high level of Rad51 is consistent with tumor progression and tumor resistance to cancer therapy [22]. Conversely, disabling the DNA repair machinery may enhance the sensitivity of cancers to CDDP.

The present study focuses on the function of Rad51 as a regulator of CDDP sensitivity. We tested the ability of short interfering RNA (siRNA) to inhibit the expression of Rad51. siRNA has been evaluated as an attractive and effective tool for suppressing the target protein by specifically digesting its mRNA [23,24]. siRNA is superior to antisense oligonucleotides and ribozymes in terms of efficiency and specificity [25,26]. However, finding a suitable delivery system for siRNA has been problematic [27]. We have been developing a highly efficient gene delivery system with minimum toxicity by converting viruses into non-viral vectors. We incorporated plasmid DNA into inactivated HVJ (hemagglutinating virus of Japan, Sendai virus) particles to form a HVJ envelope vector. By the strong fusion activity, DNA inside the envelope vector can be directly introduced into the cytoplasm of various types of cells both *in vitro* and *in vivo*. The HVJ envelope vector is also very effective for drug delivery [28,29]. siRNA was successfully introduced into pancreatic islet cell lines using the HVJ envelope vector [30]. In the present study, siRNA against human Rad51 enhanced the sensitivity of cancers to CDDP both *in vitro* and *in vivo*.

Materials and methods

HVJ

HVJ was amplified in chorioallantoic fluid of 10- to 14-day-old chick eggs and was purified by centrifugation and inactivated by UV irradiation (99 mJ/cm^2) as previously described [28]. Inactivated virus cannot replicate, but its capacity for viral fusion remains intact.

Cell culture

Human cancer cells and normal human diploid fibroblasts (NHDF) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics.

Rad51 cDNA transfer and cell survival assay

The Rad51 open reading frame sequence was subcloned into the expression vector using the Gateway system (Invitrogen, San Diego, CA, USA), amplified, and transfected into HeLa cells (3×10^5 cells) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The next day, the cells were passaged in 12-well plates (2×10^4 cells/well). Forty-eight hours after transfection, the cells were treated with 0–4 μg/ml CDDP (Nihon Kayaku, Tokyo, Japan) for 3 h. Then, 48 h later, cell survival was assessed by a modified MTT assay (Dojindo, Tokyo, Japan) as described elsewhere [31].

HVJ envelope vector-mediated siRNA transfection *in vitro*

An inactivated HVJ suspension (6×10^9 particles) was mixed with 60 μl of 40 μM Rad51 siRNA (5′-GAGCUUGACAAACUACUUC-3′) solution (Dharmacon, Lafayette, CO, USA) and 6 μl of 2% Triton X-100. Scrambled siRNA (5′-GCGCGCUUUGUAGGATTCG-3′) solution (Dharmacon) was used as a control. After centrifugation (18 500 *g*, 15 min) at 4°C, the supernatant was removed and HVJ envelope vector that included siRNA was suspended in 120 μl of phosphate-buffered saline (PBS). The incorporation rate of siRNA was approximately 20% of total siRNA initially used. Unincorporated siRNA was reduced to an undetectable level by this process. For *in vitro* transfection of HVJ that contained siRNA, 1×10^5 cancer cells were seeded in 6-well plates 1 day before transfection. Protamine sulfate (5 μl, 5 mg/ml; Nacalai Tesque, Kyoto, Japan) and 500 μl of medium were added to 20 μl (1×10^9 particles) of HVJ that contained

siRNA. Approximately 80 pmol siRNA were delivered to 1×10^5 cells. The cell culture medium was removed, and the HVJ envelope vector was added to each well. Thirty minutes later, the medium containing the vector was replaced with fresh medium.

Western blot analysis

The harvested human cancer cells were lysed in lysis buffer (1% SDS, 20 mM Tris-HCl (pH 8), 135 mM NaCl, 10% glycerol, and a protease inhibitor mixture (Roche, Basel, Switzerland)). After adding 2× sample buffer (0.1 M Tris-HCl (pH 6.8), 4% SDS, 12% 2-mercaptoethanol, 20% glycerol, and 0.01% bromphenol blue), 30 μg of protein were separated by 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk and subsequently probed with antibodies, anti-human Rad51 (Santa Cruz, Santa Cruz, CA, USA), anti-β-actin (Abcam, Cambrige, UK), and anti-GAPDH (Ambion, Austin, TX, USA). Proteins were detected with horseradish peroxidase labeled anti-goat (Santa Cruz) or anti-mouse (Amersham, Piscataway, NJ, USA) antibodies and the enhanced chemiluminescence reagent (Amersham).

Northern blot analysis

Total RNA was isolated from HeLa cells using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total RNA (15 μg/lane) was separated in a formaldehyde/1.5% agarose gel, transferred to Hybond N+ membrane (Amersham), and then hybridized with ^{32}P-labeled Rad51 and G3PDH cDNA probes.

Colony forming assay

Twenty-four hours after HVJ envelope vector-mediated siRNA transfection to HeLa cells *in vitro*, the cells were seeded in a 6-cm dish at a density of 10^3 cells/dish and treated with 0–0.1 μg/ml CDDP for 3 h. After 7 days, the colonies were fixed with methanol and stained with Giemsa (Nacalai Tesque). Then, the colonies were counted. The percentage of colony-forming cells after CDDP treatment was calculated and compared to the untreated control group.

