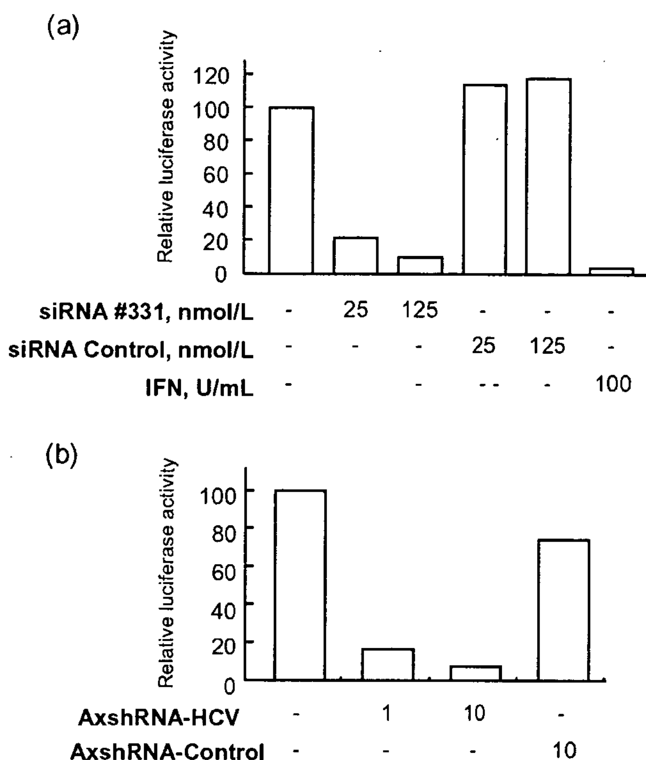

Figure 6 Effects of an siRNA and adenovirus expressing shRNA on HCV-JFH1 cell culture. (a) The siRNA #331, the siRNA-Control[14], (b) AxshRNA-HCV or AxshRNA-Control were, respectively, transfected or infected onto HV-JFH1-infected Huh7.5.1 cells. Seventy-two hours of the transfection or infection, expression level of HCV-RNA was quantified by real-time RT-PCR. The assays were repeated twice, and consistent results were obtained. IFN, recombinant interferon-alpha 2b.

Suppression of HCV-IRES-mediated translation *in vivo* by adenovirus expressing shRNA

The effects of the shRNA expression on the expression of the viral structural proteins *in vivo* were investigated using conditional HCV cDNA-transgenic mice, CN2-29.[28] Adenoviruses, AxshRNA-HCV, AxshRNA-Control or AxCAw1 were injected into CN2-29 mice in combination with AxCANCre, an adenovirus expressing *Cre* DNA recombinase. The mice were killed on the fourth day after the injection, and the hepatic expression of the HCV core protein was measured. The expressed amounts of the core protein were 143.0 ± 56.2 pg/mg and 108.5 ± 42.4 pg/mg in AxCAw1 and AxshRNA-Control-infected mice, respectively, and the expressed amount was significantly lower in mice injected with AxshRNA-HCV (28.7 ± 7.0 pg/mg, $P < 0.05$, Fig. 7a). Similarly, the induced expression of HCV core protein was not detectable by immunohistochemistry in AxshRNA-HCV infected liver tissue (Fig. 7c). Staining of a host cellular protein, albumin, was not obviously different between the liver infected with AxCAw1, AxshRNA-HCV and AxshRNA-Control (Fig. 7d). The expression levels of two ISG, IFN-beta and Mx1, in the liver tissue were not significantly different between individuals with and without injection of the adenovirus vectors (Fig. 7b). These results indicate specific shRNA silencing of HCV structural protein expression in the liver.

Discussion

The requirements to achieve a high efficiency using RNAi are: (i) selection of target sequences that are the most susceptible to RNAi; (ii) persistence of siRNA activity; and (iii) efficient *in vivo* delivery of siRNA to cells. We have used an shRNA sequence that was derived from a highly efficient siRNA (siRNA331), and constructed a DNA-based shRNA expression cassette that showed competitive effects with the synthetic siRNA (Fig. 2).[14] The shRNA-expression cassette does not only allow extended half-life of the RNAi, but also enables use of gene-delivery vectors, such as virus vectors. As shown in the results, a retrovirus vector expressing shRNA-HCV could stably transduce cells to express HCV-directed shRNA, and the cells acquired protection against HCV subgenomic replication (Fig. 3). An adenovirus vector expressing shRNA-HCV resulted in suppression of HCV subgenomic and protein expression by around three logs to almost background levels (Fig. 4). Consistent results were obtained by using an HCV cell culture (Fig. 6). More importantly, we have demonstrated *in-vivo* effects on viral protein expression in the liver using a conditional transgenic mouse model (Fig. 7). These results suggest that efficient delivery of siRNA could be effective against HCV infection *in vivo*.

An obstacle to applying siRNA technology to treat virus infections is that viruses are prone to mutate during their replication.[32] HCV continuously produces mutated viral strains to escape immune defense mechanisms. Even in a single patient, the circulating HCV population comprises a large number of closely related HCV sequence variants called quasispecies. Therefore, siRNA targeting the protein-coding sequence of the HCV genome, which have been reported by others,[15–19] may vary considerably among different HCV genotypes, and even among strains of the same genotype.[33] Our shRNA sequence targeted the 5′-UTR of HCV RNA, which is the most conserved region among various HCV isolates.[33] In addition, the structural constraints on the 5′-UTR, in terms of its requirement to direct internal ribosome entry and translation of viral proteins, might not permit the evolution of escape mutations. Our preliminary results have shown that the siRNA-HCV suppressed replication of an HCV genotype 2a replicon[34] to the same extent as the HCV 1b replicon.

Although the siRNA techniques rely on a high degree of specificity, several studies report siRNA-induced non-specific effect that may result from induction of ISG responses.[18,31] These effects may be mediated by activation of double-strand RNA-dependent protein kinase, toll-like receptor 3,[35] or possibly by a recently identified RNA helicase, RIG-I.[36] It remains to be determined whether these effects are generally induced by every siRNA construct. Sledz *et al.* have reported that transfection of two siRNA induced cellular interferon responses,[37] while Bridge *et al.* report that shRNA-expressing plasmids induced an interferon response but transfection of synthetic siRNA did not.[31] Speculatively, these effects on the interferon system might be construct dependent. Our shRNA-expression plasmids and adenoviruses did not activate ISG responses *in vitro* (Fig. 5a,b) or *in vivo* (Fig. 7b). We have preliminarily detected phosphorylated PKR (P-PKR) by western

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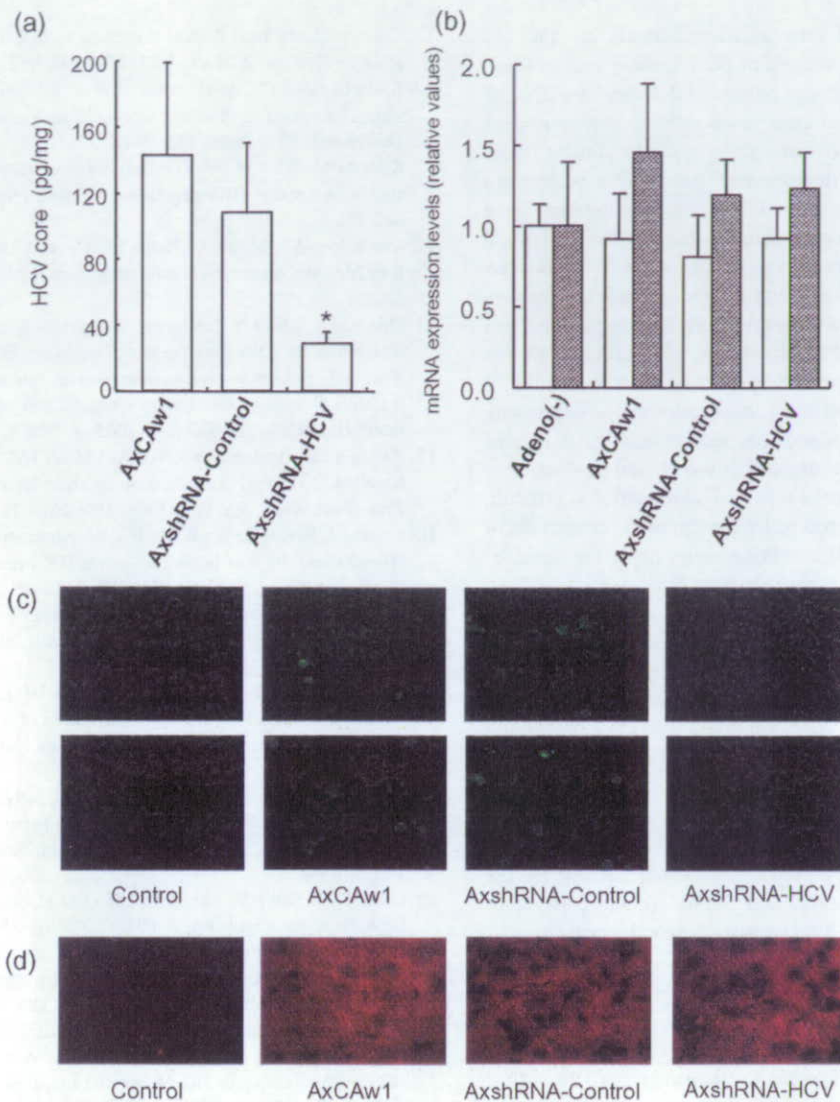

Figure 7 Effects of a recombinant adenovirus expressing shRNA on HCV core protein expression in CN2-29 transgenic mice. CN2-29 transgenic mice were administered with 1×10^9 PFU of AxCANCre combined with 6.7×10^8 PFU of AxshRNA-HCV, AxshRNA or AxCAw1. The mice were killed on day 4 after injection. (a) Quantification of HCV core protein in liver. Liver tissues were homogenized and used to determine the amount of HCV core protein. Each assay was done in triplicate, and the values are displayed as mean ± SD. Asterisk indicates *P*-value of less than 0.05. (b) Expression levels of mouse interferon-beta (white bars) and Mx1 (shaded bars) mRNA in the mouse liver tissue were quantified by the real-time RT-PCR analyses. Primers used were as follows: mouse interferon-beta sense, 5′-ACA GCC CTC TCC ATC AAC TA-3′; mouse interferon-beta antisense, 5′-CCC TCC AGT AAT AGC TCT TC-3′; mouse Mx1 sense, 5′-AGG AGT GGA GAG GCA AAG TC-3′; mouse Mx1 antisense, 5′-CAC ATT GCT GGG GAC TAC CA-3′; mouse beta-actin sense, 5′-ACT CCT ATG TGG GTG ACG AG-3′; mouse beta-actin antisense, 5′-ATA GCC CTC GTA GAT GGG CA-3′. Adeno (-) denotes mice without adenovirus administration. (c) Immunofluorescence microscopy of HCV core protein in the liver tissue. Liver sections of mice were stained using rabbit anticore polyclonal antibody and normal rabbit IgG as a negative control. The upper photographs were obtained at 400× magnification, and the lower photographs were at 1000×. (d) Immunofluorescence microscopy of albumin in liver. Liver sections from the mice were fixed and stained using rabbit antialbumin antibody and normal rabbit IgG as a negative control.

blotting, and found no apparent increase of P-PKR (data not shown). These results indicate that these target sequences and structures are of sufficient specificity to silence the target gene without eliciting non-specific interferon responses.

