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Resolution of liver cirrhosis using vitamin A–coupled liposomes to deliver siRNA against a collagen-specific chaperone

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There are currently no approved antifibrotic therapies for liver cirrhosis. We used vitamin A–coupled liposomes to deliver small interfering RNA (siRNA) against gp46, the rat homolog of human heat shock protein 47, to hepatic stellate cells. Our approach exploits the key roles of these cells in both fibrogenesis as well as uptake and storage of vitamin A. Five treatments with the siRNA-bearing vitamin A–coupled liposomes almost completely resolved liver fibrosis and prolonged survival in rats with otherwise lethal dimethylnitrosamine-induced liver cirrhosis in a dose- and duration-dependent manner. Rescue was not related to off-target effects or associated with recruitment of innate immunity. Receptor-specific siRNA delivery was similarly effective in suppressing collagen secretion and treating fibrosis induced by CCl_4 or bile duct ligation. The efficacy of the approach using both acute and chronic models of liver fibrosis suggests its therapeutic potential for reversing human liver cirrhosis.

Liver cirrhosis, or fibrosis, the ultimate pathological feature of all forms of chronic hepatic damage, is responsible for much morbidity and mortality worldwide. The principal cell type responsible for liver fibrosis is the hepatic stellate (HS) cell, a resident perisinusoidal cell that takes up vitamin A from circulation and stores it. When stimulated by reactive oxygen intermediates or cytokines, HS cells become activated and are transformed to proliferative, fibrogenic and contractile myofibroblasts[1], which synthesize and secrete procollagen, which accumulates as insoluble collagen after its terminal domains are cleaved by procollagen peptides, causing fibrosis. The collagen-specific chaperone, heat shock protein 47 (HSP47), facilitates collagen secretion by ensuring proper triple-helix formation of procollagen in the endoplasmic reticulum and has also been implicated in translational regulation of procollagen synthesis[2,3].

The demonstration that liver fibrosis in animals[4] and humans[5] can regress when collagen synthesis is inhibited suggests that fibrosis can be reversed, most likely by the activity of matrix metalloproteinases. Various therapeutic approaches to inhibit collagen synthesis or activate matrix metalloproteinases have been investigated in animal models[6,7]. However, none have yet been applied clinically, mainly because of side effects resulting from an inability to specifically target particular molecules and/or cells.

The specific association of HSP47 with a diverse range of collagen types[3,8,9] makes it an excellent candidate for targeting HS cell–mediated collagen secretion using siRNA. To enhance the specificity of such a strategy, we reasoned that encapsulating the siRNA in vitamin A–coupled liposomes should target it preferentially to HS cells, which have a remarkable capacity for vitamin A uptake, most likely through receptors for retinol binding protein (RBP).

Using three animal models of liver cirrhosis—involving induction by dimethylnitrosamine (DMN), CCl_4 or bile duct ligation—our histological analysis shows that intravenous (i.v.) injection of vitamin A–coupled liposomes carrying siRNA against mRNA encoding rat gp46, a homolog of HSP47, (VA-lip-siRNAgp46), rapidly resolves liver fibrosis. As cells analogous to HS cells apparently play an essential role in causing fibrosis associated with chronic pancreatitis[10] and laryngeal fibrosis[11], our approach may find value in treating fibrotic conditions in organs besides the liver.

RESULTS

siRNAgp46 suppresses gp46 expression and collagen secretion

We first elucidated the effects of three siRNAs (types A, B, C) on the abundance of gp46 transcripts (**Fig. 1a** and **Supplementary Fig. 1** online) in normal rat kidney (NRK) cells, previously used to study collagen production[12]. All three siRNAs suppressed accumulation of gp46 mRNA (**Supplementary Fig. 1b**) and gp46 protein (**Fig. 1a**), but the greatest efficacy, observed using type A siRNA (siRNAgp46A), led us to select it for subsequent experiments. Transducing NRK cells with various dosages of siRNAgp46A revealed an apparent dose-dependent suppression of gp46 expression with almost complete suppression at 50 nM (**Fig. 1b**). We then used proline incorporation assays to test whether siRNAgp46A could inhibit collagen secretion. The concentration of newly synthesized collagen in the culture medium of NRK cells

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transduced with siRNAgp46A was significantly reduced compared with parental cells and cells transfected with randomly selected siRNAs (siRNArandom) ($P < 0.01$) (**Fig. 1c**). Collagen secretion from cultured NRK cells was measured by Sirius red dye binding and spectrophotometry[13]. Significantly less collagen was secreted by siRNAgp46A-treated NRK cells than by non-treated NRK or NRK cells treated with siRNArandom ($P < 0.01$) (**Fig. 1d**).

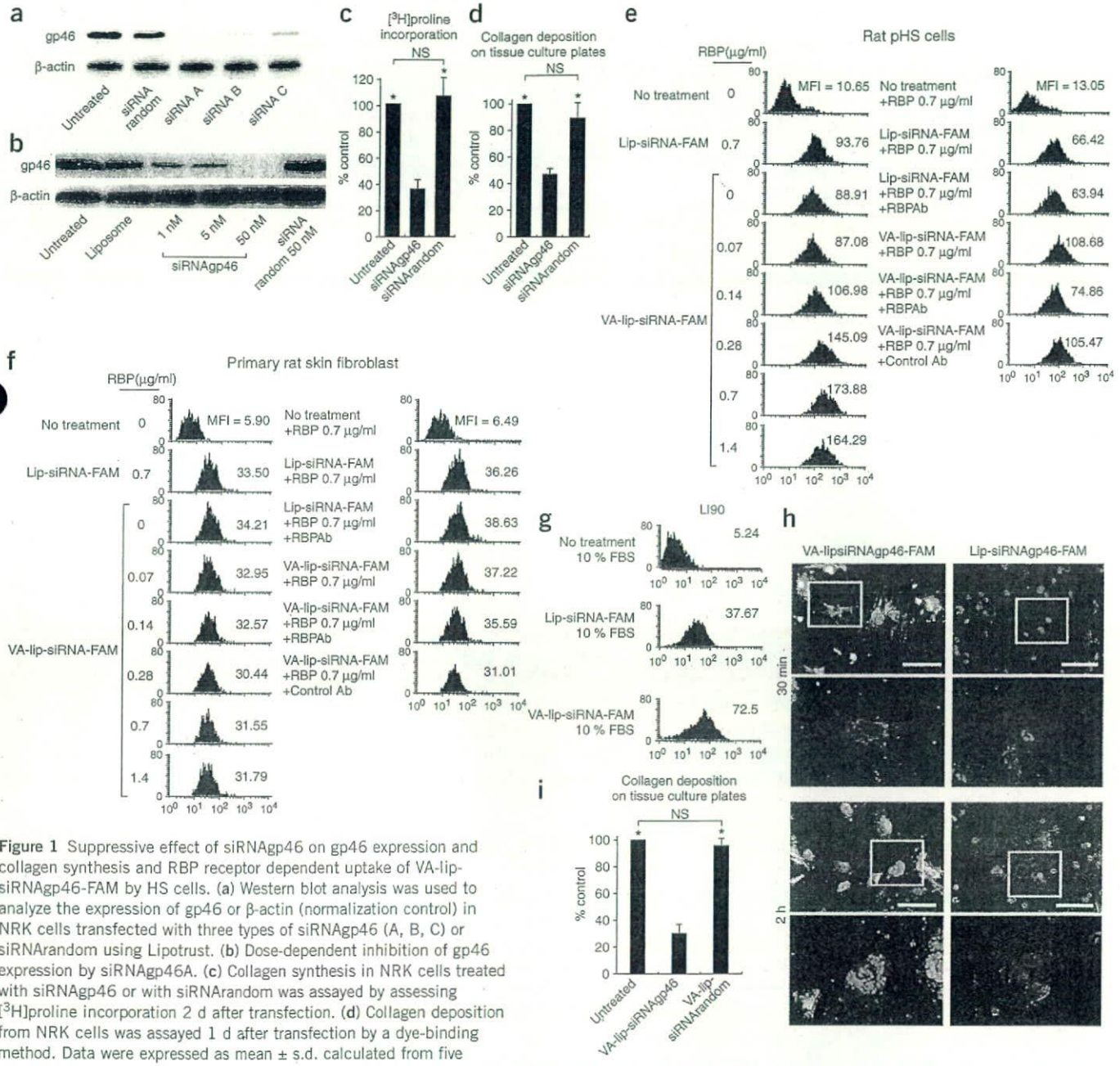

Figure 1 Suppressive effect of siRNAgp46 on gp46 expression and collagen synthesis and RBP receptor dependent uptake of VA-lip-siRNAgp46-FAM by HS cells. (**a**) Western blot analysis was used to analyze the expression of gp46 or β-actin (normalization control) in NRK cells transfected with three types of siRNAgp46 (A, B, C) or siRNArandom using Lipotrust. (**b**) Dose-dependent inhibition of gp46 expression by siRNAgp46A. (**c**) Collagen synthesis in NRK cells treated with siRNAgp46 or with siRNArandom was assayed by assessing [^{3}H]proline incorporation 2 d after transfection. (**d**) Collagen deposition from NRK cells was assayed 1 d after transfection by a dye-binding method. Data were expressed as mean ± s.d. calculated from five transfections and as a percentage of untreated control. *$P < 0.01$ versus siRNAgp46. NS, not significant. (**e,f**) Representative FACS patterns of rat pHS cell (**e**) and primary rat skin fibroblast (**f**) treated with vitamin A-free liposomes carrying siRNAgp46-FAM (lip-siRNAgp46-FAM) or VA-lip-siRNAgp46-FAM in the presence of various concentrations (0.07–1.4 μg/ml) of RBP and of the cells treated with lip-siRNAgp46-FAM or VA-lip-siRNAgp46-FAM in 0.7 μg/ml RBP with or without anti-RBP antibody. Irrelevant mouse monoclonal antibody served as a negative control. (**g**) Representative FACS patterns of LI90 cells treated with lip-siRNAgp46-FAM or VA-lip-siRNAgp46-FAM in the presence of 10% FBS. Mean fluorescence intensity (MFI) is indicated. Five independent experiments were carried out in each of (**e–g**), and the results were essentially the same. (**h**) Representative fluorescent images of the intracellular distribution of FAM-labeled siRNA. Rat pHS cells were treated with VA-lip-siRNAgp46-FAM or lip-siRNAgp46-FAM. At 30 min, the medium was replaced with fresh medium. At the time indicated, cells were fixed and analyzed by fluorescence microscopy to determine the relative intracellular distribution of the siRNA-FAM (green). Pictures taken at original magnification (× 200, upper panel) and magnified images corresponding to the areas enclosed in boxes are presented in lower panel. Scale bars, 100 μm. One representative image of five is shown. (**i**) Rat pHS cells treated with VA-lip-siRNAgp46 or with VA-lip-siRNArandom and collagen deposition on tissue culture plates were assayed 1 d after transfection by the dye-binding method. Data were expressed as mean ± s.d., calculated from five transfections and as a percentage of untreated control. *$P < 0.01$ versus VA-lip-siRNAgp46. NS, not significant.

Optimal ratio of vitamin A to liposome

To determine the optimal vitamin A/liposome ratio for transduction of siRNAgp46 into HS cells, we incubated liposomes carrying siRNAgp46 coupled to carboxyfluorescein (FAM) (lip-siRNAgp46-FAM) with vitamin A in fractionated molar ratios ranging from 1:2 to 16:1. After removing free vitamin A by ultrafiltration, transduction efficiency by the vitamin A–bound lip-siRNAgp46-FAM (VA-lip-siRNAgp46-FAM) of rat primary HS cells (pHS cells) was examined using fluorescence-associated cell sorting (FACS). The highest mean fluorescence intensity (MFI) levels were observed at a molar vitamin A/liposome ratio of 2:1 in the incubation solution (**Supplementary Fig. 2** online). We used complexes at this incubation molar ratio, which corresponded to 0.65 mol vitamin A per mol cationic lipid in liposomes, in all subsequent experiments.

Confirmation of RBP binding to VA-lip-siRNAgp46

We used gel filtration to separate VA-lip-siRNAgp46 and vitamin A–free lip-siRNAgp46, both of which had been incubated with RBP. RBP from the VA-lip-siRNAgp46 preparation eluted with liposomes in the void volume. In contrast, RBP from lip-siRNAgp46 preparations eluted after the void volume, in fractions not containing liposomes (**Supplementary Fig. 3** online). This confirms binding of RBP to VA-lip-siRNAgp46.