CDDP sensitivity in cultured cells by Rad51 siRNA transfer

Forty-eight hours after transfer of siRNA, the cells were treated with 0.1, 0.3 and 1.0 μg/ml CDDP for 3 h. Then, 48 h later, cell number was counted using a particle counter (Coulter Corporation, Miami, FL, USA). To assess apoptosis, cells treated with Rad51 siRNA and CDDP were harvested and stained with fluorescent isothiocyanate-labeled Annexin V (Becton Dickinson, San Diego, CA, USA) for 20 min at room temperature. The labeled cells were analyzed with FACScan (Becton Dickinson).

In vivo experiments

Viable HeLa cells (5×10^6 cells) were resuspended in 100 μl of PBS and intradermally injected into the right flanks of 6-week-old male SCID mice (Charles River Japan, Yokohama, Japan). The inactivated HVJ suspension (6×10^9 particles) was mixed with 60 μl of 250 μM Rad51 siRNA solution and 6 μl of 2% Triton X-100. Scrambled siRNA solution was used as a control. After centrifugation (18 500 *g*, 15 min) at 4 °C, the supernatant was removed and the HVJ envelope vector containing siRNA was suspended in 120 μl of PBS. Seven days after tumor inoculation, 100 μl (5×10^9 particles) of HVJ envelope vector containing siRNA were injected into the tumor. Approximately 2.5 nmol siRNA were delivered to the tumor mass in a mouse. The injection

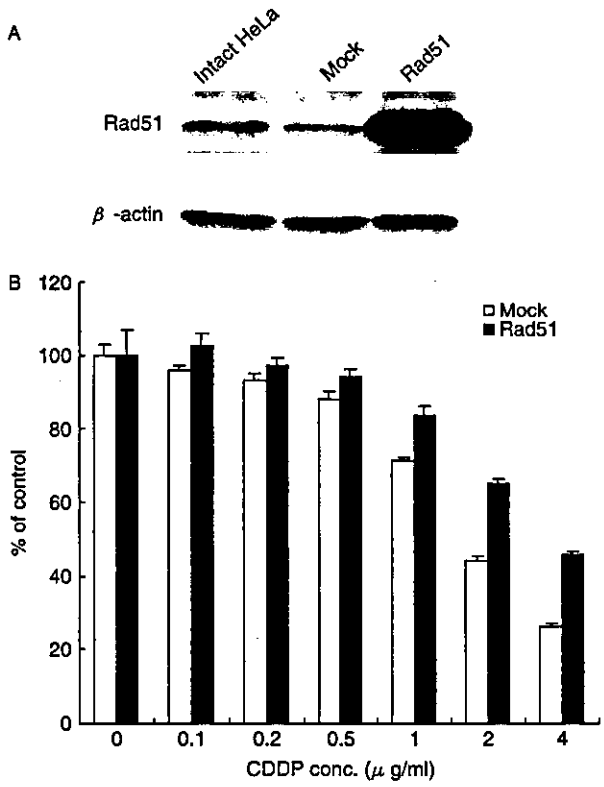


Figure 1. (A) Detection of human Rad51 transcript 48 h after the transfection of human Rad51 cDNA driven by the CMV promoter. Mock sample indicates HeLa cells transfected with a plasmid that did not contain Rad51 cDNA. Intact HeLa indicates HeLa cells that were not transfected. (B) Cell survival was detected by a modified MTT assay after treatment with 0–4 μg/ml CDDP for 3 h. The ordinate indicates the ratio of viable cells treated with various concentrations of CDDP to initial cell number. The mean value ± standard deviation from triplicate samples is shown

was repeated at 2-day intervals until each mouse received a total of three injections. At the time of the second siRNA injection, 200 μg of CDDP were intraperitoneally injected. Tumor size was measured every 2 days, and the tumor volume was calculated using the simplified formula for a rotational ellipse ($l \times w^2 \times 0.5$). All animals were treated in a humane fashion in accordance with the guidelines of the Animal Committee of Osaka University.

Results

To determine what factors induced by CDDP contribute to the repair of DNA damage, we examined the gene expression of repair genes in cells treated with CDDP. The protein level of Rad51, which is involved in homologous recombination repair, increased 1.57 ± 0.4 times more with CDDP than that without CDDP (data not shown). However, the expression level of Ku70, which is involved in non-homologous end joining, was not changed (0.9 ± 0.3 times) by CDDP treatment.

We examined whether Rad51 expression resulted in resistance to CDDP. To increase the expression of Rad51, HeLa cells were transfected with the human Rad51 gene driven by the cytomegalovirus (CMV) promoter (Figure 1A). When cell proliferation was measured by a modified MTT assay, Rad51-transfected HeLa cells cultured with various concentrations of CDDP were more viable than control cells that had undergone only a mock transfection (Figure 1B). The experiment was repeated three times, and similar results were obtained.

To enhance sensitivity to CDDP, we attempted to suppress Rad51 expression with siRNA. When Cy3-labeled siRNA was delivered to HeLa cells using the HVJ envelope vector, the efficiency was 80–100% (data not shown). Rad51 transcripts were not detected by Northern blot analysis 1 day after siRNA delivery, whereas scrambled siRNA did not reduce the transcript level (Figure 2A). We tested five different siRNAs for Rad51, but the only effective siRNA was a 19-mer from no. 321 of the Rad51 mRNA sequence. The other four siRNAs (19-mers from nos. 89, 462, 828, and 989) did not suppress Rad51 expression (data not shown). Two different antisense oligonucleotides against human Rad51 did not reduce the expression of human Rad51 (Figure 2B). These oligonucleotides had the same sequence as mouse Rad51 antisense oligonucleotides that had been used for suppression of Rad51 [32]. Rad51 protein was not detected by Western blots for 4 days after siRNA transfer. A small amount of Rad51 protein began to reappear on day 5 (Figure 2C). When Rad51 siRNA was introduced into HeLa cells, the growth of the cells was suppressed and the viability was 70% less than cells treated with scrambled siRNA (Figure 3A). The growth of cells treated with scrambled siRNA was not significantly different compared to that of cells treated with HVJ-E containing PBS. When HeLa cells were incubated with 0.02 μg/ml CDDP for 3 h after the delivery of Rad51 siRNA, the survival of the cells was reduced by 90% when compared to equivalent cells that were not exposed to CDDP (Figure 3B). More than 90% of colonies were formed with the same concentration of CDDP when scrambled siRNA was transferred into HeLa cells. Accordingly, with Rad51