Beside the canonical action of siRNA, a sequence-specific cleavage of target mRNA, the siRNA could act as a micro-RNA that suppresses translational initiation of mRNA,[38] or it could mediate transcriptional gene silencing.[39] Regarding our *in-vivo* experiments, it was difficult to differentially analyze the effect of siRNA at individual sites of action because post-translational effect of siRNA concomitantly destabilizes target mRNA, which leads to apparent decrease of mRNA transcripts.

Efficiency and safety of gene transfer methods are the key determinants of the clinical success of gene therapy and an unresolved problem. There are several reports of delivery of siRNA or siRNA-expression vectors to cells *in vivo*;[12,40,41] however, gene delivery methods that are safe enough to apply to clinical therapeutics are currently under development. Adenovirus vectors are one of the most commonly used carriers for human gene therapies.[42–44] Our present results demonstrate that the adenoviral delivery of shRNA is effective in blocking HCV replication *in vitro* and virus protein expression *in vivo*. Adenovirus vectors have several advantages of efficient delivery of transgene both *in vitro* and *in vivo* and natural hepatotropism when administered *in vivo*. The AxshRNA-HCV specifically blocked expression of HCV structural proteins in a conditional transgenic mouse expressing those proteins. The current adenovirus vectors may cause inflammatory reactions in the target organ,[45] however, and produce neutralizing antibodies which make repeated administration difficult. These problems may be overcome by the improved constructs of virus vectors with attenuated immunogenicity or by the development of non-viral carriers for gene delivery.[46]

In conclusion, our results demonstrate the effectiveness and feasibility of the siRNA expression system. The efficiency of adenovirus expressing shRNA that target HCV suggests that delivery and expression of siRNA in hepatocytes may eliminate the virus and that this RNA-targeting approach might provide a potentially effective future therapeutic option for HCV infection.

Acknowledgments

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References

1 Alter MJ. Epidemiology of hepatitis C. *Hepatology* 1997; **26**: 62S–65S.

2 Hadziyannis SJ, Sette H Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann. Intern. Med.* 2004; **140**: 346–55.

3 Fire A, Xu S, Montgomery M, Kostas S, Driver S, Mello C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 1998; **19**: 806–11.

4 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; **411**: 494–8.

5 Coburn GA, Cullen BR. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *J. Virol.* 2002; **76**: 9225–31.

6 Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. *Nature* 2002; **418**: 435–8.

7 Gitlin L, Karelsky S, Andino R. Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* 2002; **418**: 430–4.

8 Ge Q, Filip L, Bai A, Nguyen T, Eisen HN, Chen J. Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc. Natl. Acad. Sci. USA* 2004; **101**: 8676–81.

9 Wang C, Pflugheber J, Sumpter R Jr *et al.* Alpha interferon induces distinct translational control programs to suppress hepatitis C virus RNA replication. *J. Virol.* 2003; **77**: 3898–912.

10 Klein C, Bock CT, Wedemeyer H *et al.* Inhibition of hepatitis B virus replication in vivo by nucleoside analogues and siRNA. *Gastroenterology* 2003; **125**: 9–18.

11 Konishi M, Wu CH, Wu GY. Inhibition of HBV replication by siRNA in a stable HBV-producing cell line. *Hepatology* 2003; **38**: 842–50.

12 McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA. RNA interference in adult mice. *Nature* 2002; **418**: 38–9.

13 Shlomai A, Shaul Y. Inhibition of hepatitis B virus expression and replication by RNA interference. *Hepatology* 2003; **37**: 764–70.

14 Yokota T, Sakamoto N, Enomoto N *et al.* Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep.* 2003; **4**: 602–8.

15 Kapadia SB, Brideau-Andersen A, Chisari FV. Interference of hepatitis C virus RNA replication by short interfering RNAs. *Proc. Natl. Acad. Sci. USA* 2003; **100**: 2014–18.

16 Kronke J, Kittler R, Buchholz F *et al.* Alternative approaches for efficient inhibition of hepatitis C virus RNA replication by small interfering RNAs. *J. Virol.* 2004; **78**: 3436–46.

17 Randall G, Grakoui A, Rice CM. Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. *Proc. Natl. Acad. Sci. USA* 2003; **100**: 235–40.

18 Seo MY, Abrignani S, Houghton M, Han JH. Letter to the editor: small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7. *J. Virol.* 2003; **77**: 810–12.

19 Wilson JA, Jayasena S, Khvorova A *et al.* RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. *Proc. Natl. Acad. Sci. USA* 2003; **100**: 2783–8.

20 Guo JT, Bichko VV, Seeger C. Effect of alpha interferon on the hepatitis C virus replicon. *J. Virol.* 2001; **75**: 8516–23.

21 Tanabe Y, Sakamoto N, Enomoto N *et al.* Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. *J. Infect. Dis.* 2004; **189**: 1129–39.

22 Maekawa S, Enomoto N, Sakamoto N *et al.* Introduction of NS5A mutations enables subgenomic HCV-replicon derived from chimpanzee-infectious HC-J4 isolate to replicate efficiently in Huh-7 cells. *J. Viral. Hepat.* 2004; **11**: 394–403.

23 Miyagishi M, Sumimoto H, Miyoshi H, Kawakami Y, Taira K. Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. *J. Gene Med.* 2004; **6**: 715–23.

24 Li Y, Yokota T, Matsumura R, Taira K, Mizusawa H. Sequence-dependent and independent inhibition specific for mutant ataxin-3 by small interfering RNA. *Ann. Neurol.* 2004; **56**: 124–9.

25 Kanazawa N, Kurosaki M, Sakamoto N *et al.* Regulation of hepatitis C virus replication by interferon regulatory factor-1. *J. Virol.* 2004; **78**: 9713–20.

26 Wakita T, Pietschmann T, Kato T *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 2005; **11**: 791–6.

27 Zhong J, Gastaminza P, Cheng G *et al.* Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* 2005; **102**: 9294–9.

28 Wakita T, Taya C, Katsume A *et al.* Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the Cre/loxP system. *J. Biol. Chem.* 1998; **273**: 9001–6.

29 Kashiwakuma T, Hasegawa A, Kajita T *et al.* Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). *J. Immunol. Methods* 1996; **28**: 79–89.

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30 Baglioni C, Nilsen TW. Mechanisms of antiviral action of interferon. *Interferon* 1983; **5**: 23–42.

31 Bridge A, Pebernard S, Ducraux A, Nicoulaz A, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.* 2003; **34**: 263–4.

32 Carmichael GG. Silencing viruses with RNA. *Nature* 2002; **418**: 379–80.

33 Okamoto H, Okada S, Sugiyama Y *et al.* Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. *J. Gen. Virol.* 1991; **72**: 2697–704.

34 Kato T, Date T, Miyamoto M *et al.* Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* 2003; **125**: 1808–17.

35 Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kB by Toll-like receptor 3. *Nature* 2001; **413**: 732–8.

36 Yoneyama M, Kikuchi M, Natsukawa T *et al.* The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 2004; **5**: 730–7.

37 Sledz C, Holko M, de Veer M, Silverman R, Williams, B. Activation of the interferon system by short-interfering RNAs. *Nat. Cell. Biol.* 2003; **5**: 834–9.

38 Doench JG, Petersen CP, Sharp PA. siRNAs can function as miRNAs. *Genes Dev.* 2003; **17**: 438–42.

39 Morris KV. siRNA-mediated transcriptional gene silencing: the potential mechanism and a possible role in the histone code. *Cell. Mol. Life Sci.* 2005; **62**: 3057–66.

40 Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing in vitro and in vivo. *Nat. Biotechnol.* 2002; **20**: 1006–10.

41 Zender L, Hutker S, Liedtke C *et al.* Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc. Natl. Acad. Sci. USA* 2003; **100**: 7797–802.

42 Akli S, Caillaud C, Vigne E *et al.* Transfer of a foreign gene into the brain using adenovirus vectors. *Nat. Genet.* 1993; **3**: 224–8.

43 Bajocchi G, Feldman SH, Crystal RG, Mastrangeli A. Direct in vivo gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors. *Nat. Genet.* 1993; **3**: 229–34.

44 Davidson BL, Allen ED, Kozarsky KF, Wilson JM, Roessler BJ. A model system for in vivo gene transfer into the central nervous system using an adenoviral vector. *Nat. Genet.* 1993; **3**: 219–23.

45 Yang Y, Wilson JM. Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo. *J. Immunol.* 1995; **155**: 2564–70.

46 Fleury S, Driscoll R, Simeoni E *et al.* Helper-dependent adenovirus vectors devoid of all viral genes cause less myocardial inflammation compared with first-generation adenovirus vectors. *Basic Res. Cardiol.* 2004; **99**: 247–56.

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Efficient regulation of viral replication by siRNA in a non-human primate surrogate model for hepatitis C

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Abstract

RNA interference (RNAi) represents a new technology which could offer potential applications for the therapeutics of human diseases. RNAi-mediated therapy has recently been shown to be effective toward infectious diseases in *in vitro* and rodent models, however, it remains unclear whether RNAi therapy with systemic application could be effective in primates. In this study, we examined if RNAi therapy could be effective toward infectious diseases by using a non-human primate surrogate model for hepatitis C. Administration into marmosets of cationic liposome-encapsulated siRNA (CL-siRNA) for GB virus B (GBV-B), which is most closely related to hepatitis C virus, repressed GBV-B replication in a dose-dependent manner. Especially, 5 mg/kg of the CL-siRNA completely inhibited the viral replication. Since the serum interferons (IFNs) were induced by CL-siRNA *in vivo*, inhibition of viral regulation by anti-GBV-B CL-siRNA may include an antiviral effect of IFN. However, contribution of induced IFN may be partial, since the control CL-siRNA which induced a stronger IFN response than GBV-B CL-siRNA could only delay the viral replication. Our results suggest the feasibility of systemic administration of CL-siRNA as an antiviral strategy.
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Keywords: siRNA; Hepatitis C; Marmoset; Interferon; GB virus B

RNA interference is a powerful tool for silencing gene expression and has spurred considerable interest in its experimental and therapeutic potential. RNAi has been characterized as a cellular process of post-transcriptional gene silencing. An RNaseIII-like enzyme, called Dicer, cleaves double stranded RNA (dsRNA) in to 21–23 nucleotide RNA duplex, termed small interfering RNAs (siRNAs). siRNAs are unwound in the RNA-induced-silencing-complex (RISC), and single-stranded siRNAs then act as a guide to substrate selection, leading to the cleavage of a homologous target RNA molecule [1].

Hepatitis C virus (HCV) infection contributes significantly to human morbidity and mortality worldwide. It is estimated that 40–60% of infected individuals progress to chronic liver disease, and many of these patients develop liver cirrhosis and hepatocellular carcinoma [2]. Currently,

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the only treatment available for patients with chronic HCV infections is combinational therapy with interferon (IFN) and ribavirin. The standard therapy is only effective for approximately 50% of patients with chronic HCV hepatitis [3]. Therefore, there is a great need for less complicated and more generally efficient therapeutics for HCV infection.