Sizes of vitamin A–coupled liposomes

We used dynamic light scattering to compare the average sizes of vitamin A–coupled liposome, lip-siRNAgp46, VA-lip-siRNAgp46 and RBP-bound VA-lip-siRNAgp46 preparations with those of unmodified liposomes. From largest to smallest, particle sizes were: liposome, vitamin A–coupled liposome, lip-siRNAgp46, VA-lip-siRNAgp46 and RBP-bound VA-lip-siRNAgp46 (**Supplementary Table 1** online).

Receptor-mediated uptake of VA-lip-siRNAgp46-FAM by pHS cells

To verify the specific uptake of vitamin A–coupled liposomes by HS cells via the RBP receptor, we incubated rat pHS cells with VA-lip-siRNAgp46-FAM in the presence of various concentrations (0.07 μg/ml–1.4 μg/ml) of RBP and examined FACS patterns of the cells. Because the nonspecific lipofection rate increased and became more similar to that of receptor-mediated transduction with longer transduction times (data not shown), transduction time was limited to 30 min to distinguish between receptor-mediated transduction efficacy of VA-lip-siRNAgp46 and nonspecific transduction efficacy of lip-siRNAgp46. Rat pHS cells treated with VA-lip-siRNAgp46-FAM showed increasing fluorescence intensities as RBP concentrations increased from 0.07 μg/ml to 0.7 μg/ml, and the intensity plateaued at concentrations >0.7 μg/ml RBP (**Fig. 1e**). To further confirm specific uptake of vitamin A–coupled liposome/RBP complex by RBP receptors, we added anti-RBP antibody to the incubation medium of rat pHS cells treated with VA-lip-siRNAgp46-FAM in the presence of RBP (0.7 μg/ml). The MFI of these cells was suppressed by anti-RBP antibody, to levels near those with lip-siRNAgp46-FAM (**Fig. 1e**). Preferential uptake of VA-lip-siRNAgp46-FAM relative to that of lip-siRNAgp46-FAM was not observed using primary skin fibroblasts from normal rats (**Fig. 1f**). We conducted similar experiments to those involving pHS cells, using LI90 cells (an activated human HS cell line)[14] and found essentially the same results (**Fig. 1g**).

Intracellular transport of VA-lip-siRNAgp46-FAM in rat pHS cells

To elucidate the subcellular localization of siRNAgp46-FAM after uptake by RBP receptors, we monitored VA-lip-siRNAgp46-FAM–treated rat pHS cells and lip-siRNAgp46-FAM–treated rat pHS cells using fluorescence microscopy (**Fig. 1h**). In cells treated with VA-lip-siRNAgp46-FAM, fluorescence appeared as a faint granular pattern in the cytoplasm at 30 min, and as a denser granular pattern in the perinuclear region at 2 h. In contrast, no green fluorescence was seen in the cytoplasm of cells treated with lip-siRNAgp46-FAM after 30 min. Moreover, perinuclear fluorescence at 2 h was very faint.

VA-lip-siRNAgp46 suppresses collagen secretion of pHS cells

To validate the effect of VA-lip-siRNAgp46 on collagen secretion from rat pHS cells, we assessed the amount of collagen deposited on tissue culture plates by Sirius red dye binding and spectrophotometry and found it to be significantly lower than in untreated and VA-lip-siRNArandom-treated cells ($P < 0.01$) (**Fig. 1i**).

Delivery of VA-lip-siRNAgp46-FAM to HS cells in cirrhotic rat liver

Receptor-mediated uptake of vitamin A–coupled liposomes by HS cells *in vivo* was verified in rats previously treated with DMN (12 intraperitoneal injections over 4 weeks; **Supplementary Fig. 4** online) and then injected with VA-lip-siRNAgp46-FAM (3 injections, each 0.75 mg/kg siRNA and given every other day). The tissue distribution of siRNAgp46-FAM was examined by fluorescent emission 24 h after the final injection. Fluorescence of siRNAgp46-FAM (green) was identified in liver, predominantly in the region that stained positive for α-smooth muscle actin (α-SMA, red; an indicator of activated HS cells) giving rise to areas with a merged yellow color. The yellow area in ten randomly selected high-power (×630) fields from each specimen occupied 61.2 ± 9.8% of the area stained for α-SMA (**Fig. 2a**, images i,v). By contrast, the yellow area in α-SMA–positive regions (**Fig. 2a**, image iii) was negligible (5.6 ± 2.3%) in a rat injected with lip-siRNAgp46-FAM.

To confirm the specific delivery of siRNAgp46 to HS cells *in vivo*, we performed FACS analyses of cyanine 5 (Cy5) fluorescence intensity in hepatocytes, HS cells and Kupffer cells isolated from the liver of DMN-treated rats injected with VA-lip-siRNAgp46-Cy5. We observed markedly greater accumulation of siRNAgp46-Cy5 in α-SMA–positive cells (HS cells) than in CD 163–positive cells (Kupffer cells) or albumin-positive cells (hepatocytes). Accumulation of siRNAgp46-Cy5 in HS cells was not evident in rats injected with lip-siRNAgp46-Cy5 (**Fig. 2b**).

In the lung of a rat injected with VA-lip-siRNAgp46-FAM, very few cells, other than endothelial cells (alveolar epithelium) that showed autofluorescence, were positive for fluorescent emission and the fluorescence (green) of these cells merged with the red-staining of macrophages (CD68) indicating nonspecific uptake of siRNAgp46-FAM by macrophages. Similarly, fluorescent cells in the spleen of the same rat merged with red-stained macrophages, generating a yellow signal. Analyses of lungs and spleens from rats injected with lip-siRNAgp46-FAM showed results similar to those with VA-lip-siRNAgp46-FAM (**Fig. 2c**), indicating that nonspecific uptake of siRNAgp46-FAM by macrophages also occurred in both organs. The retina, which requires vitamin A for its photoreceptor function, showed almost no staining in either of the treated rats (**Fig. 2c**).

To further elucidate the pharmacodynamics and tissue distribution of VA-lip-siRNAgp46, radiolabeled liposomes carrying siRNAgp46 with [^{3}H]vitamin A were injected, through the tail vein, into normal rats and rats 24 d after commencement of the induction of acute cirrhosis by DMN (day 24 rats; **Fig. 2d**). The circulating half-lives of [^{3}H]VA-lip-siRNAgp46 were 19 min and 61 min for DMN-treated rats and normal rats, respectively, compared with 13 min for [^{3}H]vitamin A (**Fig. 2d**, insert) in cirrhotic rats. Radioactivity was found almost exclusively in cirrhotic livers 24 h after injection (**Fig. 2d**). An equal dose of radiolabeled vitamin A–coupled liposomes carrying

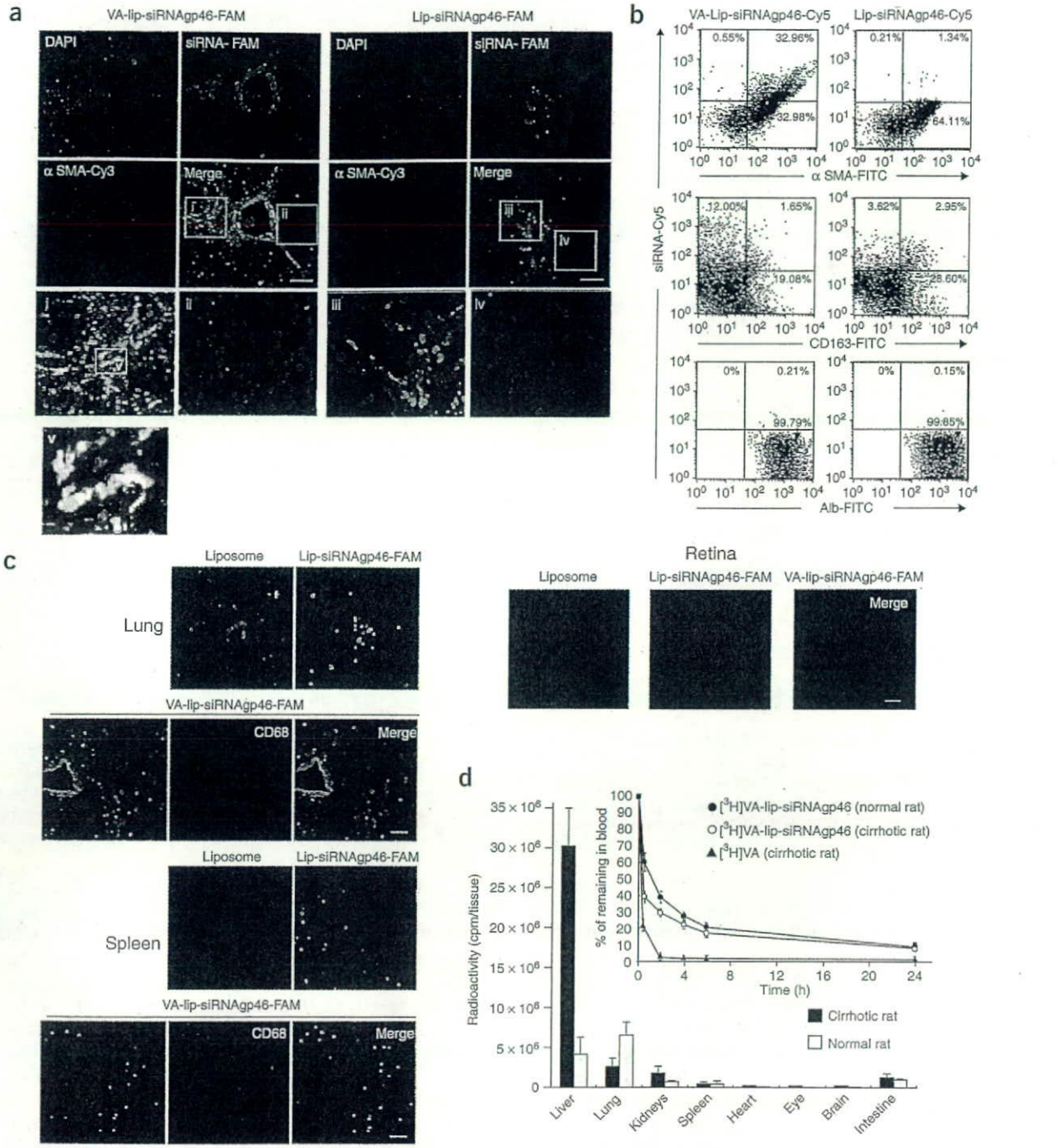

Figure 2 Specific delivery of VA-lip-siRNAgp46 to HS cells in cirrhotic rats. (**a**) Representative fluorescent images of α-SMA visualized by Cy3-conjugated anti-α-SMA antibody (red), nuclei counterstained with DAPI (blue) and siRNAgp46-FAM (green) in liver specimens from DMN-treated cirrhotic rats (day 24 rat) injected with VA-lip-siRNAgp46-FAM or lip-siRNAgp46-FAM every other day (three injections in total). The liver specimens were harvested 24 h after the last injection. Pictures taken at original magnification (×200) and magnified images corresponding to the areas enclosed in boxes are presented in i, ii and iii, iv. Image v: a high power image of i showing fluorescent staining in the cytoplasm. Scale bars, 100 μm. (**b**) Rat HS cells, Kupffer cells and hepatocytes rich fractions harvested 24 h after three injections of VA-lip-siRNAgp46-Cy5 or lip-siRNAgp46-Cy5 were stained with anti α-SMA-FITC, anti-rat CD168-FITC and anti-albumin-FITC (alb-FITC), respectively, and were analyzed by flow cytometry. (**c**) Representative immunofluorescent staining of macrophages with the Alexa 568-labeled anti-CD68 antibody (red) and siRNAgp46-FAM (green) in the lung, spleen and retina. Histologic sections from these organs were collected 24 h after i.v. injection of VA-lip-siRNAgp46-FAM, lip-siRNAgp46-FAM or liposomes. Fluorescent images of these sections were obtained using a confocal laser microscope. Scale bars, 100 μm. (**a–c**) Similar results were obtained in five independent experiments. (**d**) Representative tissue biodistribution and pharmacokinetics of [^{3}H]VA-lip-siRNAgp46 in rats. DMN-treated cirrhotic rats (day 24 rat) or normal rats ($n = 3$ per group) received a single i.v. injection of 200 μCi [^{3}H]VA-lip-siRNAgp46 via the tail vein. Tissue biodistribution was analyzed 24 h later. Data represent means ± s.d. ($n = 3$). The inserted figure shows the percentage of the injected dose remaining in the blood at the indicated times after infection. Each point represents the mean ± s.d. ($n = 3$). Similar results were obtained in two independent experiments.