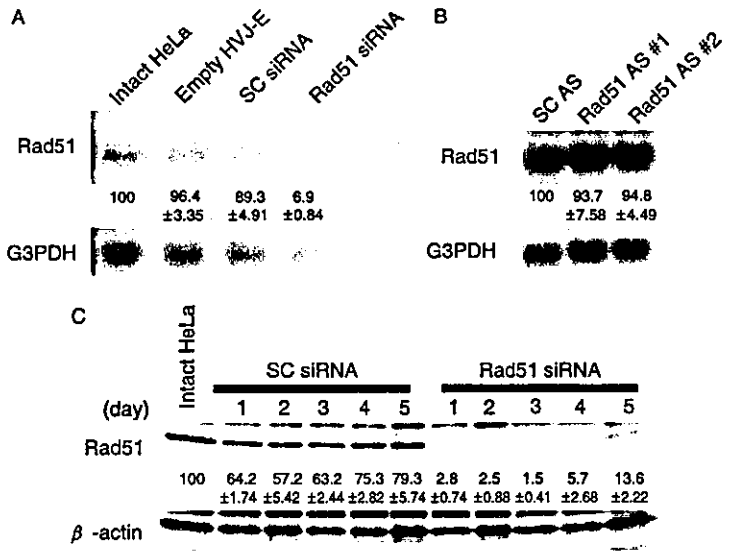


Figure 2. (A) Rad51 transcripts detected by Northern blot analysis 1 day after the delivery of Rad51 siRNA or scrambled (SC) siRNA. Rad51 mRNA in intact HeLa cells and HeLa cells treated with empty HVJ envelope vector were also measured. (B) Rad51 detection by Northern blot analysis 1 day after the delivery of two different antisense oligonucleotides (#1 and #2) against human Rad51 (Rad51 AS) or scrambled oligonucleotides (SC AS). (C) Rad51 protein detected by Western blot on days 1 to 5 after the delivery of either Rad51 siRNA or SC siRNA. These experiments were repeated twice and similar results were obtained. The ratio of Rad51 expression to G3PDH or β-actin expression was calculated by measuring the density of each band using the NIH imager. The percentage of Rad51 expression (mean ± standard deviation) is shown below each lane

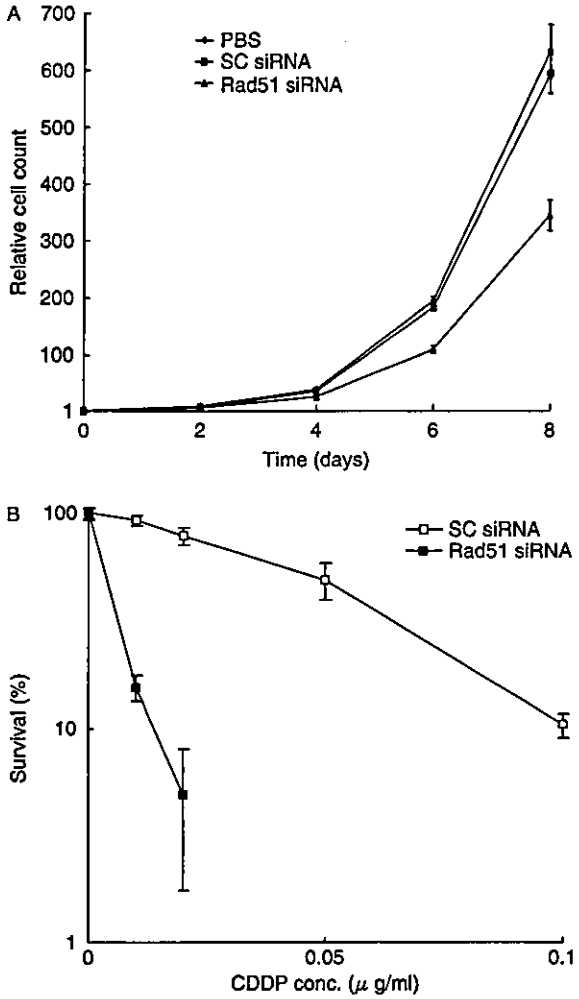


Figure 3. (A) The growth of HeLa cells detected by cell count on days 0 to 8 after the delivery of Rad51 siRNA, scrambled (SC) siRNA or PBS using the HVJ envelope vector. (B) The colony formation of HeLa cells after the delivery of either Rad51 siRNA or SC siRNA. The ordinate indicates the ratio of the number of colonies in the presence of various concentrations of CDDP to the number of colonies without CDDP after the delivery of siRNA. The mean value ± standard deviation from triplicate samples is shown at each point of both experiments. No colonies were observed at 0.05 and 0.1 μg/ml CDDP when Rad51 siRNA was delivered