We and others reported that the synthetic siRNA and the siRNA-expressing adenovirus targeting 5′-UTR of HCV genome efficiently and specifically inhibited the HCV replication *in vitro* [4–6]. Other than humans, only chimpanzees can be productively infected by HCV. Together with ethical issues it has becomes increasingly difficult to access chimpanzees for experimental studies. The new world monkeys, tamarins and marmosets, undergo hepatitis upon infection with the GBV-B, which is most closely related to HCV. The significant similarity between HCV and GBV-B at the genomic and biochemical levels led to the proposal of the GBV-B/monkey system as a good surrogate model for hepatitis C [7,8]. Taking advantage of this non-human primate surrogate model, we investigated the feasibility of siRNA-mediated therapy against infectious diseases caused by pathogenic viruses.

Materials and methods

Preparation of siRNA. The sequence of siRNA for GBV-B was cucguagaccguagcacau dTdT in the sense strand and augugcuacggucuacgagdTdT in the antisense strand which was designed to target the GBV-B RNA (Fig. 1). The sequence of control siRNA for

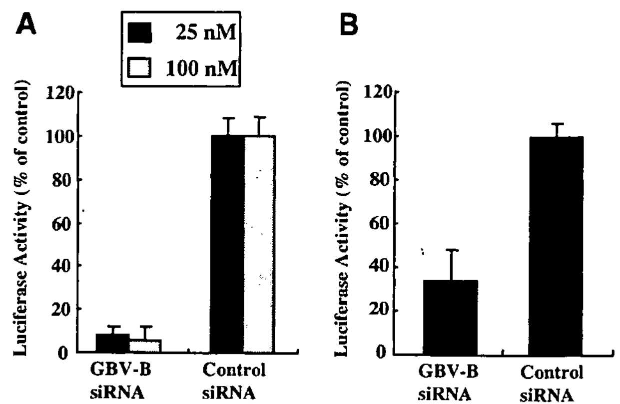

Fig. 2. Effects of the siRNA oligonucleotides on expression of GBV-B-reporter gene in culture cells. (A) and liver of mice (B).

experiments of Fig. 2A and B was uua ugc cga ucg cgu cac a dTdT in the sense strand and ugu gac gcg auc ggc aua a dTdT in the antisense strand which was designed to target beta-galactosidase RNA, and that for experiments of Figs. 3 and 4 was gct atg aaa cga tat ggg c dTdT in the sense strand and g ccc aua ucg uuu cau ugc dTdT in the antisense strand which was designed to target *firefly*-luciferase RNA. siRNA oligonucleotides were chemically synthesized and purified by reverse-phase high-performance liquid chromatography, while the unconjugated RNA oligonucleotides were purified by anion-exchange high-performance liquid chromatography. The sense and antisense strands were annealed at 95 °C for 1 min followed by slow cooling in RNAse free water. Positively charged liposomes containing cationic lipid analogue were synthesized at Nippon Shinyaku Co., as described previously [9]. To prepare CL-siRNA,

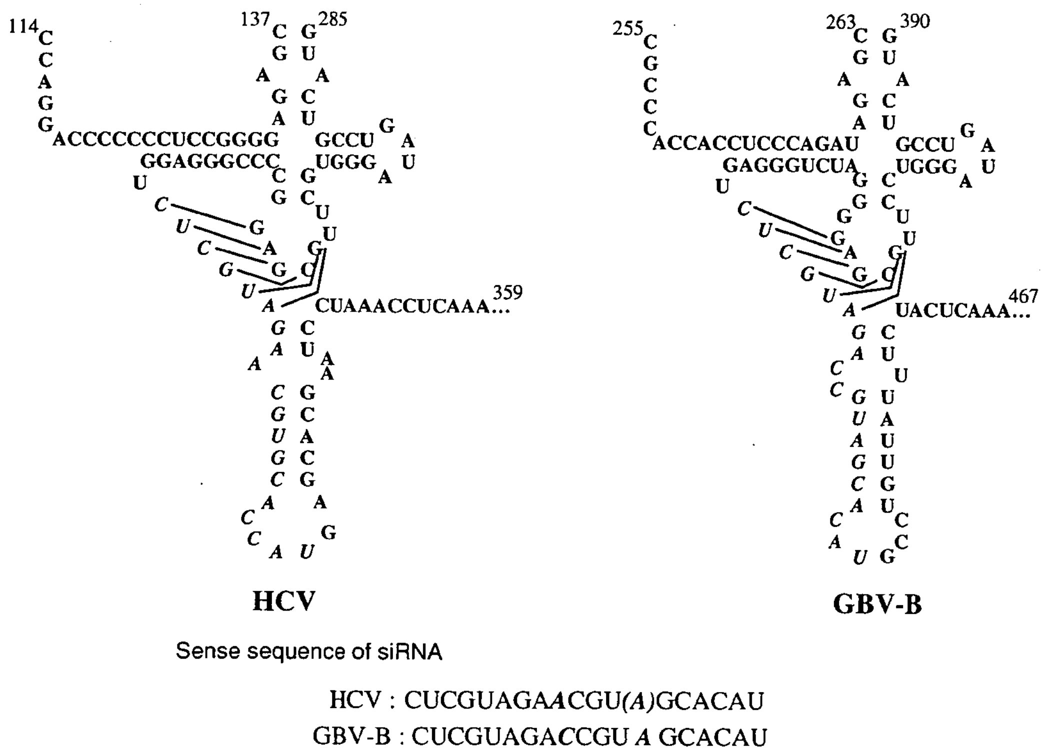

Fig. 1. Predicted secondary structure of the 5′-untranslated region around the target site (italic) in the HCV and GBV-B genome (nucleotide 114–137 and 285–359 of HCV, and 263–255 and 390–467 of GBV-B), and the sense sequences of siRNA.

annealed siRNA was added to the same volume of liposome solution with sonication. The ratio of oligonucleotide to LIC-101 was 1:16 (w/w).

Cells culture and transfection. The human embryonic kidney cell line, 293 T, was maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, Missouri) supplemented with 10% fetal calf serum at 37 °C under 5% CO_2. Transfections of the siRNA oligonucleotides and the plasmids were performed in 24-well plates using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. GBV-B-RNA-reporter gene vector, pGBV-B-Rluc, was used as a target, which expressed mRNA consisting of GBV-B 5′-untranslated region and upstream part of the core region (nucleotide 1–377) connected with upstream of *renilla* luciferase (RLuc) gene. Fifty nanograms of the pGBV-B-Rluc and 2 and 25 nM of siRNA were transiently transfected with 20 ng of *firefly* luciferase (FLuc)-expressing plasmid (pRL-RSV, Promega). The RLuc activity was adjusted by the FLuc activity, to normalize the transfection efficiency.

Luciferase assays. Luciferase activities were quantified with a luminometer (Lumat LB9501, Promega) using the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results expressed as means ± SD as percentages of controls.

Animals. Male BALB/c or ICR mice, 9 weeks of age, were obtained from CLEA Japan and subject to a 2-week quarantine and acclimation period before use. Male juvenile common marmosets (*Callithrix jacchus*) were housed in individual cages at the Tsukuba Primate Medical Center. All animal studies were conducted in accordance with the protocols of experimental procedures which were approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation and Tokyo Medical and Dental University.

In vivo efficacy experiments in mice. For the *in vivo* delivery of the siRNA to the liver of mice and monkeys, we used a novel cationic liposome that was synthesized by Nippon Shinyaku Co., Ltd. This cationic liposome was reported to be a good vehicle for the delivery of nucleic acid polymers and siRNAs to the liver when it was administered intravenously [9,10] or to the bladder by intravesical administration [11]. For the delivery of plasmid DNA to the liver of mice we used the hydrodynamic injection method in which a large volume of nucleotides solution was rapidly injected from tail vein [12]. *Three mice for each group were examined.* 5.0 mg/kg GBV-B or control CL-siRNA was administered as a regular intravenous injection from the tail vein in 0.2 ml 10% maltose over a period of 1–3 s. Three minutes later, the 50 μg of the pGBV-B-Rluc and 20 μg of pRL-RSV plasmids in a volume equivalent to 5% of the body weight were rapidly injected in 3–5 s into the mouse tail vein according to the hydrodynamic injection method. Phosphate buffer saline (PBS) was used as a carrier solution for injection. Successful injection was monitored when the conjunctiva of mouse became transiently anemic and confirmed by the luciferase activity in the liver.

In vivo efficacy experiments in monkeys. Negative control (n = 2; with or without control siRNA) and treatment group (n = 3; 1.0, 2.5 and 5.0 mg/kg of anti-GBV-B siRNA) were employed in this study. GBV-B-infectious serum obtained from a tamarin [8] was intrahepatically inoculated with the GBV-B RNA. The siRNA to GBV-B and control siRNA formulated by the cationic liposome, or just 10% maltose (sham) was administered by standard intravenous injection via the saphenous vein of the marmosets for three days. On the second day, the GBV-B infectious serum (1.3×10^9 viral RNA copies/inoculum) was directly injected to the liver of five marmosets. Blood samples were periodically collected from the femoral vein of the monkeys under anesthetization. GBV-B RNA in plasma from the monkeys was quantified by a real-time, 5′ exonuclease PCR (TaqMan) assay using a primer-probe combination that recognized a portion of the GBV-B capsid gene as previously described [8]. The Platelet cell counts were performed at FALCO Biosystems, Co., Ltd.

Measurement of IFNs in mice and monkeys. The siRNA/cationic liposome was injected from tail vein of ICR mice or saphenous vein of the marmosets. Bood samples were taken 3 h after the injection. Mouse IFN-α levels were quantified by using sandwich ELISA kits for mouse IFNs (PBL Biomedical Laboratories, Biosource). Marmoset IFN-α and -γ levels were by using sandwich ELISA kits for human and rhesus macaque IFN, respectively (U-CyTech bioscience) according to the manufacture's instructions. Assays were performed in duplicate and the results expressed as means ± SD as percentages of controls.

Results

We selected the siRNA-targeting site to the GBV-B genome from its 5′-UTR, the most conservative portion in both GBV-B and HCV genomes [13], to protect the siRNA from escape mutations of the virus [4]. The secondary structures of virus genome RNAs of HCV and GBV-B around the target site are very similar to each other, and the designed siRNA was different from the corresponding sequence of HCV by only two nucleotides (Fig. 1).

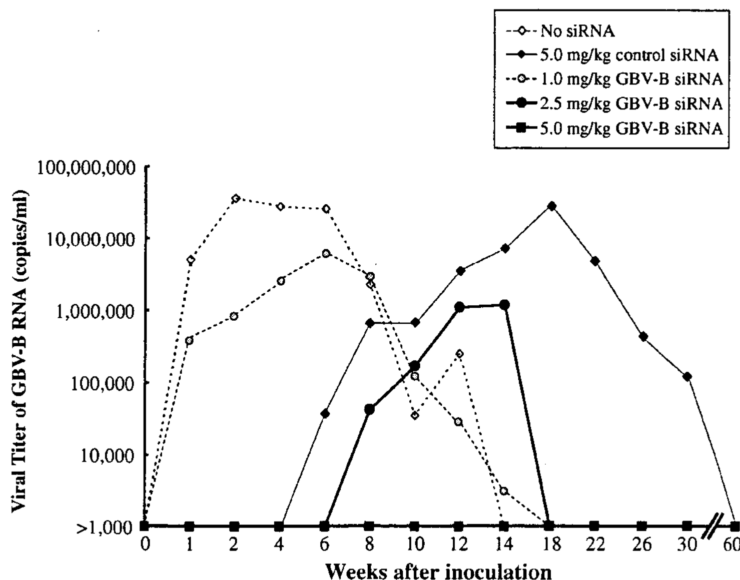

Fig. 3. Effect of the GBV-B siRNA/cationic liposome complex on replication of GBV-B in marmosets.