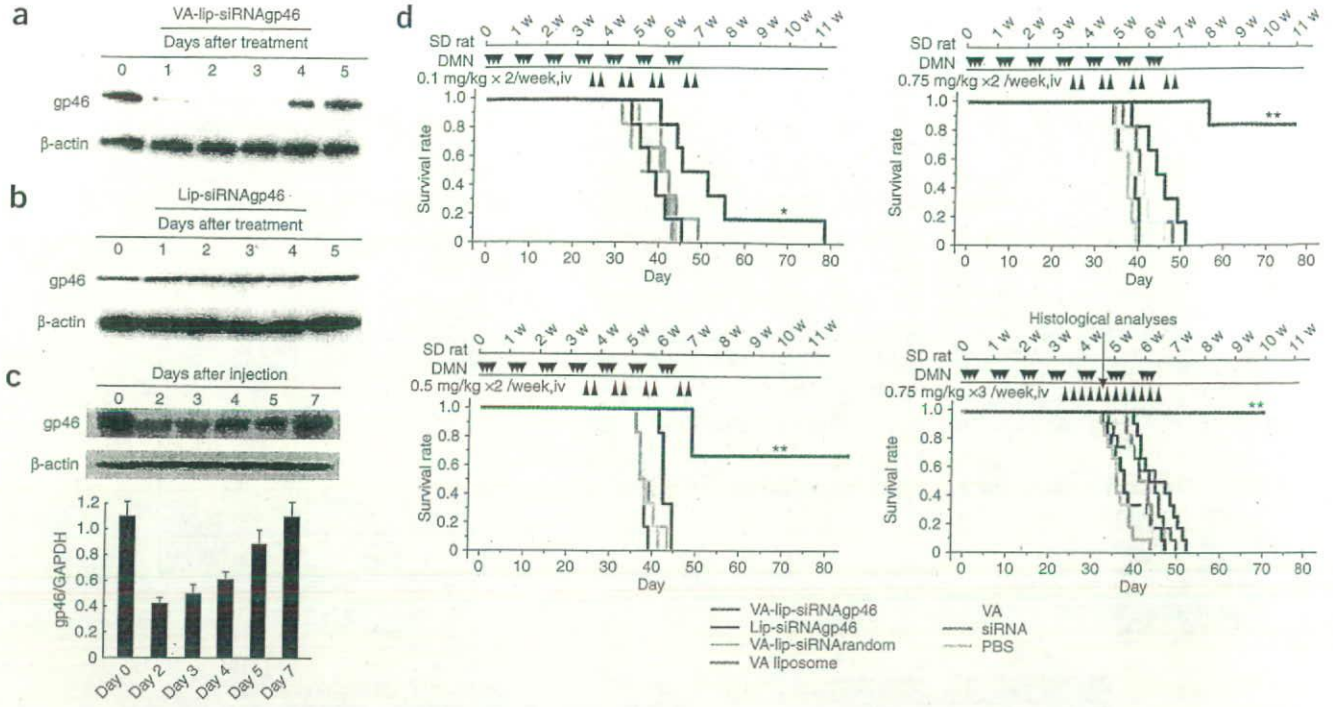

Figure 3 Effect of i.v. injected VA-lip-siRNAgp46 on cirrhotic rat survival. (**a**,**b**) Duration of suppressive effect of siRNAgp46 on gp46 expression in HS cells. Rat pHS cells were treated with VA-lip-siRNAgp46 (**a**) or lip-siRNAgp46 (**b**). At 30 min, the medium was replaced with fresh medium. At the time indicated, the expression of gp46 and β-actin (normalization control) were analyzed by western blotting. Similar results were obtained in three independent experiments. (**c**) The time course of gp46 expression in DMN-cirrhotic rats treated with VA-lip-siRNAgp46 (siRNA 0.75 mg/kg, one injection at day 24, $n = 3$). At the indicated time, western blot analysis (top) and quantitative real-time PCR (bottom) were used to analyze the expression of gp46. Liver gp46 mRNA levels were quantified relative to GAPDH mRNA. Data are mean ± s.d. from an analysis representative of three rats. (**d**) Survival of DMN-treated rats that were injected intravenously with VA-lip-siRNAgp46 at siRNA doses of 0.1, 0.5 or 0.75 mg/kg twice a week ($n = 6$ per group), or 0.75 mg/kg 3 times a week ($n = 12$ per group). In the control groups, DMN-treated rats were injected with lip-siRNAgp46, VA-lip-siRNArandom, vitamin A–coupled liposomes, vitamin A, PBS ($n = 6$ for each group of 0.1, 0.5 or 0.75 mg/kg twice a week, $n = 12$ for each group of 0.75 mg/kg 3 times a week) or siRNAgp46 ($n = 12$ for group of 0.75 mg/kg siRNA, three times a week). Life-table analyses are presented as a Kaplan-Meyer plot. (*$P < 0.05$; **$P < 0.0001$ compared with control rats).

siRNAgp46 injected into a noncirrhotic rat of equal age substantially reduced accumulation of radioactivity in all organs. This indicates that HS cells, which specifically took up VA-lip-siRNAgp46, had not proliferated to the same extent in normal livers as in cirrhotic livers.

Duration of gp46 suppression by VA-lip-siRNAgp46

To examine the duration of the effect of siRNAgp46 on gp46 expression, we treated rat pHS cells with VA-lip-siRNAgp46 and lip-siRNAgp46 *in vitro* for 30 min (**Fig. 3a,b**) and intravenously injected VA-lip-siRNAgp46 in DMN rats at day 24 (**Fig. 3c**). Both the rat pHS cells and the livers of DMN rats showed suppressed gp46 expression for at least 3 d (**Fig. 3a,c**) whereas treatment of pHS cells with lip-siRNAgp46 did not suppress gp46 expression (**Fig. 3b**).

Survival of DMN rats by i.v. treatment with VA-lip-siRNAgp46

We examined the effect of siRNAgp46 on survival of rats exposed to normally lethal DMN treatments[6] (**Fig. 3d**). In this series of experiments, treatments were begun after induction of liver cirrhosis (day 24) by 12 administrations of DMN (**Supplementary Fig. 4**). Controls treated with PBS, vitamin A, vitamin A–coupled liposome, VA-lip-siRNArandom or lip-siRNAgp46 twice weekly all died within 52 d after DMN treatment.

DMN-treated rats appeared to die from hepatic failure because they developed ascites, gastrointestinal bleeding and tarry feces (data not shown). In contrast, rats treated with VA-lip-siRNAgp46 twice weekly showed a dose-dependent (0.1, 0.5, 0.75 mg/kg) prolongation of survival time and a much higher survival rate (83.3%; 5/6 rats) at 0.75 mg/kg. Survival was 100% (12/12) when the 0.75 mg/kg dose was given three times per week (**Fig. 3d**)—a finding compatible with the observation that gp46 expression was suppressed for 72 h but restored within 4 d after siRNAgp46 treatment (**Fig. 3a,c**).

Resolution of hepatic fibrosis by siRNAgp46 in DMN-treated rats

Liver fibrosis in DMN-treated rats (day 33) that received VA-lip-siRNAgp46 (0.75 mg/kg) five times was examined using Azan-Mallory (**Fig. 4a**) and collagen I staining (**Fig. 4b**). The fibrotic area detected by computerized image analysis scoring for collagen staining was significantly smaller in specimens from VA-lip-siRNAgp46–treated rats than in control specimens ($P < 0.001$) (**Fig. 4c**); these results were consistent with quantitative RT-PCR data for procollagen type I and TIMP-1 mRNA, which showed substantial suppression of mRNA expression by VA-lip-siRNAgp46 treatment (**Fig. 4d**). Similarly, hydroxyproline levels in VA-lip-siRNAgp46–treated rats were significantly lower than in control rats ($P < 0.001$) (**Fig. 4e**). Pathological staging, that is, fibrosis and architectural alteration, was also clearly suppressed in VA-lip-siRNAgp46–treated rats (**Fig. 4f**).

The effect of siRNAgp46 on hepatic gp46 expression was also examined by staining for gp46 in liver specimens of rats injected

with VA-lip-siRNAgp46 and VA-lip-siRNArandom five times (day 33). The positively stained area (red) was substantially smaller in the former (**Fig. 4g**). Western blot analysis results were compatible with this immunohistological observation; the intensity of the gp46 band was weaker in VA-lip-siRNAgp46–treated rats than in controls (**Fig. 4h**).

The reversibility of liver cirrhosis was examined in liver specimens from day 47 and day 70 rats that stopped receiving DMN and VA-lip-siRNAgp46 treatments at day 44 and day 46, respectively. Near-complete restoration of normal hepatic architecture was observed in specimens from day 47 and day 70 rats (**Fig. 4i**), indicating the regeneration capacity of liver tissue.

Induction of apoptosis in rat HS cells by siRNAgp46

This observation led us to speculate that siRNAgp46 treatment might induce apoptotic death of HS cells. Apoptosis was determined by TUNEL staining on day 33 liver specimens from DMN-treated rats injected with VA-lip-siRNAgp46 (0.75 mg/kg five times on every other day), PBS, vitamin A alone, vitamin A–coupled liposomes, VA-lip-siRNArandom or lip-siRNAgp46; ($n = 3$ per group). TUNEL-positive cells in areas overlapping with HS cells (α-SMA positive) apparently increased in rats treated with VA-lip-siRNAgp46 versus VA-lip-siRNArandom (**Supplementary Fig. 5a** online). TUNEL-positive cells in α-SMA–positive areas (**Supplementary Fig. 5b**) significantly increased in VA-lip-siRNAgp46–treated rats relative to controls ($P < 0.01$).

In HS cell–cultivation experiments, TUNEL staining in apoptotic nuclei of rat pHS cells transfected by siRNAgp46 was greater after 3 d than in control HS cells (**Supplementary Fig. 5c**). Approximately half (48 ± 12%) of all nuclei in siRNAgp46-transfected HS cells were apoptotic—a substantial increase relative to controls ($P < 0.01$) (**Supplementary Fig. 5d**). These results suggest that siRNAgp46 not only inhibits secretion of collagen from HS cells but also abrogates HS cells in fibrotic tissue through apoptosis, culminating in the blockage of further collagen accumulation in the tissue.