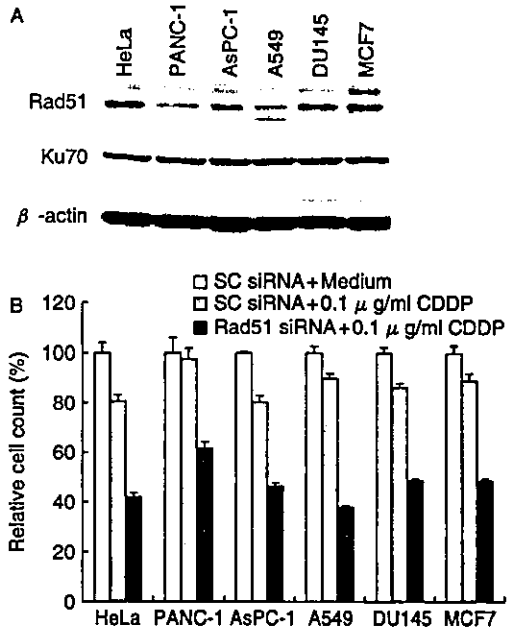


Figure 4. The increase in CDDP sensitivity in various cancer cell lines with Rad51 siRNA. (A) Rad51 and Ku70 protein levels in various cancer cell lines were detected by Western blotting. (B) siRNA was introduced into the human cancer cells using the HVJ envelope vector on day 1 after the inoculation of 10^5 cells in a 6-well plate. On day 3, cells were incubated with CDDP (0.1 μg/ml) for 3 h, and cell number was counted using a particle counter on day 5. Relative cell count indicates the ratio of cell number (the mean value of triplicate samples) treated with either scrambled (SC) or Rad51 siRNA + CDDP to that treated with SC siRNA + medium

siRNA, the number of colonies decreased to approximately 10% of that with scrambled siRNA.

We tested the effect of Rad51 siRNA on the sensitivity of CDDP in various human cancer cell lines including PANC-1 (pancreatic cancer), AsPC-1 (pancreatic cancer), A549 (lung cancer), DU145 (prostate cancer), MCF7 (mammary carcinoma), and HeLa S-3 (cervical cancer). First, the amounts of Rad51 and Ku70 in these human cancer cells were detected by Western blotting. The protein levels of Rad51 varied among cell lines while Ku70 protein levels were almost similar (Figure 4A). Then, on day 2 after the treatment with CDDP (0.1 μg/ml), the ratio of cell numbers of these cancer cell lines was examined in the presence of Rad51 siRNA or scrambled siRNA introduced using the HVJ envelope vector. Without Rad51 siRNA, more than 80% of the cells were still alive in all the cancer cell lines. Scrambled siRNA did not induce any toxicity in all the cell lines. However, with Rad51 siRNA, Rad51 protein level was reduced to less than 10% of that without siRNA in all the cell lines (data not shown), and all the cell lines were much more sensitive to CDDP. The sensitivity to CDDP increased more than 30% in all cases (Figure 4B). Thus, the enhancement of CDDP sensitivity by Rad51 siRNA appeared to be generally applicable to many cancer cells.

Next, we examined the sensitivity to CDDP in non-cancerous human cells after transfer of Rad51 siRNA. As shown in Figure 5A, the sensitivity to CDDP was not enhanced in NHDF when the concentration of CDDP increased. Then, we compared the apoptosis of NHDF to that of HeLa cells by the treatment with Rad51 siRNA in the presence or absence of 0.1 μg/ml CDDP (Figure 5B). The apoptotic cell ratio was not significantly different between HeLa cells (4.0 ± 1.1%) and NHDF (3.2 ± 0.5%) with Rad51 siRNA in the absence of CDDP. However, in the presence of CDDP, the apoptosis increased to 15.0% in HeLa cells, while it was 4.9% in NHDF.

We examined the ability of CDDP and Rad51 siRNA to suppress tumor growth in SCID mice. First, to test the gene delivery efficiency *in vivo*, we injected the HVJ

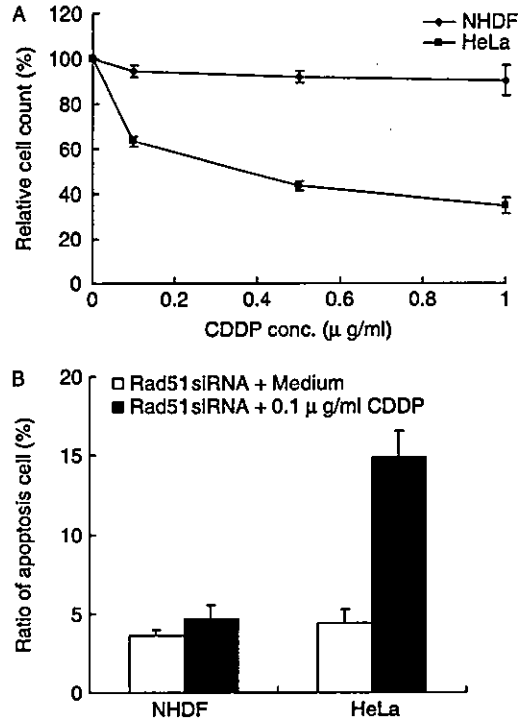


Figure 5. Rad51 siRNA did not enhance the sensitivity to CDDP in NHDF. (A) Forty-eight hours after transfer of Rad51 siRNA, the cells were treated with 0.1, 0.3 and 1.0 μg/ml CDDP for 3 h. Then, 48 h later, cell number was counted using a particle counter. Relative cell count indicates the ratio of cell number (the mean value of triplicate samples) treated with CDDP to that treated with medium alone. (B) To assess apoptosis, cells treated with Rad51 siRNA and CDDP were stained with fluorescein isothiocyanate (FITC)-labeled Annexin V and analyzed with FACScan. The ordinate indicates the ratio of labeled cells treated with Rad51 siRNA + medium or Rad51 + CDDP to that with scrambled siRNA + medium