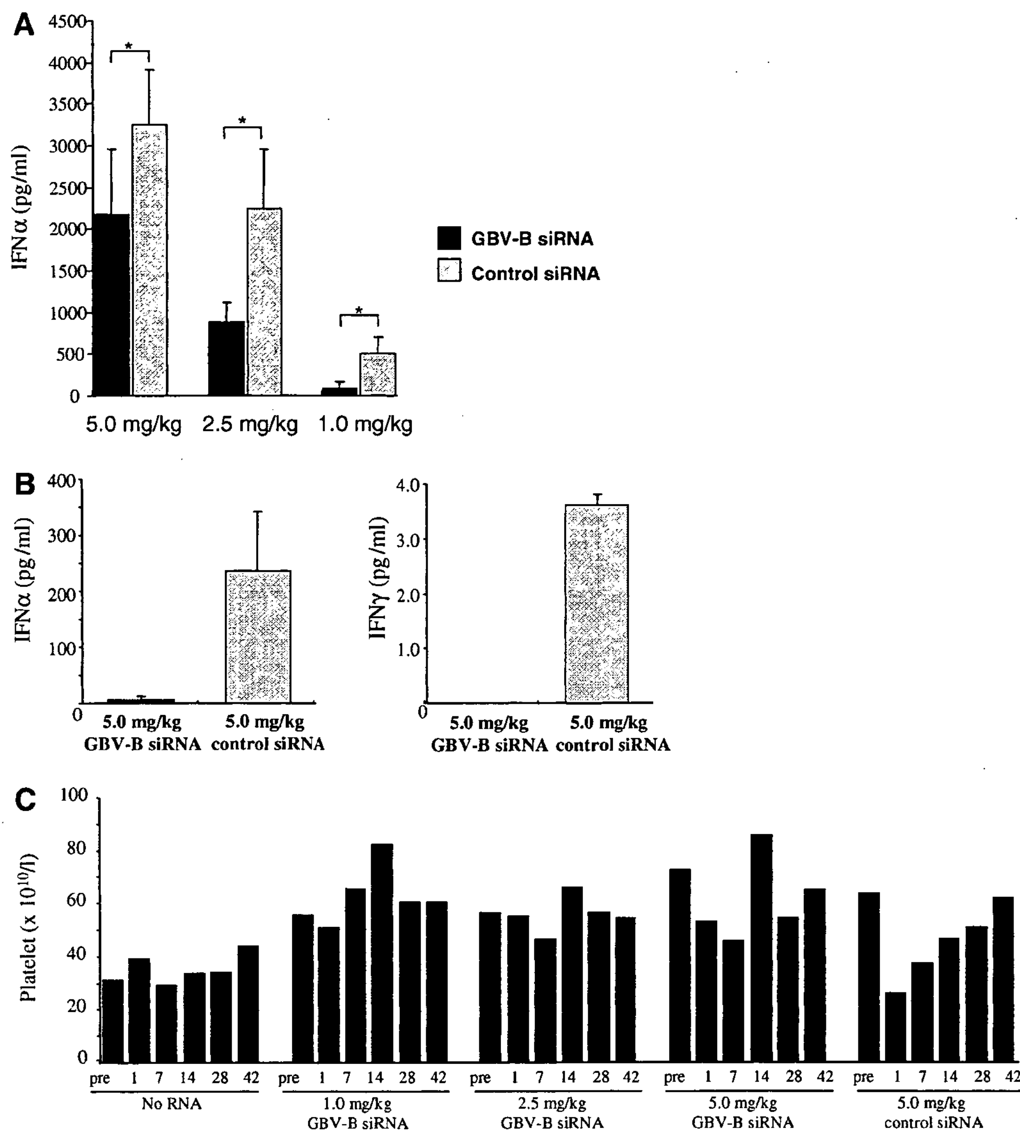

Fig. 4. Side effects of siRNA/cationic liposome complex. (A) Induction of IFN-α was evaluated by measuring mouse serum 3 h after intravenous injection of 1–5 mg/kg GVB-B or control siRNA/cationic liposome complex ($n = 3$). *<0.05 (Student t test). (B) Induction of IFN-α and γ was evaluated by measuring marmoset serum 3 h after intravenous injection of 5 mg/kg GVB-B or control siRNA/cationic liposome complex), respectively ($n = 3$). (C) Peripheral blood platelet was counted in the five marmosets examined in the same experiment shown in Fig. 3.

Effect of siRNA in vitro and in mice

First, we confirmed the efficient cleavage of GBV-B RNA by the siRNA in 293 T cells. The cells were harvested at 24 h of transfection with pGBV-B-*R*luc, pRL-RSV and siRNA oligonucleotides, and internal luciferase activities were measured and ratio of RLuc versus FLuc value was calculated. More than 90% the *R*Luc activities were inhibited by expressing co-transfected siRNA (Fig. 2A). This result clearly indicated that GBV-B siRNA efficiently inhibited the expression of GBV-B RNA in culture cells.

Next, we investigated the *in vivo* effect of siRNA formulated in the cationic liposome on silencing the viral gene expression in the liver of mice. BALB/c mice were injected intravenously from the tail vein with GVB-B CL-siRNA followed by hydrodynamically injection of pGBV-B-*R*luc and pRL-RSV. We found that intravenously administered GBV-B CL-siRNA efficiently suppressed the expression of GBV-B genome in the liver of mice (Fig. 2B).

Effect of siRNA on GBV-B replication in marmosets

The 1.0, 2.5 and 5.0 mg/kg/day of siRNA to GBV-B, 5.0 mg/kg/day of control siRNA formulated by the cationic liposome, or just 10% maltose (sham) were administered by standard intravenous injections via the saphenous vein of the marmosets for three consecutive days. On the second day, GBV-B infectious serum

(1.3×10^9 viral RNA copies/inoculum) was directly injected to the liver. Before and after the inoculation, GBV-B RNA in the serum was quantified by a real-time, 5' exonuclease PCR. In a sham-administered marmoset, the viral RNA was transiently increased in plasma after infection and the viral load reached to the peak level (3.6×10^7 copies/ml) (Fig. 3). It has been reported that GBV-B infection in marmosets as well as tamarins causes semi-acute viremia which generally ceases within 10–12 weeks post-infection [8,14,15]. This viral kinetics is consistent with the cases of HCV-infected human or chimpanzee, thus it appears to be in vivo characteristics of genus hepativirus where HCV and GBV-B belong to. Virological or immunological implication for the transient viremia is not fully addressed.

In contrast to sham-administered marmoset, we could find that the administration of CL-siRNA significantly delayed or suppressed the replication of GBV-B in a dose-dependent manner; the 5.0 mg/kg CL-siRNA completely suppressed the replication of GBV-B for more than 6 months after the infection (Fig. 3), even though the siRNA was administered only for the initial 3 days. Unexpectedly, the 5.0 mg/kg of control CL-siRNA was also able to delay the virus replication, while the peak level was comparable with that of the untreated monkey (Fig. 3).

Induction of interferons

We evaluated the induction of serum IFN-α by intravenous administration of the siRNA with cationic liposome in mice. IFN-α was induced by CL-siRNA but not by the cationic liposome nor siRNA oligonucleotide alone (data not shown). Induced IFN levels in the sera were dose-dependent and were significantly higher in mice with the *control CL-siRNA* than those with the *GBV-B CL-siRNA* (Fig. 4A).

An independent experiment using marmosets showed that single injection of 5.0 mg/kg control CL-siRNA substantially induced the serum interferon (IFN)-α and -γ, whereas the same dose of CL-siRNA induced a minimal level of IFN-α and no detectable level of IFN-γ (Fig. 4B).

In addition, a transient and mild decrease in peripheral blood platelets was more clearly observed in the marmoset treated with 5.0 mg/kg of control than 5.0 mg/kg of GBV-B CL-siRNA (Fig. 4C). There was no other remarkable abnormality related to siRNA administration in biochemical parameters indicating liver dysfunction which include alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and albumin.

Discussion

Many viruses produce some dsRNA as a byproduct of their replication [16], and RNAi serves as an important defense against viruses in plants [17]. Therefore, mammalian viruses have been expected to be a good therapeutic target of RNAi, and indeed, several animal viruses have been successfully inhibited to replicate *in vitro* [18]. Locally delivered siRNA have proven effective in abrogating infection from respiratory [19–22] and vaginal [23] viruses. Recently, systemically-delivered siRNA in mice has been successfully suppressed the expression of endogeous gene of the liver [24–26]. However, it remains to be ascertained if the RNAi-mediated gene therapy with systemically-delivered siRNA would be applicable to hepatitis virus in non-human primates. In this study, we examined if RNAi therapy could be effective toward infectious diseases by using a non-human primate surrogate model for hepatitis C. Administration into marmosets of CL-siRNA for GBV-B, which is most closely related to hepatitis C virus, repressed GBV-B replication in a dose-dependent manner. Our results suggest the feasibility of systemic administration of CL-siRNA as an antiviral strategy.

The 5.0 mg/kg GBV-B CL-siRNA dramatically inhibited the replication of GBV-B. However, control CL-siRNA also delayed the virus replication. Intravenous injection of siRNA formulated with liposomes was reported to stimulate mammalian immune system [26,27]. In relation to antiviral effect of IFNs, we therefore measured the serum IFN levels. Since the GBV-B siRNA/cationic liposome had less effect in IFN induction than the control but better antiviral effect than the control, it is possible that inhibition of the viral replication by the GBV-B siRNA/cationic liposome complex was at least in part caused by RNA interference. On the other side, it is also likely that IFN locally induced in the marmoset liver contributed the suppression of the viral replication. Because the induced level of mice serum IFN-α by GBV-B CL-siRNA was significant, although it was less than that by control CL-siRNA. Moreover, estimated IFNs level in marmoset serum was minimal but their actual levels might have been more, because the standard IFN in the ELISA was human or rhesus macaque IFN. Therefore, we considered that the antiviral effect of CL-siRNA was made by both RNA interference and induced IFNs.

In therapeutic application of siRNA to humans, general safety is a most important problem. The side effect of CL-siRNA to the liver is thought to consist of direct liver toxicity which is probably caused by its hydrophobic nature and its immuno-stimulatory effect [26–28]. Recently, Zimmermann et al. has reported that siRNA delivered systemically in a cationic liposome, stable nucleic acid lipid particles (SNALP), inhibited endogenous gene expression in the liver of the cynomolgus monkeys, which supports our notion concerning the therapeutic potential of systemically injected siRNA in primates. Although they made excellent chemical modifications to siRNA oligonucleotides to reduce IFN induction, their siRNA complex produced a considerable liver damage with a marked increase of transaminases at the dose (2.5 mg/kg) of maximal suppression effect. This indicated that the therapeutic window of their siRNA complex is overlapped with its toxic window. In contrast, our CL-siRNA induced much less liver damage, since even the 5.0 mg/kg of our CL-siRNA did not show a marked liver damage, but induced a sub-

stantial immune responses. A number of recent studies revealed that siRNA/cationic liposome complex has an immunological effects of siRNAs including the induction of proinflammatory cytokines and type I IFNs (IFN-α and IFN-β) through activation of RNA-sensing immunoreceptors including three members of the Toll-like receptor (TLR) family (TLR3, TLR7 and TLR8) [29]. Detection of siRNA molecules could trigger antiviral innate defense mechanisms including the induction of type I IFNs. In fact, double strand RNA molecule, poly I/C, was reported to eliminate the virus in GBV-B-infected tamarin hepatocytes by activating TLR3 [30,31]. These knowledges lead us to postulate that it is one of sophisticated strategy for siRNA to inhibit hepatitis virus to use this immuno-stimulatory side effect as an antiviral innate defense, only if the systemic side effects are tolerable.