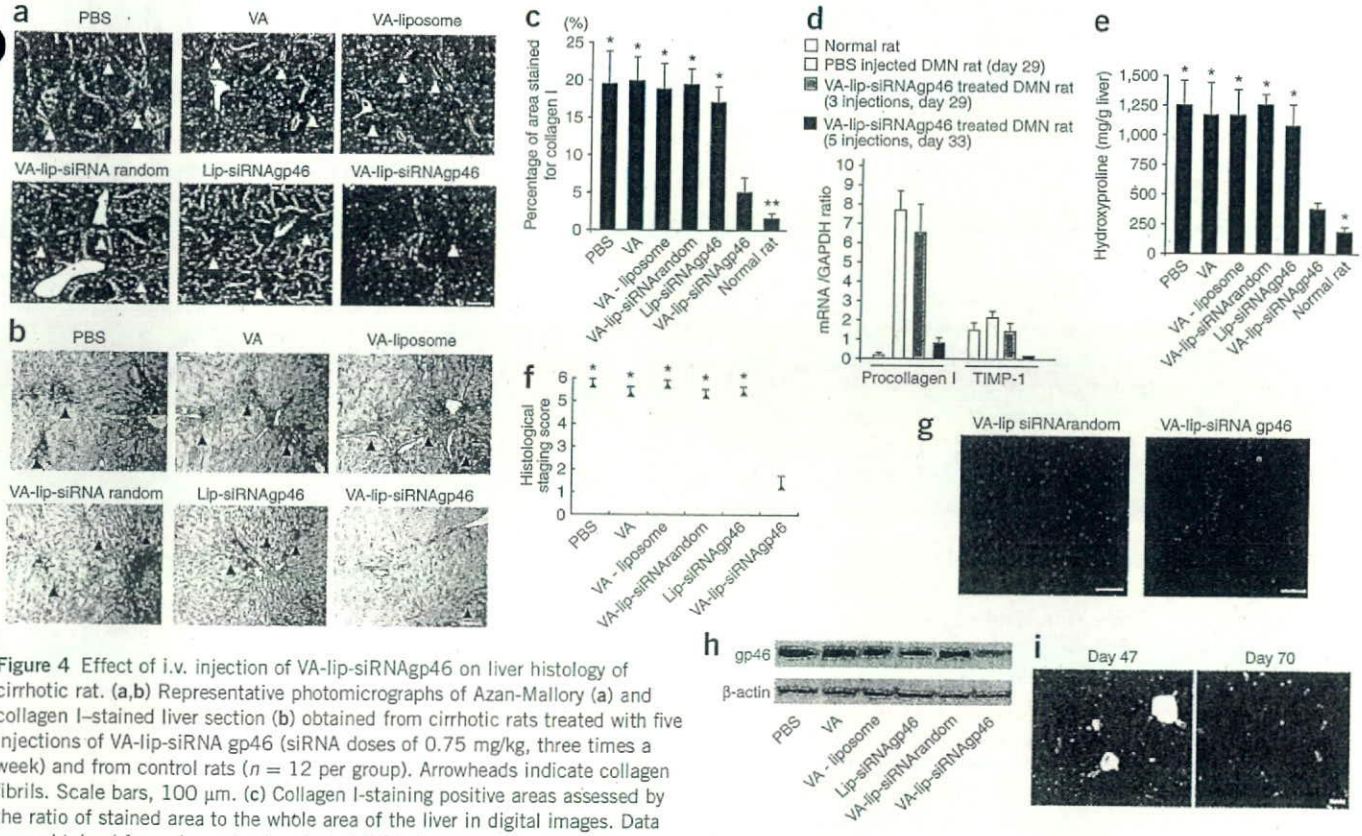

Figure 4 Effect of i.v. injection of VA-lip-siRNAgp46 on liver histology of cirrhotic rat. (**a**,**b**) Representative photomicrographs of Azan-Mallory (**a**) and collagen I–stained liver section (**b**) obtained from cirrhotic rats treated with five injections of VA-lip-siRNA gp46 (siRNA doses of 0.75 mg/kg, three times a week) and from control rats ($n = 12$ per group). Arrowheads indicate collagen fibrils. Scale bars, 100 μm. (**c**) Collagen I-staining positive areas assessed by the ratio of stained area to the whole area of the liver in digital images. Data were obtained from six randomly selected fields in each 12-rat group and represent the mean ± s.d. $*P < 0.001$; $**P < 0.01$ versus VA-lip-siRNAgp46. (**d**) The expression of procollagen I and TIMP-1 mRNA in normal rats ($n = 3$), DMN-treated cirrhotic rats treated with three injections of PBS ($n = 3$), DMN-cirrhotic rats treated with three injections of VA-lip-siRNAgp46 ($n = 3$), and DMN-cirrhotic rats treated with five injections of VA-lip-siRNAgp46 ($n = 3$) were quantified by real-time PCR. Expression was normalized as the ratio to GAPDH mRNA, a housekeeping gene. Data are mean ± s.d. from one analysis representative of three different rats. (**e**) Hydroxyproline content in the livers. Mean ± s.d. of 12 rats per group. $*P < 0.001$ versus VA-lip-siRNAgp46. (**f**) Semiquantitative scoring analysis for histological staging. Liver tissues were obtained from rats treated as described in **a**. Values are means ± s.d. ($n = 12$ per group). $*P < 0.001$ versus VA-lip-siRNAgp46. (**g**) Representative immunofluorescent staining patterns for gp46 in cirrhotic liver of rats (day 24 rat) injected with VA-lip-siRNArandom ($n = 3$) or VA-lip-siRNAgp46 ($n = 3$) three times every other day obtaining 24 h after the last injection. Immunofluorescent images of gp46 stained with Alexa 568-labeled anti-gp46 antibody (red) and of nuclei counterstained with DAPI (blue) were obtained using a confocal laser microscope. Scale bars, 100 μm. Western blot analysis of gp46 in the liver homogenates (**h**) showed essentially the same results as the histological analyses; that is, suppression of the gp46 band by VA-lip-siRNAgp46 treatment. Results from each group of three rats were essentially similar. (**i**) Representative Azan-Mallory-stained liver section from day 47 and day 70 rats. Results from each group of six rats were essentially similar. Scale bars, 200 μm.

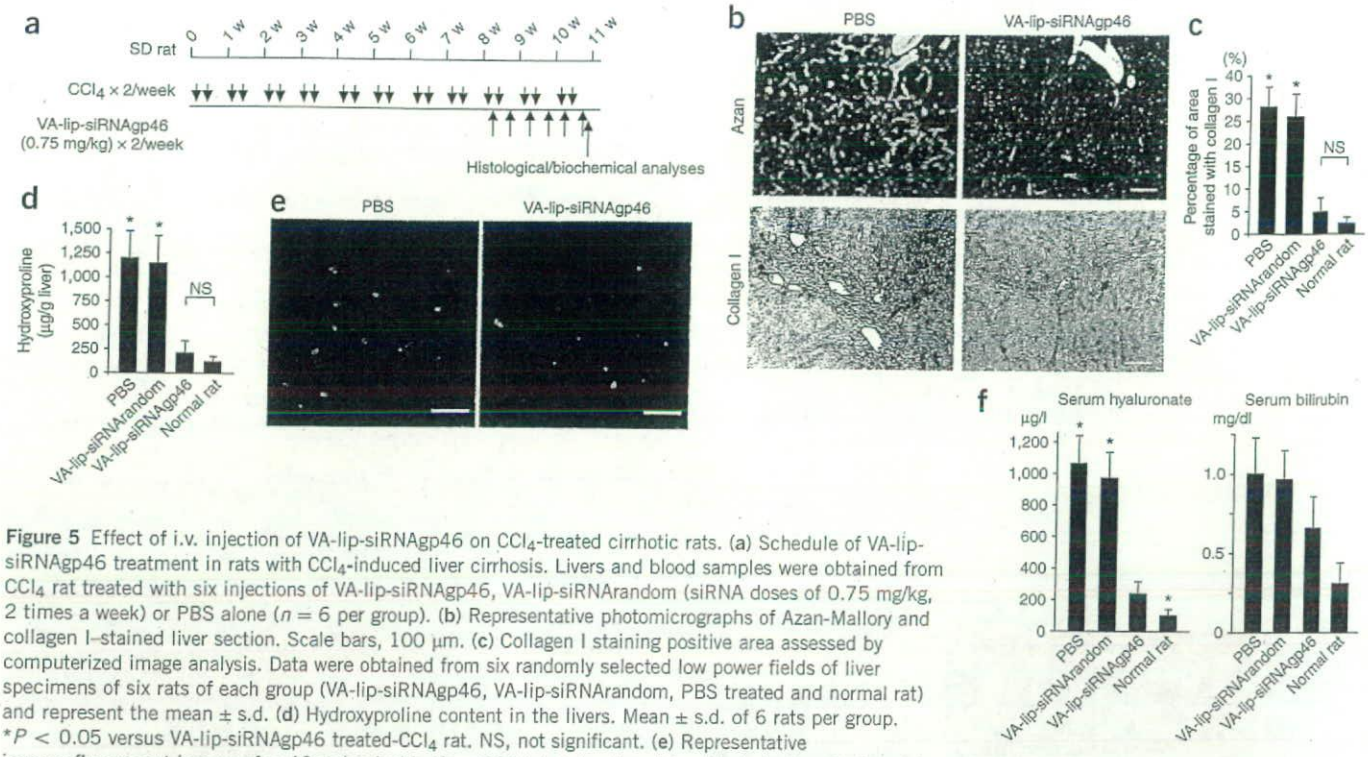

Figure 5 Effect of i.v. injection of VA-lip-siRNAgp46 on CCl_4-treated cirrhotic rats. (**a**) Schedule of VA-lip-siRNAgp46 treatment in rats with CCl_4-induced liver cirrhosis. Livers and blood samples were obtained from CCl_4 rat treated with six injections of VA-lip-siRNAgp46, VA-lip-siRNArandom (siRNA doses of 0.75 mg/kg, 2 times a week) or PBS alone ($n = 6$ per group). (**b**) Representative photomicrographs of Azan-Mallory and collagen I–stained liver section. Scale bars, 100 μm. (**c**) Collagen I staining positive area assessed by computerized image analysis. Data were obtained from six randomly selected low power fields of liver specimens of six rats of each group (VA-lip-siRNAgp46, VA-lip-siRNArandom, PBS treated and normal rat) and represent the mean ± s.d. (**d**) Hydroxyproline content in the livers. Mean ± s.d. of 6 rats per group. $^*P < 0.05$ versus VA-lip-siRNAgp46 treated-CCl_4 rat. NS, not significant. (**e**) Representative immunofluorescent images of gp46 stained with Alexa 568-labeled anti-gp46 antibody (red) and of nuclei counterstained with DAPI (blue) were obtained using a confocal laser microscope. Scale bars, 100 μm. Results from each group of three rats were essentially similar. (**f**) Total bilirubin and hyaluronate levels in the sera of rats. Values are the mean ± s.d. for each group ($n = 6$ per group). $^*P < 0.05$ versus VA-lip-siRNAgp46 treated-CCl_4 rats.

Effects of siRNAgp46 treatment on liver function

VA-lip-siRNAgp46 (0.75 mg/kg, five times on every other day) significantly attenuated the elevation of serum bilirubin and hyaluronate in VA-lip-siRNAgp46–treated cirrhotic rats relative to control cirrhotic rats at day 33, during DMN exposure ($P < 0.01$). At day 70, bilirubin, hyaluronate, alanine aminotransferase (ALT) and albumin levels in DMN rats were nearly or completely normalized by VA-lip-siRNAgp46 treatment (**Supplementary Fig. 6** online).

Effects of siRNAgp46B and siRNAgp46C on DMN cirrhosis

To eliminate the possibility that the *in vitro* and *in vivo* results obtained by treatment with siRNAgp46A resulted from off-targeting effects, we tested the effect of the two other siRNAs for gp46, siRNAgp46B and C, on cirrhosis in DMN-treated rats. Prolonged survival, reduced histological fibrosis and suppression of hydroxyproline accumulation were clearly associated with exposure to either siRNA (**Supplementary Fig. 7** online), indicating that the effects of siRNAgp46A were not related to off-targeting.

VA-lip-siRNAgp46 does not trigger immune reaction

Interferon-α (IFN-α) mRNA expression in the liver, spleen and lung remained normal after VA-lip-siRNAgp46 treatment (**Supplementary Table 2** online). As siRNAβgal728 complexed to lipofectamine-2000 reportedly induces immune reactions in mammals[15] (**Supplementary Methods** online), we also tested the serum concentrations of TNF-α and IL-12 in rats treated with VA-lip-siRNAgp46 or siRNAβgal728 complexed to lipofectamine-2000. Serum concentrations of TNF-α and IL-12 remained normal after VA-lip-siRNAgp46 treatment, in contrast to the significantly elevated values in rats treated with siRNAβgal728 complexed to lipofectamine-2000 ($P < 0.05$) (**Supplementary Table 2**).

Collagenase activity in liver of siRNAgp46-treated cirrhotic rats

To assess collagenolytic activity in the cirrhotic liver, we measured collagenase activity in liver homogenates from DMN rats. Collagenase activity in DMN-treated rats injected with PBS or VA-lip-siRNAgp46 three times (day 29) was as high as in normal liver and remained high after five treatments with VA-lip-siRNAgp46 (day 33) (**Supplementary Table 3** and **Supplementary Methods**).

Effect of VA-lip-siRNAgp46 on CCl_4-induced cirrhosis

Serial intraperitoneal treatment of rats with CCl_4 (16 times over 8 weeks) causes nonlethal liver cirrhosis[4]. We used this additional model of chemically induced cirrhosis to further assess the antifibrotic potential of siRNAgp46 and VA-lip-siRNAgp46 by administering them twice weekly for 3 weeks (**Fig. 5a**). The effect of siRNAgp46 was essentially the same as in DMN-treated rats with respect to shrinkage of the fibrotic area (**Fig. 5b,c**), suppression of hydroxyproline levels (**Fig. 5d**) and gp46 expression (**Fig. 5e**). Bilirubin and hyaluronate levels were also improved in CCl_4-treated cirrhotic rats treated with VA-lip-siRNAgp46 (**Fig. 5f**).