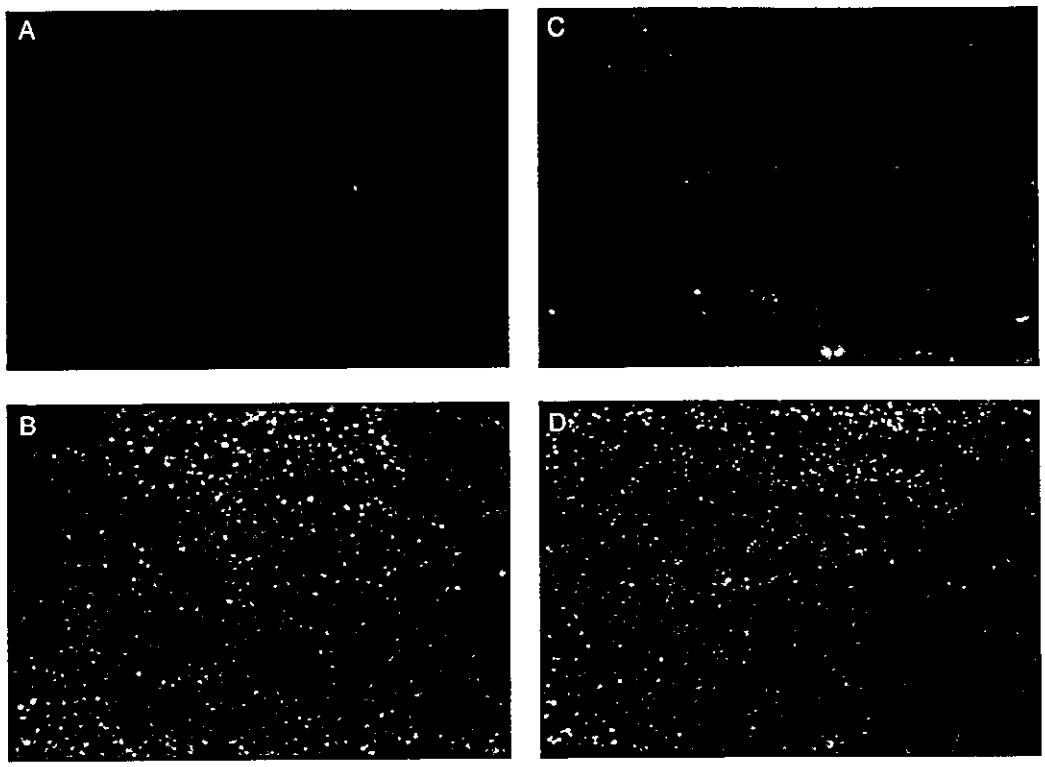
envelope vector containing fluorescein isothiocyanate (FITC)-labeled oligodeoxynucleotides (FITC-ODN) into HeLa cell-derived tumors. As shown in Figure 6, the number of FITC-labeled cells and cells stained with Hoechst in randomly selected fields of three independent experiments were counted. They were 1227/2256, 616/1360, and 769/1424 cells. Thus, the delivery efficiency of FITC-ODN to HeLa cell tumors *in vivo* was 51.5 ± 5.2% (mean ± standard deviation). Next, Rad51 siRNA was delivered to tumors using the HVJ envelope vector. Western blot analysis showed that the level of Rad51 transcript was reduced to approximately 25% of that in intact HeLa tumors (Figure 7). Intraperitoneal injection of 200 μg of CDDP on day 2 transiently suppressed tumor growth, but tumors began to grow again 8 days after the treatment. To enhance the anti-tumor effect of CDDP, Rad51 siRNA delivered by the HVJ envelope vector was injected into the tumors on days 0 and 2. However, the suppression of tumor growth was not significant when compared to CDDP treatment alone (data not shown). Finally, Rad51 siRNA was injected into tumor mass on days 0, 2, and 4, and CDDP was injected into the abdominal cavity on day 2. This combination treatment significantly reduced the growth of HeLa tumors when compared to other treatment groups (Figure 8). Thus, the combination of CDDP and Rad51 siRNA is an effective anti-cancer protocol.

Discussion

We enhanced the sensitivity of cancer cells to CDDP by completely suppressing Rad51 with siRNA. The combination of CDDP and siRNA caused the regression of human tumors in mice. These results support the theory that DNA damage induced by CDDP can be repaired by Rad51. Our results suggest that CDDP-induced DNA damage can be repaired by homologous recombination of DNA double-strand breaks. We succeeded in suppression of Ku70 proteins in HeLa cells using Ku70 siRNA, but the sensitivity to CDDP was not enhanced in HeLa cells (data not shown). An antisense Ku70 study supports our observation [18]. Although we have not applied siRNA technology to suppress another factors such as Ku80 and DNA protein kinase (DNA-PK) which are also involved in non-homologous DNA end joining, it has been reported that silencing of DNA-PK or Ku86 by siRNA enhances sensitivity to radiation and anti-cancer drugs such as methyl methanesulfonate and bleomycin, but not to DNA cross-linking agents such as cisplatin and chlorambucyl [32–34]. Moreover, cisplatin killing is mediated by kinase activity of the Ku70, Ku80 and DNA-PK complex [35]. However, another report indicates that novel inhibitors of DNA-PK, vanillins, sensitize cells to cisplatin [36]. Thus, the involvement of DNA-PK in cisplatin sensitivity is still controversial. A comparative study of Rad51 siRNA and DNA-PK siRNA in cisplatin sensitivity should be conducted.

siRNA very effectively suppressed Rad51 expression. A previous study found that antisense oligodeoxynucleotides against mouse Rad51 enhanced the radiosensitivity of malignant glioma [37]. Although the target sequence of the antisense oligonucleotides is the same in humans and mice, the antisense oligonucleotides to human Rad51 did not suppress human Rad51 mRNA (Figure 2). As shown in Figure 2, Rad51 protein completely disappeared for 4 days after the siRNA transfer. We have never observed such complete loss of target protein using either antisense oligonucleotides or ribozymes. However, only one of five siRNA constructs effectively suppressed Rad51 expression. The system for predicting effective siRNA sequences should be improved.