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References

[1] G.J. Hannon, RNA interference, Nature 418 (2002) 244–251.
[2] W.R. Kim, The burden of hepatitis C in the United States, Hepatology 36 (2002) S30–S34.
[3] J.M. Mchutchison, Hepatitis C advances in antiviral therapy: what is accepted treatment now? J. Gastroenterol. Hepatol. 17 (2002) 431–441.
[4] T. Yokota, N. Sakamoto, N. Enomto, H. Tanabe, M. Maekawa, M. Miyagishi, K. Taira, M. Watanabe, H. Mizusawa, Inhibition of intracellular hepatitis C Virus replication by synthetic and vector-derived small interfering RNAs, EMBO Rep. 4 (2003) 602–608.
[5] M.Y. Seo, S. Abrignani, M. Houghton, J.H. Han, Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7, J. Virol. 77 (2003) 810–812.
[6] M. Korf, D. Jarczak, C. Beger, M.P. Manns, M. Kruger, Inhibition of hepatitis C virus translation and subgenomic replication by siRNAs directed against highly conserved HCV sequence and cellular HCV cofactors, J. Hepatol. 43 (2005) 225–234.
[7] A. Martin, F. Bodola, D.V. Sangar, K. Goettge, V. Popov, R. Rijnbrand, R.E. Lanford, S.M. Lemon, Chronic hepatitis associated with GB virus B persistence in a tamarin after intrahepatic inoculation of synthetic viral RNA, Proc. Natl. Acad. Sci. USA 100 (2003) 9962–9967.
[8] K. Ishii, S. Iijima, N. Kimura, Y.-J. Lee, N. Ageyama, S. Yagi, K. Yamaguchi, N. Maki, K. Mori, S. Yoshizaki, S. Machida, T. Suzuki, N. Iwata, T. Sata, K. Terao, T. Miyamura, H. Akari, GBV-B as a pleiotropic virus: distribution of GBV-B in extrahepatic tissues in vivo, Microb. Infect. 9 (2007) 515–521.
[9] K. Hirabayashi, J. Yano, T. Inoue, T. Yamaguchi, K. Tanigawara, G.E. Smyth, K. Ishiyama, T. Ohgi, K. Kimura, T. Irimura, Inhibition of cancer cell growth by polyinosinic-polycytidylic acid/cationic liposome complex: a new biological activity, Cancer Res. 59 (1999) 4325–4333.
[10] J. Yano, K. Hirabayashi, S. Nakagawa, T. Yamaguchi, M. Nogawa, I. Kashimori, H. Naito, H. Kitagawa, K. Ishiyama, T. Ohgi, T. Irimura, Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer, Clin. Cancer Res. 10 (2004) 7721–7726.
[11] M. Nogawa, T. Yuasa, S. Kimura, M. Tanaka, J. Kuroda, K. Sato, A. Yokota, M. Koizumi, T. Maekawa, Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer, J. Clin. Invest. 115 (2005) 978–985.
[12] F. Liu, Y.K. Song, D. Liu, Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA, Gene Ther. 6 (1999) 1258–1266.
[13] A.A. Kolylhalov, K. Mihalik, S.M. Feinstone, C.M. Rice, Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication in vivo, J. Virol. 74 (2000) 2046–2051.
[14] J.R. Jacob, K.C. Lin, B.C. Tennant, K.G. Masnfield, GB virus B infection of the common marmoset (*Callithrix jacchus*) and associated liver pathology, J. Gen. Virol. 85 (2004) 2525–2533.
[15] R.E. Lanford, D. Chavez, L. Notvall, K.M. Brasky, Comparison of tamarins and marmosets as hosts for GBV-B infections and the effect of immunosuppression on duration of viremia, Virology 311 (2003) 72–80.
[16] B.L. Jacobs, J.O. Langland, When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA, Virology 219 (1996) 339–349.
[17] M.T. Mcmanus, Small RNAs and immunity, Immunity 21 (2004) 747–756.
[18] J.N. Leonard, D.V. Shaffer, Antiviral RNAi therapy: emerging approaches for hitting a moving target, Gene Ther. 13 (2006) 532–540.
[19] V. Bitko, A. Musiyenko, O. Shulyayeva, S. Barik, Inhibition of respiratory viruses by nasally administered siRNA, Nat. Med. 11 (2005) 50–55.
[20] W. Zhang, H. Yang, X. Kong, S. Mohapatra, H. San Juan-Vergara, G. Hellermann, S. Behera, R. Singam, R.F. Lockey, S.S. Mohapatra, Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene, Nat. Med. 11 (2005) 56–62.
[21] B.J. Li, Q. Tang, D. Cheng, C. Qin, F.Y. Xie, Q. Wei, J. Xu, Y. Liu, B.J. Zheng, M.C. Woodle, N. Zhong, P.Y. Lu, Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque, Nat. Med. 11 (2005) 944–9451.
[22] S.M. Tompkins, C.Y. Lo, T.M. Tumpey, S.L. Epstein, Protection against lethal influenza virus challenge by RNA interference in vivo, Proc. Natl. Acad. Sci. USA 101 (2004) 8682–8686.
[23] D. Palliser, D. Chowdhury, Q.Y. Wang, S.J. Lee, R.T. Bronson, D.M. Knipe, J. Lieberman, An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection, Nature 439 (2006) 89–94.
[24] J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R.K. Pandey, T. Racie, K.G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, H.P. Vornlocher, M. Manoharan, H.P. Vornlocher, Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs, Nature 432 (2004) 173–178.
[25] E. Song, P. Zhu, S.K. Lee, D. Chowdhury, D.M. Dykxhoorn, Y. Feng, D. Palliser, D.B. Weiner, P. Shankar, W.A. Marasco, J. Lieberman, Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors, Nat. Biotechnol. 23 (2005) 709–717.

[26] D.V. Morrissey, J.A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C.S. Shaffer, L.B. Jeffs, A. Judge, I. Maclachlan, B. Polisky, Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs, Nat. Biotechnol. 23 (2005) 1002–1007.

[27] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. Mcclintock, I. Maclachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, Nat. Biotechnol. 23 (2005) 457–462.

[28] T.S. Zimmeemamm, A.C. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M.N. Fedoruk, J. Harborth, J.A. Heyes, L.B. Jeffs, M. John, A.D. Judge, K. Lam, K. McClintock, L.V. Nechev, L.R. Palmer, T. Racie, I. Rohl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A.J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H.P. Vornloche, I. MacLachlan, RNAi-mediated gene silencing in non-human primates, Nature 441 (2006) 111–114.

[29] M. Schlee, V. Hornung, G. Hartmann, siRNA and isRNA: two edges of one sword, Mol. Ther. 14 (2006) 463–470.

[30] R.E. Lanford, D. Chavez, B. Guerra, J.Y.N. Lau, Z. Hong, K.M. Brasky, B. Beames, Ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes, J. Virol. 75 (2001) 8074–8081.

[31] K. Li, Z. Chen, N. Kato, M. Gale Jr., S.M. Lemon, Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes, J. Biol. Chem. 280 (2005) 16739–16747.

19 RNAiの神経変性疾患への応用

横田　隆徳

要旨

遺伝性神経変性疾患においてその変異遺伝子自体をsiRNA（short interfering RNA）で治療するといった究極の遺伝子治療を目ざした基礎研究が進行している．さらに孤発性神経変性疾患においてもその機序の解明に伴い，判明したキーとなる分子をターゲットとしたsiRNAによる治療戦略も始まった．モデルマウスを用いた治療実験では良好な結果が得られている．神経細胞へのデリバリーの方法やoff-target効果，shRNA（short hairpin RNA）毒性など，まだまだ解決すべき問題点も多いが，siRNAの高い抑制効果からその神経変性疾患への応用が急速に進展していくことは間違いないものと思われる．

キーワード

- siRNA
- shRNA
- Argonauteファミリー
- 遺伝子治療

19-1 ● はじめに

RNAi（RNA interference. RNA干渉ともいう）は二本鎖RNAによって配列特異的に遺伝子の発現が抑制される現象で，1998年にA. Z. FireとC. C. Melloらによって報告され，2006年のノーベル生理学医学賞に選ばれた．RNAiはいかなる遺伝子に対してもデザインできて，その標的遺伝子の発現抑制効果は他の核酸医薬であるアンチセンス核酸の$10^{3\sim7}$倍，リボザイムの$10^{2\sim5}$倍（自験）高いといわれている．しかもその配列特異性も高く，1塩基の違いの認識も可能であり，医療分野におけるその臨床応用については発見当初から大きく期待されていた．それは，RNAiライブラリーをはじめとする創薬におけるツールといった側面と，siRNAを直接核酸医薬として疾患に適応するという二つの方面から行われている．siRNAを用いた遺伝子治療の研究はすでにウイルス性疾患，悪性腫瘍などで急速に進んでいる．ここでは，siRNAおよびshRNA（short hairpin RNA）の核酸医薬としての開発の研究現状と問題点について，神経変性疾患への適応を中心について概説したい．

19-2 • RNA 干渉とは

長い二本鎖 RNA によって誘導される遺伝子発現抑制である RNAi 現象は植物から昆虫，哺乳動物に至るまで広く保存して観察され，元来真核細胞に備わった抗ウイルス機構として知られていた．細胞内に導入された二本鎖 RNA は Dicer とよばれる RNaseⅢ核酸分解酵素ファミリーによって 21～24mer の短い 3′突出型の二本鎖の siRNA に分解される．siRNA は Dicer の結合タンパク質である TRBP（human immunodeficiency virus transacting response RNA-binding protein）によって Argonaute2（AGO2）にリクルートされ[1]，RLC（RISC-loading complex）を形成する[2]．siRNA の二本鎖のうちパッセンジャー鎖（センス鎖）は AGO2 によりその中央部で切断されて取除かれ，ガイド鎖（アンチセンス鎖）のみの一本鎖化が起こる．一本鎖となった siRNA は他のいくつかのタンパク質を伴って RNA タンパク質複合体である RISC 複合体（RNA induced silencing complex）を構成する[3]．この RISC 複合体がガイド鎖に相補的な配列をもつ標的 RNA にアクセスして，その中央で分解する[3]．しかし，哺乳動物における二本鎖 RNA の導入は PKR や RIG-I や 2′,5′-oligosynthetase の活性化による非特異的な翻訳抑制や RNA の分解をひき起こし，ホストの細胞が死んでしまうため，分子生物学的手法としても遺伝子治療の方法としても大きな妨げになっていた．しかし，2001 年に，RNAi 機構の中間産物である siRNA を合成して用いることによってこれらの副反応が回避され，効率的で特異的な遺伝子発現抑制が可能となった[4]．さらに，siRNA 配列を短い 9 mer のループ配列でつないだ stem 型のパリンドロミックな配列である shRNA を polⅢ系のプロモーター下に挿入した siRNA 発現 DNA プラスミドも開発され，ウイルスベクターやトランスジェニックマウスのトランスジーンとして用いられている[5]．