Effect of VA-lip-siRNAgp46 on bile duct ligation–induced cirrhosis

To verify that our modality is also effective for cirrhosis induced by chronic continuous stimulation with bile regurgitation, we administered VA-lip-siRNAgp46 to rats that underwent bile duct ligation[16] (BDL; **Fig. 6a**). Histological improvement of fibrosis (**Fig. 6b,c**) and suppression of hydroxyproline levels (**Fig. 6d**), serum bilirubin and hyaluronate levels (**Fig. 6e**) were clearly observed at day 45 after five injections of VA-lip-siRNAgp46 (0.75 mg/kg, every other day). These effects were maintained until day 67 by consecutive injection of the same agents twice a week (**Fig. 6f–i**), although BDL stimulation

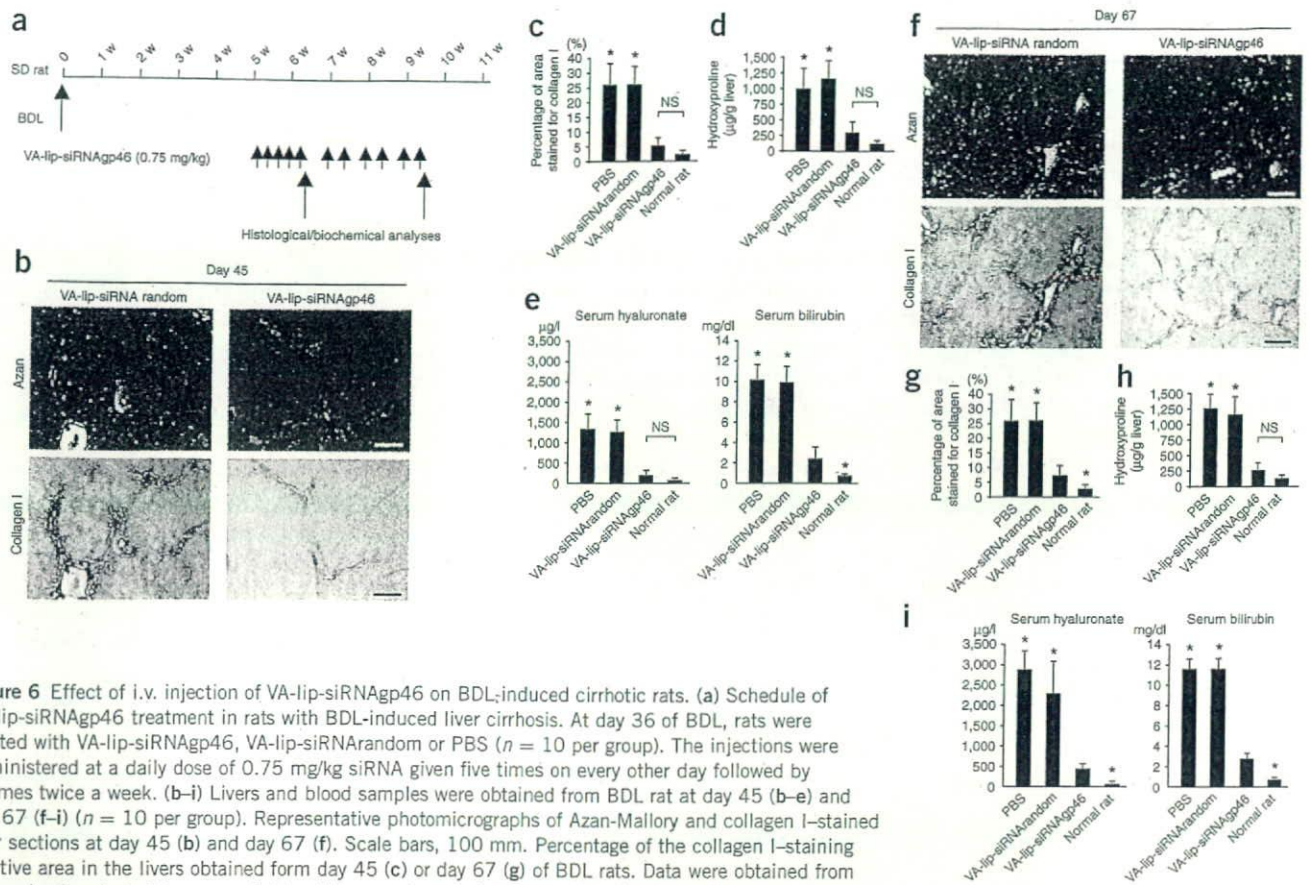

Figure 6 Effect of i.v. injection of VA-lip-siRNAgp46 on BDL-induced cirrhotic rats. (**a**) Schedule of VA-lip-siRNAgp46 treatment in rats with BDL-induced liver cirrhosis. At day 36 of BDL, rats were treated with VA-lip-siRNAgp46, VA-lip-siRNArandom or PBS ($n = 10$ per group). The injections were administered at a daily dose of 0.75 mg/kg siRNA given five times on every other day followed by 6 times twice a week. (**b–i**) Livers and blood samples were obtained from BDL rat at day 45 (**b–e**) and day 67 (**f–i**) ($n = 10$ per group). Representative photomicrographs of Azan-Mallory and collagen I–stained liver sections at day 45 (**b**) and day 67 (**f**). Scale bars, 100 mm. Percentage of the collagen I–staining positive area in the livers obtained form day 45 (**c**) or day 67 (**g**) of BDL rats. Data were obtained from six randomly selected low power fields of liver specimens of ten rats of each group (VA-lip-siRNAgp46, VA-lip-siRNArandom, PBS treated and normal rat) and represent the mean ± s.d. Hydroxyproline content in the livers obtained form day 45 (**d**) or day 67 (**h**) of BDL rats. Data were obtained from ten rats in each group and represent the mean ± s.d. Total bilirubin and hyaluonate levels in the sera of rats obtained form day45 (**e**) or day 67 (**i**). Values are the mean ± s.d. for each group ($n = 10$ per group). $^*P < 0.05$ versus VA-lip-siRNAgp46-treated-BDL rat.

persisted as evidenced by marked bile duct proliferation accompanied by fibrosis in PBS- or VA-lip-siRNArandom–treated groups.

DISCUSSION

The inadequate target specificity of most approaches to treat liver fibrosis has limited their suitability for clinical use[17]. Our two-pronged strategy to ensure specificity involves, first, targeting a collagen-specific chaperone molecule (gp46) with siRNA and, second, delivery of siRNA specifically to collagen-producing liver cells, using vitamin A–coupled liposomes. This enabled us to resolve hepatic collagen deposition in rat models involving induction of cirrhosis by either DMN or CCl_4 treatments or bile duct ligation. Survival of DMN-treated rats was prolonged in a dose-and duration-dependent manner, indicating a biologically specific effect of siRNAgp46 treatment.

Off-target effects[18] and immune responses, such as induction of interferon (IFN-α) driven by the interaction with Toll-like receptor (TLR)3 or TLR 7/8 (ref. 15), are two issues often associated with the use of siRNA that can lead to misinterpretation of siRNA experiments. We used three independent siRNAs against the same target (gp46) mRNA and found comparable gene silencing efficacy and antifibrotic effects *in vivo* (**Fig. 1a** and **Supplementary Fig. 7**), suggesting that the phenotype observed with downregulation of gp46 was indeed related to gp46 knockdown and not a bystander effect of the siRNA sequence. To circumvent immune responses such as induction of IFN-α, we used siRNA with a 2-nucleotide 3′ overhang, shown to impair activation of the transcription factor IRF3 (ref. 19). In addition, our siRNA did not contain the 5′ triphosphate of the T7-transcript (manufacturer's information), which reportedly plays a role in IFN induction[20]. In fact, there was no elevation of either IFN-α mRNA in the liver or TNF-α and IL-12 in the circulation of rats treated with VA-lip-siRNAgp46. However, as the modifications of siRNA described above may not prevent the triggering of all immune responses, it is possible that the low immune activation in the present investigation was relevant to the RBP receptor–mediated uptake of siRNA, in addition to the modifications of siRNA structure.

Although specificity is important, another critical factor in the use of siRNAs as therapeutic agents is the efficacy of suppression of the target molecule. As synthetic RNA duplexes 25–30 nucleotides in length are up to 100-fold more potent than corresponding conventional 21-mer siRNA[21], we used a 27-nucleotide RNA duplex with 2-nucleotide 3′ overhangs.

Another factor influencing the efficacy of siRNA treatment is the duration of gene silencing, which is mainly governed by dilution resulting from cell division. In rapidly dividing tumor cells, recovery of protein levels suppressed by siRNAs to pretreatment levels occurs within <1 week, whereas in slow-dividing fibroblasts or in nondividing hepatocytes, it takes >3 weeks[22]. We confirmed that levels of gp46

in HS cells treated with siRNA remained suppressed for at least 72 h *in vitro* and *in vivo* and used 48–72 h as the siRNA injection interval to demonstrate *in vivo* efficacy.

The *in vivo* siRNA dose used in our study (0.75 mg/kg per single injection) was less than doses previously shown to have *in vivo* therapeutic effects[23–25]. This may be related to the use of vitamin A–coupled liposomes to preferentially deliver siRNAgp46 to HS cells. It has long been debated whether transfer of retinol from plasma RBP/retinol complex to cellular RBP in HS cells is receptor driven or proceeds by passive diffusion[26]. Findings from most recent studies are consistent with receptor-driven uptake of retinol by HS cells[27–30], although coexistence of direct positioning into the hydrophobic part of cell membranes and receptor-mediated uptake has not been entirely ruled out[31].

Consistent with the receptor-based theory, specific uptake of vitamin A–coupled liposomes by HS cells after binding to plasma RBP was shown in *in vitro* studies assessing RBP concentration–dependence, the effect of RBP antibody (**Fig. 1e,f**), and subcellular localization (**Fig. 1h**). Notably, siRNA-FAM fluorescence in the cirrhotic liver of rats injected intravenously with vitamin A–coupled liposomes was mainly observed in areas with HS cells (identified by α-SMA staining) but not in parenchymal areas. Along with the finding that the area where FAM-florescence and HS cells were merged was markedly greater than in rats treated with vitamin A–free liposomes, this is also compatible with the notion that vitamin A–coupled liposomes are specifically taken up into HS cells by RBP receptors. The observation that liver fibrosis underwent regression and that survival time was significantly prolonged only in animals treated with vitamin A–coupled liposomes carrying siRNAgp46 further supports the vitamin A–receptor theory (Breslow-Gehan Wilcoxon test, $P < 0.0001$).

Incidentally, our results also confirm that activated HS cells, which become free of vitamin A deposition during the activation process[1], take up vitamin A as effectively as resting HS cells, which store vitamin A[32]. Both an activated HS cell line (LI90) and primary rat liver HS cells activated by *in vitro* cultivation[33] expressed markedly enhanced FAM fluorescence when they were incubated with vitamin A–coupled liposomes instead of vitamin A–free liposomes (**Fig. 1e,g**).

VA-lip-siRNAgp46-FAM distribution was negligible in retina, presumably because the eye is an isolated system with a strong blood-retinal barrier[34]. It was slightly evident in spleen and liver, which showed uptake of VA-lip-siRNAgp46 in a vitamin A receptor–independent manner to the reticuloendothelial system (macrophages), although these cells are not primarily collagen-producing cells and therefore should not be affected by nonspecific uptake of siRNAgp46.

When organ distribution of radiolabeled vitamin A–coupled liposomes was examined, prominent uptake of radioactivity was seen only in cirrhotic livers. The radioactivity in other organs was essentially the same in both DMN-treated and normal rats, which indicated that the delivery of vitamin A–coupled liposomes was indeed specific to HS cells possessing RBP receptors, and that delivery to other tissues occurred in a nonspecific manner, probably through nonspecific engulfment by macrophages.