When siRNA was delivered using the HVJ envelope vector, the efficiency was almost 100% in cultured cells, and Rad51 expression was completely prevented for 4 days after the delivery. siRNA very effectively suppresses gene expression, especially when an efficient delivery system is used. However, even when the HVJ envelope vector was used, the efficiency of a single siRNA injection into a tumor was only 50%. One limitation of synthetic siRNA is that its effect is transient, probably because



Color Figure - Print and Online

Figure 6. Detection of FITC-labeled ODN in tumors derived from HeLa cells in SCID mice. HVJ envelope vector containing unlabeled ODN (A, B) or FITC-ODN (C, D) was injected into tumors. FITC was detected in A and C. Hoechst 33 258 was used to counterstain the nucleus (B and D). The experiments were repeated three times and representative photos are shown

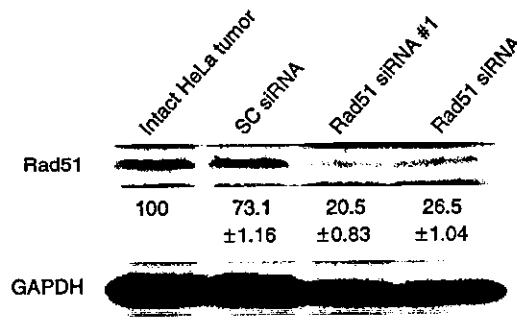


Figure 7. Rad51 transcript was detected by Western blot analysis after the delivery of either Rad51 siRNA or scrambled (SC) siRNA. The samples were isolated from two mice (#1 and #2) injected with the same Rad51 siRNA. This experiment was repeated twice and similar results were obtained. The percentage of Rad51 expression (mean ± standard deviation) below in each lane was calculated as described in Figure 2

the siRNA is gradually diluted after cell division. The use of lentivirus vector or retrovirus vector to insert siRNA expression DNA into the host chromosome has been proposed [38,39]. However, we believe that a combined treatment of synthetic siRNA and CDDP is sufficient for cancer treatment, because the cells that received Rad51 siRNA and CDDP in this study died in a few days. An important factor in the success of the combination treatment is the consecutive delivery of synthetic siRNA. Indeed, three injections of Rad51 siRNA into the tumor were more effective for tumor regression than two injections. The immunogenicity of the HVJ envelope vector is much less than that of native HVJ because of the inactivation of the viral genome. Consecutive injection is feasible with this vector system [28].

Rad51 siRNA enhanced the sensitivity to another anti-cancer drug, bleomycin, which can induce DNA double-strand breaks. The enhancement of bleomycin sensitivity by Rad51 siRNA was almost similar to that in a CDDP experiment (M. Ito and Y. Kaneda, unpublished data). It has been reported that Rad51 is also involved in the sensitivity of cancers to other anti-cancer drugs, such as etoposide (VP16) and imatinib mesylate (Gleevec) [40,41]. Since only Rad51 siRNA decreased cancer cell viability (Figure 4A), Rad51 siRNA can also enhance the sensitivity of cancer cells to other drugs which do not induce DNA double-strand breaks. This experiment is being performed in our laboratory. Furthermore, although Rad51 expression levels varied from cell line to cell line, all the cancer cells became very sensitive to CDDP in combination with Rad51 siRNA. The sensitivity of the cancer cell lines to CDDP did not appear to be related to the endogenous Rad51 protein level. These results suggest that the combination of CDDP with Rad51 siRNA will be generally applicable to various human cancers.

The enhancement of CDDP sensitivity by Rad51 siRNA was observed only in HeLa cells, not in NHDF. Similarly, apoptosis by Rad51 siRNA and CDDP increased in