キーワード解説

- **siRNA(short interfering RNA)**：21～30mer の短い 3′突出型の二本鎖の RNA．細胞内に導入された長い二本鎖 RNA は，Dicer とよばれる RNaseⅢ核酸分解酵素ファミリーによって 21～24mer の短い 3′突出型の二本鎖の siRNA に分解される．哺乳動物において，30mer 以上の長い二本鎖 RNA と異なって PKR や RIG-Ⅰや 2′,5′-oligosynthetase の活性化などの副反応を起こさない．化学合成 siRNA は RNAi の遺伝子治療の中心である．
- **shRNA（short hairpin RNA）**：siRNA 配列を短い 4～20mer のループ配列でつないだ stem 型のパリンドロミックな配列．類似の構造を有する pre-miRNA と同様に細胞内で RNase Dicer によって siRNA に分解され，RISC に取込まれ遺伝子抑制作用を示す．おもに polⅢ 系のプロモーター下に挿入した shRNA 発現 DNA はウイルスベクターやトランスジェニックマウスのトランスジーンとして用いられている．
- **Argonaute ファミリー**：PAZ ドメインと PIWI ドメインを一つずつもつことを特徴とするタンパク質で，RNA 干渉の遺伝子抑制の実行分子である．結晶解析の結果，PAZ ドメインは一本鎖の核酸に結合するドメインで，PIWI ドメインは RNaseH とよく似た構造で RNA を切断ドメインである．ヒトでは Argonaute ファミリーは AGO1-AGO4の 4 種類あり，AGO2の PAZ ドメインが siRNA の 3′末端と結合して，PIWI ドメインが siRNA と結合した標的 RNA を切断する．

19-3 ● siRNA の特異性

遺伝性疾患やがん遺伝子をsiRNAで治療しようとした場合，変異遺伝子のみを選択的に発現抑制して，野生型には作用しないことが望ましい．siRNAと基質RNAとの特異性については，一般に4塩基以上ミスマッチがあった場合でsiRNAの切断活性はおおむね消失するが，1～2塩基のミスマッチによる切断効率の低下は完全ではなく，ミスマッチの位置によってその効果は異なる．siRNA配列の5′末端側は基質との結合よりRISCとの関わりから基質を切断するルーラー（物差し）と働き，基質の認識としては中央から3′末端側のほうが重要で，したがってミスマッチによる失活効果が強いと考えられている[6]．

siRNAを臨床応用する際にも，ライブラリーを用いた遺伝子探索をする際にも，off-target効果，すなわち，ターゲットとした遺伝子以外に，用いた19塩基のsiRNAの配列に部分的にホモロジーのある別の遺伝子の発現を抑えてしまう，いわゆる交差反応が報告されている[7]．全般にその特異性はアンチセンスなどに比較してかなり高いが，それでも多くの遺伝子の発現が少なからず影響を受ける可能性がある．A. L. Jacksonらの検討で[7]，通常19塩基中15塩基以上で，最低では11塩基のホモロジーのある遺伝子においても影響があったと報告された．今後このoff-target効果の評価とその回避は重要な問題である．

19-4 ● 遺伝性神経変性疾患への応用

1 遺伝性神経変性疾患のRNAiによる遺伝子治療の基本概念

遺伝性疾患でゲノム遺伝子の変異が原因で発症する場合，遺伝子変異に起因する発症機序には変異のある遺伝子の遺伝子産物であるタンパク質の本来のもつ機能の消失または低下する場合loss of functionと変異遺伝子や変異タンパク質が新たに病的機能を獲得する場合gain of functionの二つがあることが知られている．遺伝子変異が常染色体にある場合，対立する二つのアリルの双方に遺伝子変異があって初めて発症する常染色体劣性遺伝形式の疾患の多くはloss of functionをその機序とし，一方のアリルのみで発症する常染色体優性遺伝形式の疾患の多くの場合はgain of functionであることが多い．常染色体優性遺伝の場合は野生型のアリルからは原則として正常個体の半分量の正常なタンパク質は発現しているので，本来のタンパク質の機能の影響は少ないか全くなく，変異アリルから発現した変異タンパク質が何らかの正常と異なった機能gain of adverse functionや毒性gain of toxic functionを新たに獲得することにより疾患が発症することが想定されている．*SOD1*変異による筋萎縮性側索硬化症（ALS），多くのポリグルタミン病，*APP*や*PS1*遺伝子変異によるアルツハイマー病，*α-synuclein*変異によるパーキンソン病などの常染色体優性遺伝形式を示す主要な神経変性疾患の多くにおいてgain of toxic functionがその発症機序と考えられている．このような疾患の治療を考える場合，変異したタンパク質の発現を抑制する方法があれば，その機序のいかんにかかわらず発症，進行を防止することが期待できるわけである．われわれはSOD1に対するsiRNAを過剰発現させたトランスジェニックマウスを作製して，これをALS

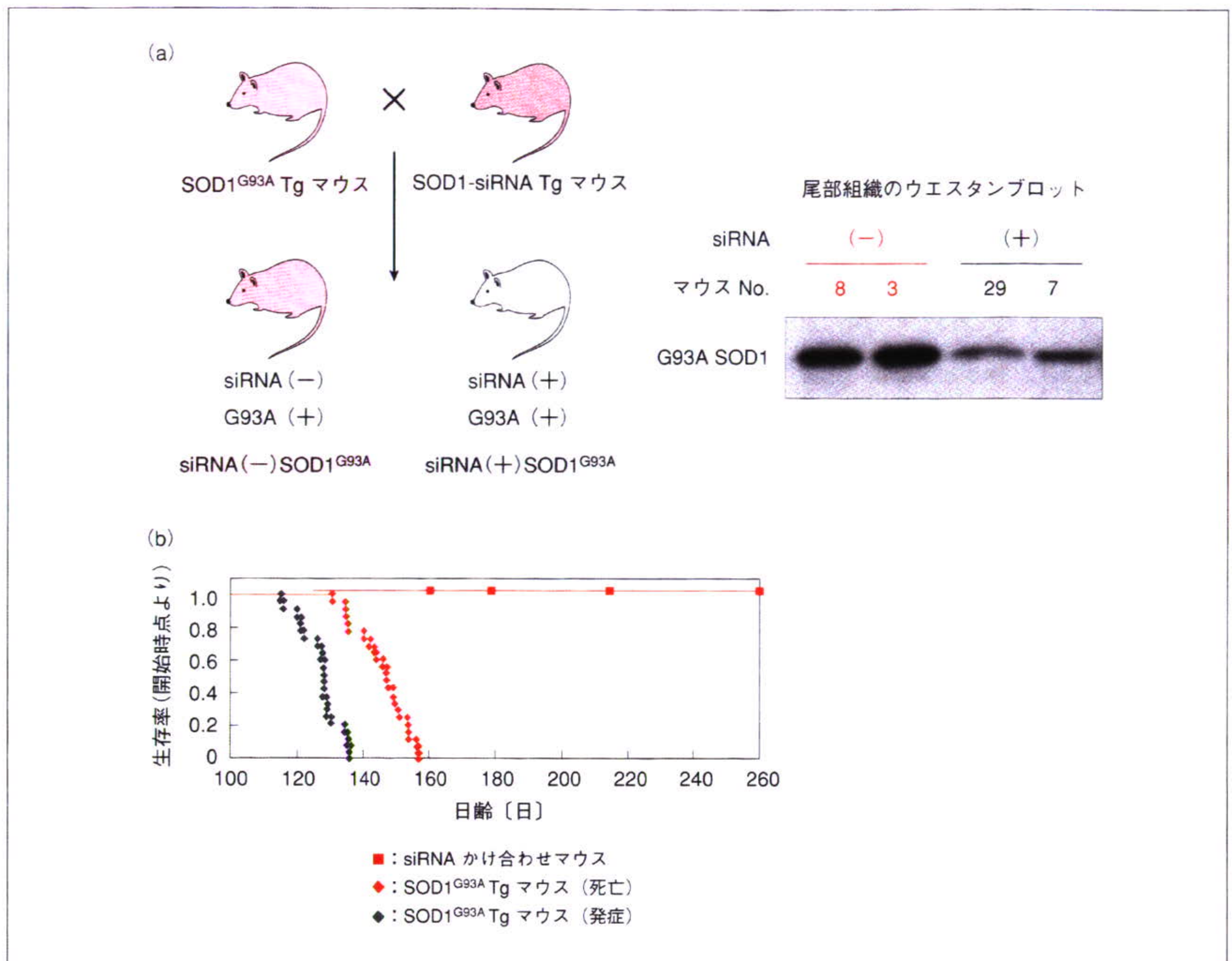

図19-1　SOD1G93A トランスジェニックマウスの遺伝子治療
(a) SOD1に対する siRNA を過剰発現させたトランスジェニックマウスを ALS のモデルマウスである G93A 変異 SOD1 トランスジェニックマウスとかけ合わせることにより，変異 SOD1タンパク質の発現を80％以上抑制することに成功した．(b) 6 月齢の時点で ALS 症状の発症は完全に抑制されている．[Saito Y, Yokota T, et al.：J. Biol. Chem., 280：42826-42830, 2005を一部改変]

のモデルマウスである G93A 変異 SOD1 トランスジェニックマウスとかけ合わせ，中枢神経の変異 SOD1タンパク質の発現を80％以上抑制することに成功した（**図19-1a**）[8]．この効果により，6 月齢の時点で ALS 症状の発症は完全に抑制されている（**図19-1b**）．これらの結果は siRNA という方法で遺伝性神経変性疾患が治療可能であることを理論的に示したと考えられる（巻頭**写真13a**）．

2 変異遺伝子特異的な RNAi 法

野生型 SOD1はノックアウトしても明瞭な神経症状は示さないので副作用はない可能性が高いが，たとえば SCA6 の場合，その原因遺伝子 α カルシウム1A チャネルのノックアウトマウスは生後 1～2 週で死亡することが知られており，正常アリルの発現抑制は新たな症状を来たす可能性が高い．したがって，優性遺伝疾患の治療には，正常アリルの発現を損なわずに，変異アリルの発現のみを抑制することが望ましい．上で述べたように，一塩基置換の場合は siRNA の配列の 5′ 末端側から 10～13 番目の塩基に変異部