It is also noteworthy that our vitamin A–coupled liposome system for the delivery of siRNA appeared to be quite efficient compared with previously reported methods using conventional liposomes. Our vitamin A–coupled liposomes produced biological effects at a dose of 0.75 mg/kg liposome, whereas in previous reports[23,24,35,36], doses of ~8–160 mg/kg were necessary. Furthermore, most other studies[37] used approximately a 1:10 ratio of solution to total blood volume (that is, 200 μl/2 ml blood in mice; 2,000 μl/20 ml blood volume in rats) to dissolve liposomes containing siRNA for a single bolus injection. However, we used only 200 μl per injection for 200-g rats (1:100 blood volume). This suggests that in our study hydrodynamic pressure did not contribute to *in vivo* transduction of siRNA, whereas in previous studies, siRNAs might have been forcibly transduced by hydrodynamic pressure, at least to some extent. This may explain why background fluorescence for FAM was weak and showed low nonspecific distribution of radioactive vitamin A–coupled liposomes to tissues other than the liver.

Various animal models of liver fibrosis have been explored[4,6,16,38,39]. We chose to focus primarily on the DMN model because it causes progressive and lethal fibrosis, which enabled us to demonstrate prolonged survival resolution of fibrosis. We confirmed these results using both the CCl_4 model, which is nonlethal and has milder features than the DMN model, and bile duct ligation models, which induces chronic cirrhosis by continuous stimulation of bile regurgitation. The consistency of the findings across all three models indicates the applicability of our approach for various types of liver cirrhosis.

In these models, regression of fibrosis occurred after five injections of siRNAgp46, whereas the animals were still being exposed to DMN, CCl_4 or BDL stimulation. The primary mechanism underlying the therapeutic effect was surmised to be the inhibition of collagen secretion by siRNAgp46 (**Fig. 1c,d,i**) and concomitant degradation of predeposited collagen by collagenase activity, which remained as high as in normal liver until fibrosis was almost completely resolved by the five treatments with VA-lip-siRNAgp46 (**Supplementary Table 3**). This finding was consistent with the notion that matrix metalloproteinases, once they are secreted from cells, form a persistent extracellular matrix–associated pool by binding to type I collagen[40,41]. In addition, HS cell apoptosis, possibly resulting from loss of anchorage sites (collagen) is considered to be a secondary mechanism for the therapeutic effect (**Supplementary Fig. 5**). Incidentally, apparent suppression of procollagen I mRNA in siRNAgp46-treated liver (**Fig. 4d**), which should not be caused by siRNAgp46 *per se*, may also be ascribed to the apoptosis of collagen-producing HS cells.

The improvement of serum bilirubin and hyaluronate levels in all three cirrhosis models further substantiates resolution of fibrosis in the portal area. Serum albumin and ALT levels, however, were not significantly improved by VA-lip-siRNAgp46 treatment in DMN-treated rats. This may result from the toxic effect of ongoing DMN treatment, as DMN-treated rats at day 70 showed restoration of normal hepatic architecture (**Fig. 4i**) and nearly complete normalization of serum albumin and ALT levels in addition to serum bilirubin and hyaluronate (**Supplementary Fig. 6**). Therefore, our modality actually reverses liver cirrhosis both histologically and functionally. This underscores its promise for clinical translation to treat liver cirrhosis.

METHODS

Cell lines. Normal rat kidney fibroblast (NRK) cells and LI90 cells (human HS cell line) were obtained from RIKEN cell bank (RIKEN Institute). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) supplemented with 10% (wt/vol) FBS, 1 mM sodium pyruvate and 4 mM L-glutamine at 37 °C in 5% CO_2.

Preparation of siRNAs. Three formulations of siRNA directed against gp46 (GenBank accession no. M69246), a rat homolog of human HSP47, were purchased from Hokkaido System Science. The sense and anti-sense strands of siRNAs were: gp46 (sequence A), beginning at nt 757, 5′-GU UCCACCAUAAGAUGGUAGACAACAG-3′(sense); 5′-P. GUUGUCUACC AUCUUAUGGUGGAACAU-3′ (antisense); gp46 (sequence B), beginning

at nt 1626, 5′-P. CCACAAGUUUUAUAUCCAAUCUAGCAG-3′ (sense), 5′-GCUAGAUUGGAUAUAAAACUUGUGGAU-3′ (antisense); gp46 (sequence C), beginning at nt 1909, 5′-CUAGAGCCAUUACAUUACAUUGACAAG-3′ (sense); 5′-UGUCAAUGUAAUGUAAUGGCUCUAGAU-3′ (antisense). siRNA-random, 5′-CGAUUCGCUAGACCGGCUUCAUUGCAG-3′(sense) and 5′-GCAAUGAAGCCGGUCUAGCGAAUCGAU-3′ (antisense).

For some experiments, gp46 siRNA (sequence A) with 6′-carboxyfluorescein (6-FAM) or cyanine 5 (Cy-5)-coupled to the 5′ end of the sense strand was used. All of these sequences were shown by BLAST search to not share sequence homology with any known rat mRNA. For IFN response experiments, siRNA targeting β-galactosidase bearing a motif eliciting interferon production (siRNAβgal728), obtained from Dharmacon, Inc., was used[15].

Transfection of siRNAs. siRNAs were introduced into NRK cells by the use of Lipotrust (Hokkaido System Science) according to the manufacturer's protocol.

Western blot analysis. Protein extracts of cells or liver specimens were resolved over 4/20 SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, probed with antibodies against HSP47 (gp46) (Stressgen) or β-actin (Cell Signaling), then with peroxidase-coupled antibodies as the secondary antibody (Oncogene Research Product). Lastly, they were visualized with ECL (Amersham Life Science).

Assay for collagen synthesis. Collagen synthesis in NRK cells was assessed by proline incorporation assay[42]. Briefly, 1×10^5 NRK cells were seeded in 6-well plates with culture medium containing 10% FBS. After 24 h, the cells were transfected with 50 nM of siRNAgp46 using Lipotrust and incubated for 24 h. Thereafter, the medium was changed to OPTI-MEM containing [2,3-^{3}H]-l-L-proline (5 μCi/ml, NEN). After 24 h, two 200-μl aliquots of culture supernatant were removed from each well.

Samples of supernatant were used to quantify total newly synthesized [^{3}H]proline-labeled protein production. The amount of noncollagen proteins and collagenase-digestible protein was measured in the second aliquot using collagenase (*Clostridium histolyticum*, Sigma). The radioactivity was measured by scintillation counting. The amount of collagen present in each culture supernatant was determined by subtracting the counts per minute (c.p.m.) obtained from the aliquot used to measure noncollagen protein from the c.p.m. obtained from the aliquot used to measure total protein. Data are expressed as the percent of collagen produced relative to control cultures.

Preparation of VA-coupled liposomes carrying siRNAgp46. Cationic liposomes (Lipotrust) containing *O,O′*-ditetradecanoyl-*N*-(α-trimethylammonioacetyl) diethanolamine chloride (DC-6-14) (**Supplementary Fig. 2a**) as a cationic lipid[43], cholesterol and dioleoylphosphatidylethanolamine at a molar ratio of 4:3:3 (which has shown high transfection efficiency under serum-containing conditions for *in vitro* and *in vivo* gene delivery[43,44]) were purchased from Hokkaido System Science. The liposomes were manufactured using the freeze-dried empty liposomes method described previously[43] and prepared at a concentration of 1 mM (DC-16-4) by addition of double-distilled water (DDW) to the lyophilized lipid mixture under vortexing before use. To prepare VA-coupled liposomes, 200 nmol of vitamin A (retinol, Sigma) dissolved in DMSO was mixed with the liposome suspensions (100 nmol as DC-16-4) by vortexing in a 1.5 ml tube at 25 °C. To prepare VA-coupled liposomes carrying siRNAgp46 (VA-lip-siRNAgp46), a solution of siRNAgp46 (580 pmol/μl in DDW) was added to the retinol-coupled liposome solution with stirring at 25 °C. The ratio of siRNA to DC-16-4 was 1:11.5 (mol/mol) and the siRNA to liposome ratio (wt/wt) was 1:1. Any free vitamin A or siRNA that was not taken up by liposomes were separated from liposomal preparations using a micropartition system (VIVASPIN 2 concentrator 30,000 MWCO PES, VIVASCIENCE). The liposomal suspension was added to the filters and centrifuged at 1,500*g* for 5 min 3 times at 25 °C. Fractions were collected and the material trapped in the filter was reconstituted with PBS to achieve the desired dose for *in vitro* or *in vivo* use.

Isolation of rat HS cells and skin fibroblasts. Rat HS cells were isolated by digestion with pronase-collagenase followed by centrifugation in a Nycodenz gradient, as described previously[45]. The cells were then cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. After 2 d, cell debris and nonadherent cells were removed by washing and the medium was changed every 2 or 3 d thereafter. The purity was assessed by microscopy, by intrinsic vitamin A autofluorescence and by immunocytochemistry using a monoclonal antibody against desmin (1:25, Dako). Cell viability was examined by Trypan blue exclusion. Both the cell purity and viability exceeded 95%. Cells cultured for 10 d after isolation were regarded as activated HS cells[33]. Rat skin fibroblasts were obtained according to the method described previously[46].

Quantification of collagen production. Rat pHS cells or NRK cells were plated into 6-well tissue culture plates at a density of 1×10^5 per well in DMEM with 10% FBS. After 24 h culture (day 0), NRK cells were transfected with 50 nM of siRNA using Lipotrust and Rat pHS cells were treated with VA-lip-siRNAgp46 (50 nM of siRNA) or VA-lip-siRNArandom (50 nM of siRNA). On day 1, cells were washed and collagen deposited in the wells was stained with Sirius red dye (Biocolor), as previously described[13]. Unbound dye was removed by washing and the bound complex dissolved in 0.5% sodium hydroxide. Collagen was quantified by spectrophotometry at 540 nm and results were expressed as a percentage of the untreated controls.

FACS analysis of VA-lip-siRNAgp46-FAM. Rat pHS cells or primary skin fibroblast (1×10^5 cells) were treated with VA-lip-siRNAgp46-FAM (50 nM of siRNA) in the presence of various concentrations of RBP (0.07–1.4 μg/ml, SCIPAC) and were cultivated for 30 min. For the blocking assay, 1×10^5 cells were treated with mouse anti-RBP antibody (10 μg/ml, BD Pharmingen) or negative control mouse IgG1 (10 μg/ml, Dako) for 30 min before adding VA-lip-siRNAgp46-FAM. LI90 cell were treated with VA-lip-siRNAgp46-FAM in the presence of 10% FBS and were cultivated for 30 min. The MFI of VA-lip-siRNAgp46-FAM–treated cells was assessed on a FACScalibur with CellQuest software (Becton Dickinson).

Intracellular distribution analysis of VA-lip-siRNAgp46-FAM. Rat pHS cells were plated in Lab-Tek chambered cover glasses at 1×10^4 cells/chamber. VA-lip-siRNAgp46-FAM or lip-siRNAgp46-FAM was added to cells at a final siRNA concentration of 50 nM. Cells were cultured in DMEM containing 10% FBS for 30 min, then the medium were replaced with fresh medium. At 30 min and 2 h post-treatment, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at 25 °C. After fixation, the cells were washed three times with PBS and were exposed to Prolong Gold Antifade Reagent with DAPI (Molecular Probes) for 1 min to stain nuclei. The subcellular localization of FAM-labeled siRNAgp46 was assessed using fluorescent microscopy (Keyence, BZ-8000).

Treatment of animals. Male Sprague-Dawley rats (Charles River) 4 to 5 weeks old were used. All animal procedures were approved by the Sapporo Medical College Institutional Animal Care and Use Committee. Rats were anesthetized with sodium pentobarbital (40 mg/kg body weight, intraperitoneally) and exsanguinated via the inferior vena cava.

Induction of DMN liver cirrhosis. Rats were given 1% DMN dissolved in saline (1 ml/kg body weight) intraperitoneally for 3 consecutive days per week until day 24 to induce liver cirrhosis, and in some experiments, for another 3 weeks to maintain cirrhosis[38] (**Supplementary Fig. 4**).

***In vivo* localization of VA-lip-siRNAgp46-FAM in rat liver and other organs.** DMN-treated cirrhotic rats (day 24) were injected intravenously with 1 μl/g body weight of VA-lip-siRNAgp46-FAM or lip-siRNAgp46-FAM. The injections were administered under normal pressure at a daily dose of 0.75 mg/kg siRNA given three times every other day. At 24 h after the last injection, the rats were killed by saline perfusion, and the liver and other organs (lung, spleen and retina) were harvested. Tissue specimens were immediately embedded in OCT medium and cryogenically sectioned. Sections were mounted on slides, fixed in 4% paraformaldehyde washed with PBS, permeabilized with 0.2% Triton X-100 in PBS and blocked with 1% BSA in PBS.