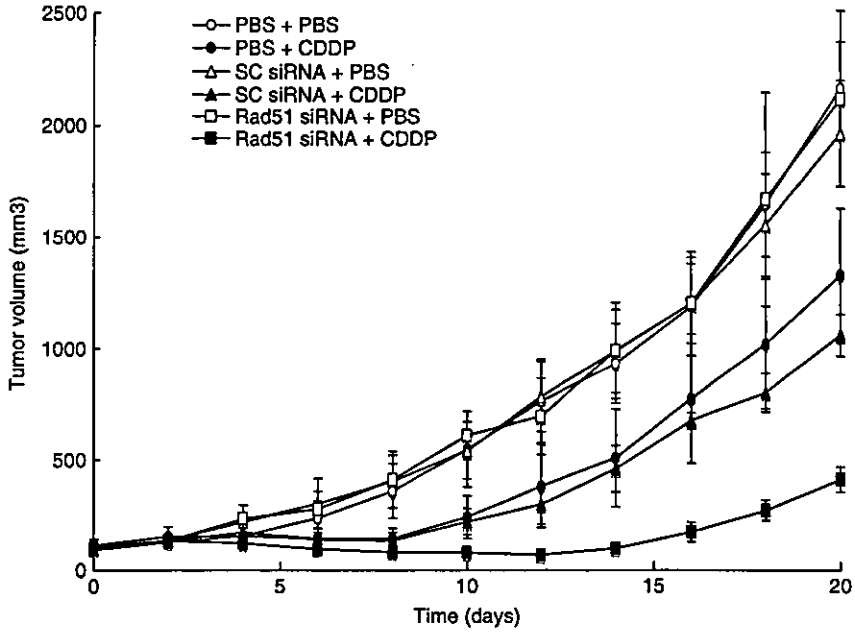


Figure 8. Tumor volume in SCID mice. Intraperitoneal injection of CDDP on day 2 transiently suppressed tumor growth *in vivo*, but tumors began to grow again 8 days after the treatment. To enhance the anti-tumor effect of CDDP, Rad51 siRNA or scrambled (SC) siRNA was injected on days 0, 2, and 4. In three groups, 200 μg of CDDP were injected into the abdominal cavity on day 2. In a negative control group, PBS was injected into both the tumor mass and peritoneal cavity. Each group contained five mice, and the representative result from three independent experiments is shown

HeLa cells, but not in NHDF. The discrepancy of CDDP sensitivity by Rad51 siRNA between NHDF and HeLa cells may be due to the difference of the CDDP uptake by the two cell lines. Indeed, the equitoxic dose of CDDP in NHDF and HeLa cells was 1.2 and 0.5 μg/ml, respectively, in our case (M. Ito and Y. Kaneda, unpublished data). Another possibility is that cell cycle difference between both cells may affect the sensitivity to CDDP in the presence of Rad51 siRNA. The precise mechanism of this different sensitivity to CDDP remains to be solved.

However, in human gene therapy, we should be very careful regarding the toxicity of Rad51 siRNA. As shown in Figure 5B, Rad51 siRNA alone induced apoptosis in both HeLa cells and NHDF, although the apoptotic cell ratio was much lower in the absence of CDDP. This may be consistent with the fact that Rad51 knockout mice are embryonic lethal [42]. To minimize the adverse effects to normal tissues, tumor-selective targeting is indispensable for cancer treatment. There are two ways to achieve selective targeting. One is the insertion of tumor-specific molecules to vectors, and another is the modification of vector size and charge. We have already reported that HVJ-cationic liposomes targeted tumor nodules in mouse peritoneum by intraperitoneal injection [43]. We are now constructing targeting vectors by modifying the HVJ envelope vector with polymers or tumor-specific single-chain antibodies.

When delivered by tumor-targeting vectors, siRNAs against genes resistant to cancer therapy hold great promise to become very effective anti-neoplastic therapeutics in combination with chemotherapy or radiotherapy.

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References

1. Baird RD, Kaye SB. Drug resistance reversal – are we getting closer? *Eur J Cancer* 2003; 39: 2450–2461.
2. Links M, Brown R. Clinical relevance of the molecular mechanisms of resistance to anti-cancer drugs. *Expert Rev Mol Med* 1999; Oct 25: 1–21.
3. Fojo T, Bates S. Strategies for reversing drug resistance. *Oncogene* 1988; 22: 7512–7523.
4. Holleran WM, DeGregorio MW. Evolution of high-dose cisplatin. *Invest New Drugs* 1988; 6: 135–142.
5. Weiss RB, Christian MC. New cisplatin analogues in development. *Drugs* 1993; 46: 360–377.
6. Silvani A, Eoli M, Salmaggi A, *et al.* Phase II trial of cisplatin plus temozolomide in recurrent and progressive malignant glioma patients. *J Neurooncol* 2003; 66: 203–208.
7. Roberts JJ, Kotsaki-Kovatsi VP. Potentiation of sulphur mustard or cisplatin-induced toxicity by caffeine in Chinese hamster cells correlates with formation of DNA double-strand breaks during replication on a damaged template. *Mutat Res* 1986; 165: 207–220.
8. Kanno S, Hyodo M, Suzuki K, Ohkido M. Effect of DNA-damaging agents on DNA replication and cell-cycle progression of cultured mouse mammary carcinoma cells. *Jpn J Cancer Res* 1985; 76: 289–296.
9. Zdraveski ZZ, Mello JA, Marinus MG, Essigmann JM. Multiple pathways of recombination define cellular responses to cisplatin. *Chem Biol* 2000; 7: 39–50.
10. Xu Z, Chen ZP, Malapesta A, *et al.* DNA repair protein level vis-à-vis anticancer drug resistance in the human tumor cell