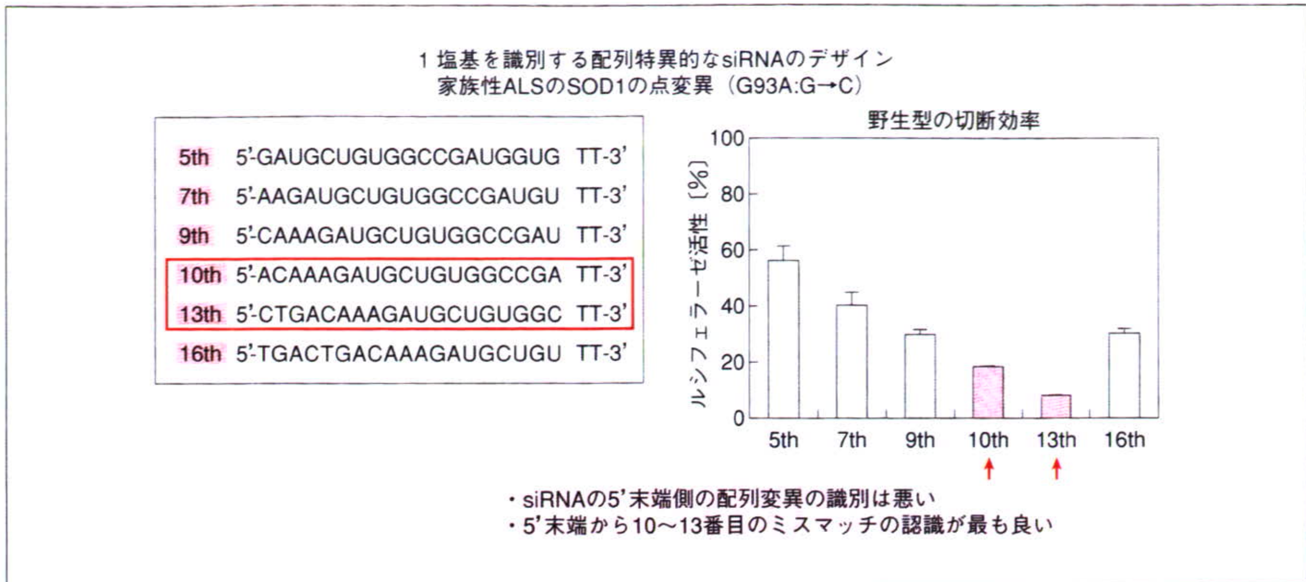

図19-2　siRNAへの標的遺伝子とのミスマッチ変異挿入位置によるsiRNA効果への影響
家族性筋萎縮性側索硬化症（家族性ALS）の遺伝子変異であるG93A SOD1（点変異G→C，赤で示した）を標的としたG93A siRNAのデザイン．G93A siRNAの5′末端側から10〜13番目の塩基に変異部位を置いた場合が最も野生型SOD1の切断効率が低下する．

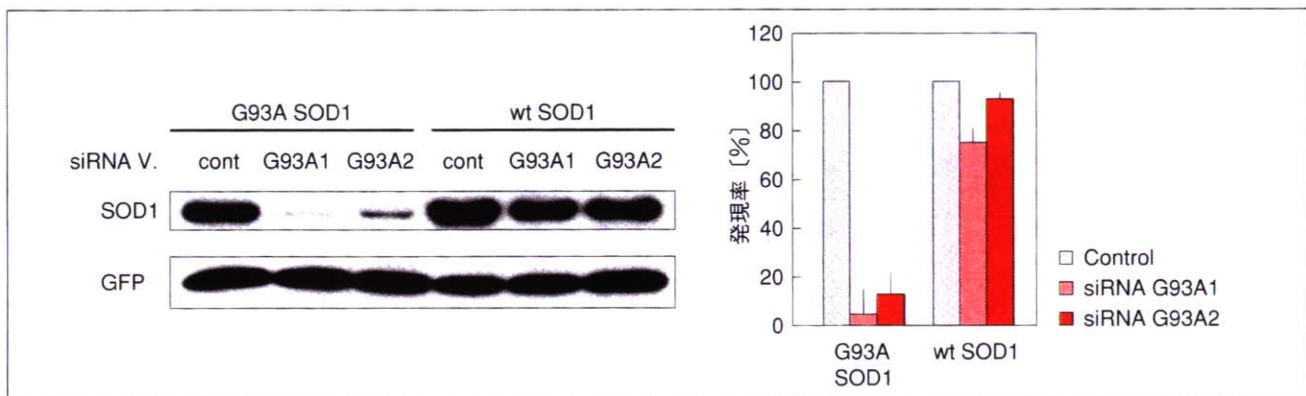

図19-3　変異SOD1に特異的に作用するsiRNA
293T細胞にG93Aまたは野生型SOD1発現ベクターとsiRNA G93A1，A2を共発現させ，野生型および変異SOD1の発現をウエスタンブロットした．siRNA G93A1，A2をともにG93A SOD1の発現を著明に抑制して，野生型SOD1の発現はほとんど抑制しなかった．［Yokota T, et al.：Biochem. Biophys. Res. Com., 314：283-291, 2004を一部改変］

位を置いた場合が最も野生型SOD1の切断効率が低下する（**図19-2**）．変異が1塩基の違いである点変異でも正常アリルと変異アリルの配列の差を認識して変異アリルのみを切断できるsiRNAの作製は可能である．**図19-3**ならびに巻頭**写真13b**に家族性筋萎縮性側索硬化症（家族性ALS）の原因遺伝子である*SOD1*の点変異G93Aを選択的に切断して正常配列にはほとんど影響しないsiRNAの例を示す[9]．同様の報告は捻転ジストニア[10]や前頭側頭型認知症frontotemporal dementia（FTD）[11]で報告されている．

ポリグルタミン病のように，繰返し配列の長さが変わることが変異である場合は，この伸長した繰返し配列そのものに対するsiRNAのデザインをすることは難しい．Machado-Joseph病（SCA3）の場合，CAGリピートの直下の下流にG/C多型があり，これはCAGリピートの繰返し配列の長さと関連している．長い繰返しをもつ病的アリル

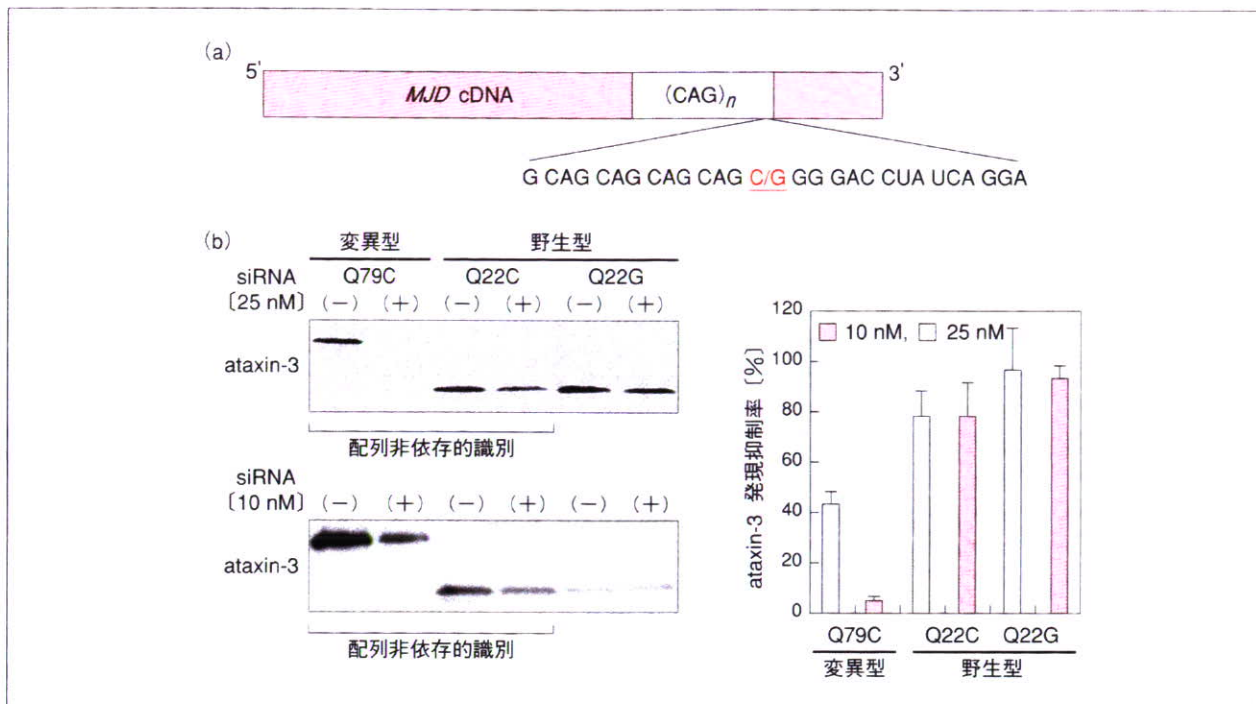

図19-4 Machado-Joseph（MJD）RNA に対する配列変異アリル特異的な一次配列非依存的な siRNA の切断
(a) Machado-Joseph 病は *MJD* 遺伝子内の CAG リピートの伸長によって発症する．CAG リピートの後には G/C 多型があり，伸長した CAG リピートをもつ変異アリルはすべて C で，正常アリルでは G/C が同頻度でみられる．(b) われわれのデザインした MJD siRNA はこの 1 塩基の差を認識して変異アリル（Q79C）を切断し，正常アリル（Q22G）は切断しなかった．加えて，驚いたことにこの MJD siRNA は Q79C と標的配列の全く同じもう一つの正常アリル（Q22C）もわずかにしか切断しなかった．［Li Y, Yokota T, et al.：Ann. Neurol., 56：124-129, 2004を一部改変］

はすべて C だが，短い繰返しをもつ正常アリルでは約半数の例で G である（**図19-4a**）[12]．そこでわれわれはこの G/C 多型の標的として siRNA を設計して，病的アリルに特異的な siRNA を作製した．ところが驚いたことにこの siRNA は多型が変異アリルと同じ C である短い CAG リピートの正常アリルもあまり切断しなかった（**図19-4b**）[12]．この機序は不明だが，CAG リピート長の変化に伴う RNA の二次構造の変化や MJD RNA の多型付近に結合する RNA 結合タンパク質の結合度の変化によって，siRNA の標的配列へのアクセスに差異が生じるためかもしれない．

しかし，*SOD1* や *presenilin1*（*PS1*）の点変異は 100 種類以上知られており，そのすべてに特異的で効率な siRNA がデザインできるわけではない．われわれはいかなる遺伝子変異に対しても特異的で有効な新しい RNAi 法を考案して（**図19-5**）[13]，現在その *in vivo* での有効性を検証中である．

19-5 ● 孤発性神経変性疾患への応用

ほとんどのアルツハイマー病，パーキンソン病や筋萎縮性側索硬化症（ALS）は家族歴のない孤発性で遺伝子異常は明らかでないが，それぞれの発症機序のキーとなる分子がわかれば，その発現を抑制することで治療が可能かもしれない．たとえば，アルツハイマー病の β-セクレターゼは有望な標的分子である．アルツハイマー病のモデル動