For the immunofluorescence study, slide-mounted liver sections were treated with monoclonal anti-α-SMA-CY3-conjugated antibody (1:400, Sigma). Other tissue sections were treated with anti-CD68 antibody (1:100,

DAKO) prelabeled with the Alexa Fluor 568 Mouse IgG_1 Labeling Kit (Molecular Probes) for 2 h at 25 °C. All slides were washed with PBS and exposed to Prolong Gold Antifade Reagent with DAPI for 1 min to stain nuclei. Multicolored fluorescent staining of tissues was analyzed by confocal laser scanning microscopy using a Radiance 2100 Rainbow (Bio-Rad) equipped with a Zeiss Axioskop 2 fluorescent microscope (Carl Zeiss MicroImaging). The fluorescence signals of liver sections were video-digitized and analyzed with a software program that automatically outlined the total stained areas with threshold settings (Photoshop 4.0; Adobe). These areas were then quantified with NIH Image 1.62 software, and the transduction efficiency was calculated from the ratio of the merged yellow color area to the total α-SMA-stained area in each section. The areas were assessed in ten randomly selected high-power fields (× 630) per specimen from each liver.

VA-lip-siRNAgp46-Cy5 delivery to different cell types in the liver. DMN-treated cirrhotic rats (day 24) were injected intravenously with VA-lip-siRNAgp46-Cy5 or lip-siRNAgp46-Cy5. The injections were administered under normal pressure in a volume of 1 μl/g body weight at a daily dose of 0.75 mg/kg siRNA given 3 times, every other day. At 24 h after the last injection, HS cells, Kupffer cells, and hepatocyte-rich fractions of rat livers were obtained by the pronase-collagenase method[47] and stained with anti-α-SMA FITC (Sigma), anti-rat CD168-FITC (Serotec) and anti-rat albumin-FITC (Cedarlane Laboratories), respectively. To detect intracellular albumin and α-SMA, cells were pretreated with Perm Buffer I (BD Biosciences). Cells were then analyzed by flow cytometry.

Tissue distribution and pharmacokinetics of radiolabeled VA-lip-siRNAgp46. Radiolabeled VA-lip-siRNAgp46 was prepared by incorporating[^{3}H]retinol ([11,12-^{3}H (N)]: 44.4Ci/mmol, Perkin Elmer) into liposomes as described above for preparation of VA-coupled liposomes carrying siRNAgp46. [^{3}H]VA-lip-siRNAgp46 (200 μCi) was administered via the tail vein under normal pressure in either DMN-treated cirrhotic rats (day 24) or normal rats and blood was collected via tail vein nick over a 24-h period. After 24 h, the rats were killed under anesthesia, the tissues were harvested, and 50-mg samples were transferred to glass scintillation vials and solubilized with Solvable (Perkin Elmer). Solubilized tissues were assayed for radioactivity by liquid scintillation counting with Hionic-Fluor (Perkin Elmer).

Quantitative RT-PCR. Total RNA was isolated using RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Total RNA (1 μg) were used for reverse transcription in total volume of 20 μl with the cDNA cycle kit (Invitrogen). All TaqMan primers mixed with probes were purchased from Applied Biosystems. Primers for the experiment were as follows: gp46 forward 5′-TTTAAGCCGCACTGGGATGAGAAGT-3′, reverse 5′-TGGGGACACCT TAGCATC-3′; procollagen, type I, alpha 1 (COL1A1), forward 5′-TAAGGGT GACAGAGGTGATGCTGGT-3′, reverse 5′-TGGGGACACCTTAGCATC-3′; tissue inhibitor of metalloproteinase 1 forward 5′-ACTCGGACCTGGTTA TAAGGGCTAA-3′, reverse 5′-TGGGGACACCTTAGCATC-3′; IFN-α forward 5′-AGAGCAGAAGTGTGGAGAGCCCTGT-3′, reverse 5′- ACAGGGCTCTC CACACTTCTGCTCT-3′; GAPDH, forward 5′-AACCCATCACCATCTTCCAG GAGCG-3′, reverse 5′-CATCGAAGGTGGAAGAGTGG-3′. GAPDH was used as a control for RNA integrity. The TaqMan reactions were performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). The results were expressed as the ratio of the number of copies of the product gene to the number of copies of the housekeeping gene (GAPDH) from the same RNA (respective cDNA) sample and PCR run.

***In vivo* siRNAgp46 treatment.** To evaluate the effect of siRNAgp46 treatment on liver cirrhosis, we conducted two sets of experiments. In the first set of experiments, 24 groups of rats were used to determine the dose- and duration-dependent effect on survival. From day 24 of DMN administration, when serious liver cirrhosis was established (**Supplementary Fig. 4**), four treatment groups of rats were injected intravenously with VA-lip-siRNAgp46 at siRNA doses of 0.1, 0.5 or 0.75 mg/kg twice a week ($n = 6$ for each group), or 0.75 mg/kg three times a week ($n = 12$ for each group).

The 20 control groups received injections of lip-siRNAgp46, VA-lip-siRNA random, vitamin A–coupled liposome, vitamin A or PBS using the same dosage schedule as the corresponding four treatment groups. In addition, injections of siRNAgp46 (0.75 mg/kg three times a week) were also performed ($n = 12$).

In a second set of experiments, six groups of rats ($n = 12$ for each group) were used for histological and liver function evaluations. Each group was treated with PBS, vitamin A alone, vitamin A–coupled liposome, lip-siRNAgp46, VA-lip-siRNArandom or VA-lip-siRNAgp46 (0.75 mg/kg siRNA) three times a week. All injections were given via the tail vein under normal pressure in a volume of 1 μl/g body weight. The livers of rats were fixed in 10% paraformaldehyde, and paraffin-embedded sections were stained with Azan-Mallory stain or with anti-collagen I antibody (1:100, Abcam) using the EnVision system (Dako), according to the manufacturer's instructions, followed by the DakoLiquid DAB (diaminobenzidine) Substrate-Chromagen System kit.

To accurately quantify collagen I-positive areas, slides from six randomly selected low-power fields (×100) per liver section of each rat were viewed by microscopy (Axioplan 2; Carl Zeiss, Inc.). Digital pictures were captured through a video archival system using a digital TV camera system (AxioCam High Resolution color, Carl Zeiss, Inc.). An automated software analysis program (KS400, Carl Zeiss, Inc.) was used to determine the percentage of stained areas in the digital photomicrographs.

For the immunofluorescence study, slide-mounted liver sections were treated with anti-gp46 antibody (1:250, Stressgen) prelabeled using an Alexa Fluor 568 Mouse IgG_{2b} Labeling Kit (Molecular Probes). When rats were killed, blood samples were also drawn and assessed for serum bilirubin, hyaluronate, albumin and ALT by standard procedures. For assessment of histological staging, a semiquantitative scoring (scores 0 to 6) was performed as described previously[48] by an independent hepatologist (Board Certified Hepatologist of the Japan Society of Hepatology) in a blind fashion using liver preparations from 12 rats from each group.

Hydroxyproline content assay. Hydroxyproline content was determined by the Jamall methods as previously reported[49]. Briefly, liver tissue (300 mg) was homogenized in 6N HCl and hydrolyzed at 110 °C for 18 h. Twenty-five microliter aliquots were dried at 60 °C. The sediment was dissolved in 1.2 ml of 50% isopropanol and incubated with 200 ml of 0.56% chloramine T Solution (Sigma) in acetate citrate buffer pH 6.0. After incubation for 10 min at 25 °C, 1 ml of Ehrlich's reagent was added and the mixture was incubated at 50 °C for 90 min. After cooling, the absorbance was measured at 560 nm.

VA-lip-siRNAgp46 treatment of CCl_4-induced rat liver fibrosis. Liver cirrhosis was generated by 8-week treatment of adult male Sprague–Dawley (200 g) rats with CCl_4 (CCl_4 in olive oil, 1:1 (vol/vol) per kg body weight by intraperitoneal injection twice weekly) as previously described[4]. At day 58 of CCl_4 treatment, rats were treated with VA-lip-siRNAgp46, VA-lip-siRNArandom (0.75 mg/kg siRNA, two times a week) or PBS alone 2 times a week. At day 76 of CCl_4 treatment, all rats were killed and liver and serum samples were prepared. Immunohistochemical evaluations, liver function evaluations and hydroxyproline content assay were performed essentially as described in the previous section.

VA-lip-siRNAgp46 treatment of BDL-induced rat liver fibrosis. Biliary hepatic fibrosis was induced by ligation of the common bile duct of male Sprague–Dawley (200 g) rats as previously described[16]. In brief, the common bile duct was identified and double ligated with 3-0 silk ligatures. Five weeks after BDL, rats were assigned to 1 of 3 treatment groups: (i) VA-lip-siRNAgp46, (ii) VA-lip-siRNArandom or (iii) PBS alone. The injections were administered under normal pressure in a volume of 1 μl/g body weight at a daily dose of 0.75 mg/kg siRNA given five times every other day followed by six times twice a week. At day 45 and 67 of BDL, rats ($n = 10$ per group) were killed and liver and serum samples were prepared for immunohistochemical evaluations, liver function evaluations and hydroxyproline content assay as described in the previous section.

Statistics. Results are presented as means (± s.d.) for each sample. Multiple comparisons between control groups and other groups were performed by Dunnnet's test. Survival curves were constructed according to the Kaplan-Meier method and tested by Breslow-Gehan-Wilcoxon analysis.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

Y.S., K. Murase and J.K. designed research, performed experiments and wrote the paper. M.K., T.S., Y.K., R.T., K.T., K. Miyanishi, T.M. and T.T. performed experiments. Y.N. designed research, wrote the paper and supervised the whole project. All authors discussed the results and commented on the manuscript.

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【総　　説】

機能性食品の安全性と有効性の評価

Evaluating Safety and Efficacy of Functional Foods

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【要　旨】
本論文では，機能性食品の安全性と有効性の評価について，日本の現状と私の考え方を記した．食品の安全性では食経験が重要であるが，機能性食品が通常の食品と比して投与量や濃度が著しく異なる場合には，過去の食経験だけでは安全とは言い難く，医薬品同等の対応が必要と考える．機能性食品の有効性の探索には観察的疫学知見を重視すべきであり，その証明には無作為割付臨床試験が重要である．

【キーワード】
機能性食品，無作為割付臨床試験，食経験，食物繊維

機能性食品とは

数十年前まで，食品の機能は主に栄養面のみを研究されていたが，1980年代頃より食品の機能について，栄養を第一次機能，嗜好を第二次機能，生体機能調整を第三次機能に分類し，特に第三次機能について特化した食品を機能性食品と称する考えが提唱され，第三次機能の研究が盛んになってきた．機能性食品の研究の進展により1991年には特定保健用食品の制度が作られ，効果を示すための規定が設けられた．

第三次機能を担う食品成分も食品に含まれるものであり，法的にも食品として扱われてきた．最近になり，機能をより高めるために，有効成分と考えられる成分を取りだして濃度を高めたり，通常では食品として供されていない部分を用いたり，食経験のほとんどない動植物やその他の物質まで用いるようになってきた．その結果，食品とは称することが困難と思われる物なども食品として市場に出回ることになり，安全性や有効性について多くの問題点が指摘されるようになってきた．

ここでは，機能性食品の安全性と有効性の評価についての現状を紹介するとともに，私の機能性食品に対する研究方針を述べる．

I. 機能性食品の安全性評価

1. 機能性食品の安全性評価の現状

医薬品が人に投与されるためには，きわめて多くの試験により安全性を確認する必要がある（表1）．試験には，細胞を用いたin vitro試験から，動物を用いたin vivo試験まで数多くの試験がある．このような試験を経て，初めて人への投与試験が行われ，それらの試験によりヒトにおける安全性と副作用を把握してから市販される．

それに対して，食品は食品衛生法に基づく規制以外には，ヒトに投与する際の規制はない．医薬品では厳しい規制があるのに対して，食品にそのような規制がないのは，食品には膨大でかつ長期間の食経験があるからである．これまで人類が摂取してきた膨大な食経験により安全性は担保されているのである．

2. 機能性食品における食経験は信用できるのか

食経験があると言っても，その食経験の期間が短かったり，きわめて遠い過去にしか摂取されていなかったりした場合には，食経験により安全性が担保されたとは言い難い．また，十分な食経験のある食品であっても，摂取量や摂取方法が異なったり，一部の成分だけを抽出したりしたものであれば，その元になった食品の食経験をもってそれらが安全と言うには問題があろう．しかし，これらについての法規制はない．この点が機能性食品の