lines of the National Cancer Institute drug screening program. *Anticancer Drugs* 2002; **13**: 511–519.
11. D'Andrea AD, Grompe M. The Fanconi anemia/BRCA pathway. *Nat Rev Cancer* 2003; **3**: 23–34.
12. Sherr CJ. Principles of tumor suppression. *Cell* 2004; **116**: 235–246.
13. Henning W, Sturzbecher H-W. Homologous recombination and cell cycle checkpoints: Rad51 in tumor progression and therapy resistance. *Toxicology* 2003; **193**: 91–109.
14. Huang J, Dynan WS. Reconstitution of the mammalian DNA double-strand break end-joining reveals a requirement for an Mre11/Rad50/NBS1-containing fraction. *Nucleic Acids Res* 2002; **30**: 667–674.
15. Freit P, Canitrot Y, Muller C, *et al.* Cross-resistance to ionizing radiation in a murine leukemic cell line resistant cis-dichlorodiammineplatinum(II): role of Ku autoantigen. *Mol Pharmacol* 1999; **56**: 141–146.
16. Myint WK, Ng C, Raaphorst GP. Examining the non-homologous repair process following cisplatin and radiation treatments. *Int J Radiat Biol* 2002; **78**: 417–424.
17. Britten RA, Kuny S, Perdue S. Modification of non-conservative double-strand break (DSB) rejoining activity after the induction of cisplatin resistance in human tumour cells. *Br J Cancer* 1999; **79**: 843–849.
18. Omori S, Takiguchi Y, Suda A, *et al.* Suppression of a DNA double-strand break repair gene, Ku70, increases radio- and chemosensitivity in a human lung carcinoma cell line. *DNA Repair* 2002; **29**: 299–310.
19. Husain A, He G, Venkatraman ES, Spriggs DR. BRCA1 up-regulation is associated with repair-mediated resistance to cis-diamminedichloroplatinum(II). *Cancer Res* 1998; **58**: 1120–1123.
20. Aloyz R, Xu ZY, Bello V, *et al.* Regulation of cisplatin resistance and homologous recombinational repair by the TFIIH subunit XPD. *Cancer Res* 2002; **62**: 5457–5462.
21. Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. *J Biol Chem* 2000; **275**: 23 899–23 903.
22. Raderschall E, Stout K, Freier S, Suckow V, Schweiger S, Haaf T. Elevated levels of Rad51 recombination protein in tumor cells. *Cancer Res* 2002; **62**: 219–225.
23. Tijsterman M, Plasterk RH. Dicers at RISC; the mechanism of RNAi. *Cell* 2004; **117**: 1–3.
24. Dorsett Y, Tuschl T. siRNAs: applications in functional genomics and potential as therapeutics. *Nat Rev Drug Discov* 2004; **3**: 318–329.
25. Miyagishi M, Hayashi M, Taira K. Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev* 2003; **13**: 1–7.
26. Yokota T, Miyagishi M, Hino T, *et al.* siRNA-based inhibition specific for mutant SOD1 with single nucleotide alternation in familial ALS, compared with ribozyme and DNA enzyme. *Biochem Biophys Res Commun* 2004; **314**: 283–291.
27. Sioud M. Ribozyme- and siRNA-mediated mRNA degradation: a general introduction. *Methods Mol Biol* 2004; **252**: 1–8.
28. Kaneda Y, Nakajima T, Nishikawa T, *et al.* HVJ (hemagglutinating virus of Japan) envelope vector as a versatile gene delivery system. *Mol Ther* 2002; **6**: 219–226.
29. Oshima K, Shimamura M, Mizuno S, *et al.* Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats. *FASEB J* 2004; **18**: 212–214.
30. Itoh Y, Kawamata Y, Harada M, *et al.* Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* 2003; **422**: 173–176.
31. Ahn J-D, Morishita R, Kaneda Y, *et al.* Inhibitory effects of novel AP-1 decoy oligodeoxynucleotides on vascular smooth muscle cell proliferation in vitro and neointimal formation in vivo. *Circ Res* 2002; **90**: 1325–1332.
32. Collis SJ, Swartz MJ, Nelson WG, DeWeese TL. Enhanced radiation and chemotherapy-mediated cell killing of human cancer cells by small inhibitory RNA silencing of DNA repair factors. *Cancer Res* 2003; **63**: 1550–1554.
33. Belenkov AL, Paiement J-P, Panasci LC, Monia BP, Chow TYK. An antisense oligonucleotide targeted to human Ku86 messenger RNA sensitizes M059 malignant glioma cells to ionizing radiation, bleomycin, and etoposide but not DNA cross-linking agents. *Cancer Res* 2002; **62**: 5888–5896.
34. Peng Y, Zhang Q, Nagasawa H, Okayasu R, Liber HL, Bedford JS. Silencing expression of the catalytic subunit of DNA-dependent protein kinase by small interfering RNA sensitizes human cells for radiation-induced chromosome damage, cell killing, and mutation. *Cancer Res* 2002; **62**: 6400–6404.
35. Jensen R, Glazer P. Cell-interdependent cisplatin killing by Ku/DNA-dependent protein kinase signaling transduced through gap junctions. *Proc Natl Acad Sci U S A* 2004; **101**: 6134–6139.
36. Durant S, Karran P. Vanillins – a novel family of DNA-PK inhibitors. *Nucleic Acids Res* 2003; **31**: 5501–5512.
37. Ohnishi T, Taki T, Hiraga S, Arita N, Morita T. In vitro and in vivo potentiation of radiosensitivity of malignant gliomas by antisense inhibition of the Rad51 gene. *Biochem Biophys Res Commun* 1998; **245**: 319–324.
38. Liu CM, Liu DP, Dong WJ, Liang CC. Retrovirus vector-mediated stable gene silencing in human cell. *Biochem Biophys Res Commun* 2004; **313**: 716–720.
39. Abbas-Terki T, Blanco-Bose W, Deglon N, Pralong W, Aebischer P. Lentiviral-mediated RNA interference. *Hum Gene Ther* 2002; **13**: 2197–2201.
40. Russell JS, Brady K, Burgan WE, *et al.* Gleevec-mediated inhibition of Rad51 expression and enhancement of tumor cell radiosensitivity. *Cancer Res* 2003; **63**: 7377–7383.
41. Hansen LT, Lundin C, Spang-Thomsen M, Peterson LN, Helleday T. The role of Rad51 in etoposide (VP16) resistance in small cell lung cancer. *Int J Cancer* 2003; **105**: 472–479.
42. Lim DS, Hasty P. A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53. *Mol Cell Biol* 1996; **16**: 7133–7143.
43. Miyata T, Yamamoto S, Sakamoto K, Morishita R, Kaneda Y. Novel immunotherapy for peritoneal dissemination of murine colon cancer with macrophage inflammatory protein-1β mediated by a tumor-specific vector, HVJ-cationic liposomes. *Cancer Gene Ther* 2001; **8**: 852–860.