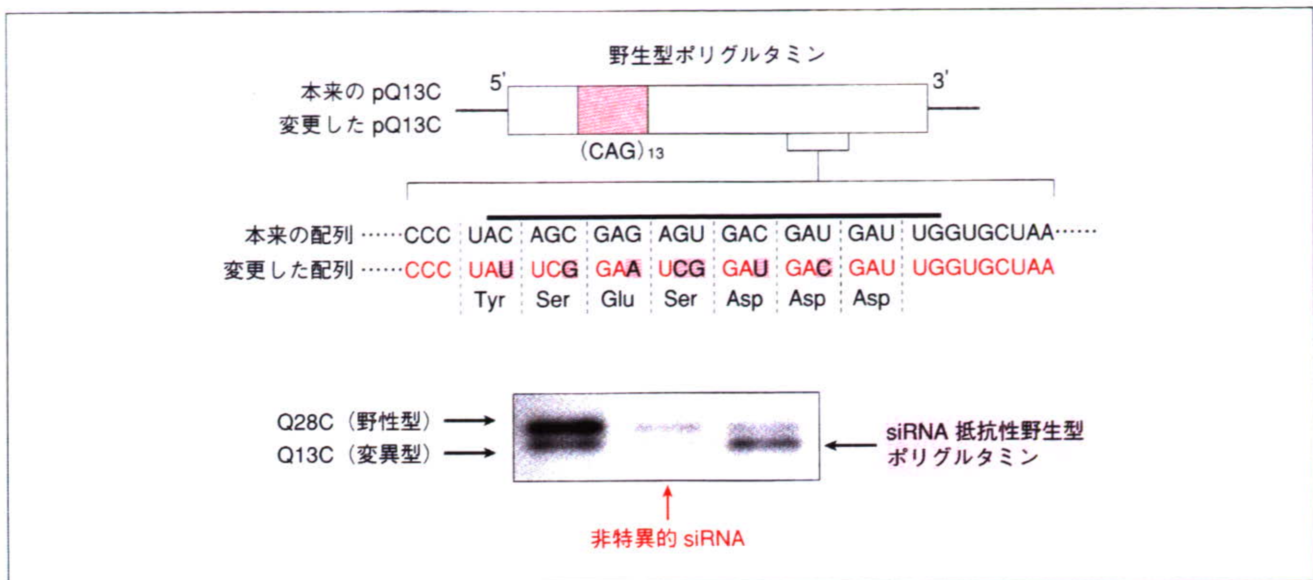

図19-5　いかなる変異に対しても変異アリル特異的な RNAi 法
SCA6の原因遺伝子はαカルシウム1Aチャンネル遺伝子内のCAGリピートの異常伸長だが変異アリル特異的な siRNA を設計することは不可能である．そこで，非特異的な siRNA によって変異型と野生型，両者の mRNA の発現を抑制し，その siRNA で切断されないように作製した cDNA を利用して野生型タンパク質を戻す方法によって，変異アリル特異的な遺伝子発現抑制が可能となる．［Kubodera T, Yokota T, et al.：Oligonucleotides, 15：298-302, 2005を一部改変］

物はAβのワクチン治療やその抗体の受動免疫により老人斑の生成を抑制し，認知障害も軽減しえたと報告されている．Aβはアミロイド前駆タンパク質 APP からβ-セクレターゼとγ-セクレターゼによって切り出されて生成される．presenilin1（PS1）などからなるγ-セクレターゼは Notch などの他の重要な分子も基質としているため，その機能を抑制すると問題が出るが，β-セクレターゼの本体といわれる BACE1 のノックアウトマウスは特別の異常を示さない．われわれも BACE1 に対する siRNA を作製して，培養細胞系でAβ産生を抑制できることを示した（**図19-6**）．今後，広範な神経細胞に siRNA を持続的に導入することが可能となれば，画期的な治療方法になるかもしれない．最近，BACE1 に対する siRNA を発現するレンチウイルスを用いてスウェーデン型変異 APP を過剰発現させたトランスジェニックマウスの海馬に直接注入して，老人斑の沈着を減少させ，認知機能障害を改善させたとの報告がなされた[14]．また，他の基質への影響なくγ-セクレターゼ機能を低下させる可能性のある APP アダプター分子 X11aαと X11aβをターゲットとした siRNA でAβ産生を抑制できる可能性や，ミクログリアの活性化をカテプシンBに対する siRNA で抑制し，神経細胞毒性を軽減したとの報告もされている．

19-6 • siRNA の *in vivo* へのデリバリー

siRNA は細胞質で RISC に取込まれて切断活性を発揮することより，siRNA のデリバリーは細胞膜さえ越えればよく，遺伝子治療によく使われる発現 DNA ベクターのように核にアクセスする必要がない．A. P. McCaffrey[15] らはマウスの尾静脈から 10～50 μg の合成 siRNA を体重の 5～10％の大量の PBS 溶液で 5～7 秒の短時間で注入する

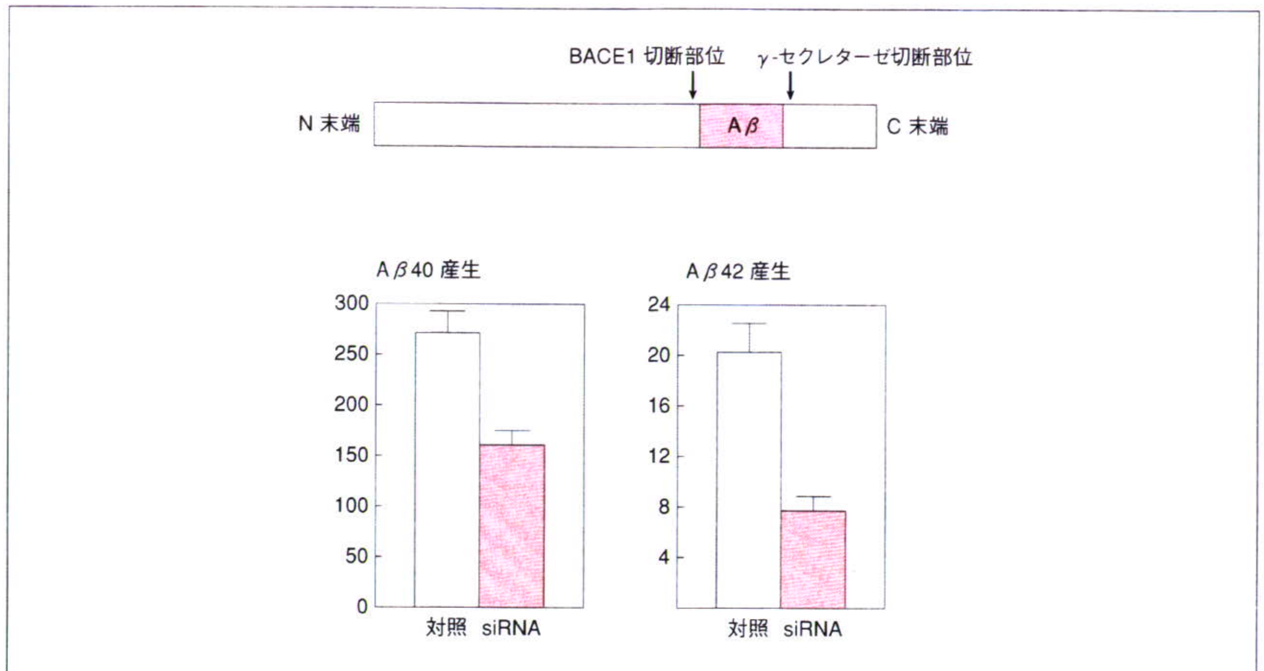

図19-6 孤発性アルツハイマー病に対する siRNA による治療
APPsw 安定発現培養細胞株において BACE1に対する siRNA により培養液中に分泌される Aβ 産生を抑制した.

ハイドロダイナミックス導入法で，マウスの肝細胞に siRNA の導入に成功した．さらに最近このハイドロダイナミックス導入法で導入された Fas やカスパーゼ-8に対する合成 siRNA で，マウスに誘発された激症肝炎による死亡率を低下させたとの報告がされた．通常静脈内の全身投与方法でも，合成 siRNA を疎水性でプラスに荷電したカチオニックリポソームに包み込むことにより，とくにがん細胞において合成 siRNA を導入させることが可能である[16]．ただ，近年 siRNA/カチオニックリポソーム複合体はI型インターフェロンや TNF α や IL-6 などのサイトカインを誘導することが判明して，副作用や非特異的な発現抑制効果の原因になりうる可能性が問題になっている[17]．siRNA/カチオニックリポソーム複合体が細胞に導入される際に，複合体がまず細胞のエンドソームに取込まれ，エンドソーム内に発現している Toll-like 受容体を介してインターフェロン誘導がされることが判明している．siRNA/カチオニックリポソーム複合体によるインターフェロンは配列依存的であり，その明確な法則は明らかでないが，これを回避する方法として，siRNA への種々の化学修飾が試みられている[18]．また，siRNA のセンス鎖の 3′ 末端にコレステロール[19] を直接共有結合させて，siRNA を導入しようとする試みもされている．さらに，抗体[20] や RNA アプタマー[21] を siRNA 結合させて，導入細胞の受容体を介した siRNA の新しいデリバリーも報告されている．しかし，これらのいずれの方法でも siRNA を脳血管関門を越えて，静脈注射で中枢神経系にデリバリーすることは困難で，siRNA による神経疾患治療の大きな問題になっている．

その点で最近，化学修飾した合成 siRNA を直接の脳室内に 1 ～ 2 週間持続注入することによって脳室の表層に近い海馬や大脳基底核においての標的遺伝子の発現を 50 %

程度抑制したとの報告がなされ[22]，注目されている．siRNAをカチオニックリポソームに包埋して同様に長期脳室内持続注入して，脊髄，後根神経節や視床下部などにおいて有効に導入に成功したとの報告もある[23),24]．また，神経細胞へ導入のため細胞導入シグナルペプチドの利用も注目されている[25]．さらに，血液脳関門を通過するようにトランスフェリン受容体に対するモノクローナル抗体を，神経細胞に導入するためにインスリンに対するモノクローナル抗体を結合させたペグ化免疫リポソームを作製して，全身投与することにより神経細胞へ導入させようとする試みも始まっている[26]．

長期の抑制効果にはウイルスベクターが必要となる．ヘアピン型siRNA発現ベクターコンストラクト（shRNA）をアデノウイルスやレンチウイルス，レトロウイルス，アデノ随伴ウイルスなどのウイルスベクターに組み込んで作製したsiRNA発現ウイルスベクターを用いて，***in vivo***の細胞へのsiRNA導入の報告が次々とされている[27]．とくに最近開発されたアデノ随伴ウイルスの新しい血清型8型（AAV-8）は非常に高い遺伝子導入効率があり期待されている．しかし，最近，AAV-8を用いたshRNAの全身投与にて，遅発性の致死的な重篤な肝障害があるとの重要な報告がされた[28]．この肝障害は導入したsiRNAの発現量に依存し，複数の異なった標的遺伝子に対するshRNAにおいて生じており，複数のmicroRNA（miRNA）の低下を伴っていた．これは細胞内でshRNAと内因性のmiRNAの前駆体であるpre-miRNAが共通のプロセス機構であって，核から細胞質への移行タンパク質であるエクスポーティン-5 exportin-5をshRNAが競合することにより，miRNAの成熟化プロセスが障害されて成熟miRNAが低下するためと考察されている．われわれもマウスにおいて同様の肝障害を経験しており，今後のshRNA発現ベクターを用いた遺伝子治療の問題になる可能性がある．

19-7 • おわりに

siRNA/shRNAの核酸医薬としての臨床応用の研究には，上で述べたように解決すべき課題はまだ多くある．しかし，基礎研究は爆発的に進んでおり，最も大きな問題であるデリバリー方法にも急速な進歩がある．非常に近い将来に，難治性疾患での新しい治療法の開発にsiRNAの利用が突破口になると期待している．

●文 献

1）Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R：TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature. 436：740-744, 2005.

2）Tomari Y, Zamore PD：Perspective：machines for RNAi. Genes Dev., 19：517-529, 2005.

3）Rand TA, Petersen S, Du F, Wang X：Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell, 123：621-629, 2005.

4）Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD：Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell, 123：607-620, 2005.

5）Elbashir S, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T：Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature, 411：494-498, 2001.

6）Brummelkamp TR, Bernards R, Agami R：A system for stable expression of short interfering RNAs in mammalian cells. Science, 29：550-553, 2002.

7）Amarzguioui M, Holen T, Babaie E, Prydz H：Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res., 31：589-595, 2003.