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表 1 医薬品と食品の安全性評価の違い

医薬品	食　品
構造式決定と有害作用の理論的検証	膨大でかつ長期間の食経験
	味覚
細胞やレセプターを用いた in vitro 試験	食欲
in vitro 毒性試験	
変異原性試験	食品衛生法に基づく規制
染色体異常試験	有害物質・異物のないこと
動物やヒト組織を用いた ex vivo 試験	微生物汚染のないこと
動物実験に投与する in vivo 試験	指定外添加物のないこと
安全性薬理試験	食品・添加物等の規定基準の遵守
単回投与毒性試験	
反復投与毒性試験	
特殊毒性試験	
遺伝毒性試験	
発癌試験・発癌抑制試験	
生殖発生毒性試験	
薬物動態試験	
第 I 相単回投与試験	
第 I 相反復投与試験	
第 II 相臨床試験（探索的臨床試験）	
第 III 相臨床試験（検証的臨床試験）	
第 IV 相臨床試験（市販後臨床試験）	

安全性について，最も危惧されるところである．

このように，現在，機能性食品として開発されている製品の多くは食経験だけで安全性を担保することは困難と考えられるため，特定保健用食品では，変異原性試験や染色体異常試験，急性毒性試験，亜急性毒性試験，ヒト過剰摂取試験（28 日），ヒト長期摂取試験（90 日）などが義務づけられている．しかし，医薬品で求められるような慢性毒性試験や市販後臨床試験などは義務づけられておらず，医薬品並みの十分な安全性を確保しているとは言い難い．

また，食品として販売されているため，消費者も薬を摂取する時ほどには副作用に敏感になっておらず，副作用の把握が遅れる心配もある．

食経験による安全性の担保を補助するものとして，味覚や食欲はきわめて重要な仕組みと私は考えている．ヒトも動物であるので，食欲や味覚を駆使して体に必要で安全な物を摂取している．腐った物や毒物は，異様な味のため摂取しないですむし，食欲に関しても，今，体が必要な物を判断する能力の一つと考える．しかし，健康に良いとか，病気が治るなどの情報があれば，美味しくないものや食べたくないものも無理をして摂取することになる．従って，そのような付加情報を持つ食品は，ヒトが安全を保つための仕組みである味覚や食欲の機能を破壊していることになるため，そのような付加情報を持つ食品はより一層の安全性を確保しておく必要があろう．

3. 機能性食品の安全性確保のために

これまでに書いたように，食経験が十分でない動植物を用いる場合や，食経験を持つ食品であってもその成分を高濃度に抽出したり，加工法を変えたりする場合には，医薬品同等の試験を行うことが望まれる．しかし，現実には，医薬品と同じ条件の試験は行われていない．

現状に即した対応としては，企業の安全性確保のモラルの向上，消費者からの苦情に対する適切な対応とそれらの事例の公表，国民生活センターなどを用いた有害事象の集計とその公表などが必要と考える．大学や研究所の研究者は，どうしても機能性食品の有効性についての研究が中心になることが多いが，有害事象に関しても注意してデータを収集して公表をすることも重要であろう．

II. 機能性食品の有効性評価

1. 食品の持つ機能はどのようにして見いだされるのか

栄養面，嗜好面以外の機能を食品が持つことを発見するきっかけは様々であるが，多くは観察的疫学研究，体験談，研究者や生産者の発案などから見いだされる．こ

表 2　機能性食品の有効性評価

疫学的研究
〈観察的疫学研究〉
I　記述疫学
国別比較，癌登録，経時的変化など
II　分析疫学
症例対照研究
コホート研究
〈実験的疫学研究〉
III　介入疫学
単群投与試験
用量設定試験
無作為割付臨床試験
a. 非盲検試験
b. 二重盲検試験
a. 中間代理指標をエンドポイントとする試験
b. 疾病発生をエンドポイントとする試験
基礎研究
機序仮説の検討
細胞やレセプターを用いた in vitro 試験
動物やヒト組織を用いた ex vivo 試験
動物実験に投与する in vivo 試験

れらのきっかけから食品に機能が期待された場合，その有効性を科学的に評価するためにいろいろな研究が行われる（表 2）．これらの研究も，安全性評価と同様に医薬品の開発を模して行われることが多いが，医薬品の有効性評価と大きく異なるのは，機能性食品の有効性評価では，観察的疫学研究が重視されることである．医薬品では，市販後の適応拡大研究などにおいて観察的疫学研究手法が用いられることがあるが，新薬は摂取経験がないので観察的疫学研究は当然できない．

有効性評価に関する基礎的な研究については，医薬品と機能性食品とはよく類似している．まず，有効性機序の仮説を立案し，その仮説を検証するための基礎的な研究を行う．細胞や動物を用いた研究により，様々な生物学的指標が仮説通りの動きを示すかどうかを検討する．そして，疾患モデル動物を用いた有効性評価を行う．ここで注意が必要なのは，疾患モデル動物での反応が，ヒトでの反応と異なることが起こりえることである．その傾向は，医薬品よりも食品でより顕著かもしれない．なぜならば，動物により食事内容は大きく異なっており，機能性食品は，本来，ヒトが摂取しているものであるので，通常では食していないモデル動物では，ヒトにおける反応とは異なる可能性が考えられるからである．実際，発癌物質と発癌予防候補物質を投与してその予防効果を調べる実験系において，様々な物質の発癌予防効果が検討されてきた．この実験系で発癌予防効果が期待される物質が多数発見されているが，ヒトで同じ効果が証明されたものはきわめて少ない．

さらに，ヒトでは有効でない物質が有効と判断される危険性だけではなく，ヒトでは有効な物質が動物実験では有効でないと判断される危険性もあるため，疾患モデル動物による研究結果の解釈は，慎重にすべきである．

これらより，機能性食品の開発に関しては，観察的疫学研究を重視するのが良いと考える．その結果，有効性が強く期待されれば，介入疫学研究を実施することになる．この介入疫学研究も医薬品開発で行われる臨床試験に類似している．ヒトに投与する試験であるので，安全性に関しては十分に確認した上で実施することが重要である．

2. 単群投与試験

観察的疫学研究や動物実験による基礎研究により，有効性が期待され，安全性も高いと判断された場合，ヒトに投与してその効果をみる試験が行われる．一般には候補食品を少数例に短期間だけ投与して有効性の感触を確かめることから行うであろう．しかし，少数例に短期間だけ投与したような試験は，無作為割付試験を行う前のパイロット試験的な情報としては有用であるが，消費者にその効果を公表できるような確実性の高い知見にはなり得ない．なぜならば，このような検討では，少数例のため偶然の可能性が高いこと，有効と感じたヒトだけがその印象を述べるバイアスが発生しやすいこと，異常のあったヒトを対象とすると正常に向かう平均値への回帰現象が起こること，摂取していなくても自然に改善していたかもしれないこと，摂取しているという気持ちの影響を受けること（プラシーボ効果），判定者（医師）の判断に恣意が入る可能性があること，などにより本当は効果のないものでも効果があるように示される可能性があるからである．

また，癌の治療効果などでは，診断や治療内容が正確に患者に伝わっていない可能性についても注意する必要がある．「末期の癌がいわゆる健康食品により消失した」などの体験談では，本当は癌でなかったり，抗癌剤を併用していたりする可能性があり得る．また，進行癌であっても，自然消失したり進行がきわめて遅かったりすることもあり，そのような症例の患者がいわゆる健康食品を摂取していた時に，効果があったように勘違いすることもある．進行癌患者でいわゆる健康食品を服用していても，死亡した患者の声は販売者には伝わらないので，販売者には効果があったように感じやすいことも注意が必要である．

効果をみる最初の投与試験は，単群投与試験になるこ

とが多いが，上述するような誤った判断をしないために，投与した対象者全例を確実に登録することや，第三者委員会などを設置して監査ができる体制を設けることなどが必要である．

そのような工夫を行っても，プラシーボ効果や自然経過の影響などは取り除くことはできないため，無作為割付臨床試験を行うことが重要である．

3. 無作為割付臨床試験

無作為割付試験は二重盲検試験で行うことが望ましいが，食品の場合，機能を期待する候補食品と外見上見分けがつかない機能を持たない食品（無機能食品）を作成するのが困難なことが多い．また，有効成分が分かっていない時には，機能を持たないと考えて作成した食品にも機能を持つ可能性もある．どうしても無機能食品を作成するのが困難な場合には，無作為割付して非投与群になった参加者には候補食品を投与しない方法を用いるしかないが，その場合でもエンドポイントの評価者には，なるべくどちらの群に入っているか分からないようにして判定したり，生物学的指標など客観的な指標をエンドポイントとして採用したりするなどの工夫が必要である．

エンドポイントは，目標とする疾病の発生をエンドポイントとすることが望ましいが，疾病の発生をエンドポイントとした場合，目標症例数や試験期間が膨大になることも多く，倫理的問題も発生するため，適切な中間代理指標を用いることも多い．具体的には，心筋梗塞に対する血中コレステロール値，心血管疾患に対する血圧，大腸癌に対する大腸腺腫，前立腺癌に対する血中PSA値などである．中間代理指標を用いる際に重要なことは，その中間代理指標が疾病発症を予測する指標であり，それを変化させることにより将来の疾病発症も減少することが証明されていることがある．中間代理指標が疾病発症の指標になり得ることを，機序の面からも論理的に説明できることが望ましい．

機能性食品の効果をみるための臨床試験を開始するにあたり，最も重要な情報は安全性に関する情報である．前述したように，医薬品開発のための臨床試験では，きわめて詳細な安全性試験が実施されるが，機能性食品の臨床試験においては食経験があるとの理由で，医薬品のような厳密な安全性試験は行われないことが多い．安全性のところで記したように，機能性食品はきわめて高濃度にしたり，食経験の少ないものを用いたりすることも多いため，安易に食経験に頼らず，必要に応じて医薬品同等の安全性把握をすることが必要である．

以下に，食物繊維の大腸癌予防効果の評価を例に挙げて，具体的な機能性食品の有効性評価方法を紹介する．

III. 食物繊維による大腸癌予防効果の評価

1. 食物繊維と大腸癌の観察的疫学研究

食物繊維と大腸癌の関係については，英国の外科医である Burkitt がアフリカの人々に大腸疾患が少ないのは多くの食物繊維を摂取しているから，と考えた[1] のが最初とされている．

その後の検討により，各国の食物繊維の摂取量と大腸癌の発生率に負の相関があることが報告された．また，本邦では食物繊維の摂取量の減少とともに大腸癌が増加している．しかし，これらの記述疫学による知見だけでは食物繊維と大腸癌の関係を確定することはできない．なぜならば，国や時代が異なれば，食物繊維だけではなく，その他のいろいろな因子も異なり，どの変化が大腸癌の発生に影響を与えているか分からないからである．観察的な方法で，食物繊維が大腸癌の発生を予防している知見の確かさを高めるためには，分析疫学研究である症例対照研究やコホート研究を行う必要がある．

症例対照研究では，大腸癌に罹患した患者とそれに対応する健常な対照者を抽出し，それらの過去の食物繊維の摂取量をアンケートして比較する方法が考えられる．そのような方法で行われた多数の症例対照研究の成績を集計した検討では，食物繊維の摂取は大腸癌の発生に対し予防的に働くことが報告されている[2]．しかし，症例対照研究は，対照集団の設定による影響や，思いだし調査の不確かなど，得られた成績の信頼性が低い．それに対して，十分な規模で行われ高度に精度管理されたコホート研究の結果はより信頼性が高い．

コホート研究は，数万人の集団に対して食事調査を行い食物繊維の摂取量を把握した後に，数十年間にかけて大腸癌の発生を調査し，大腸癌が発生した者と発生しなかった者のエントリー時点での食物繊維の摂取量を比較する方法であり，これにより得られた結果は，信頼性が高いと考えられている．しかし，コホート研究は，費用が膨大にかかり，結果が出るのに長期間が必要なため，あまり多くの研究はされていなかったが，最近になり，いくつかのコホート研究の結果が報告されるようになった．

1999 年に報告された研究[3] は，34 歳から 59 歳の米国の看護師 88,757 人に対して半定量食事頻度調査票による食事調査を行い，その後，16 年間，追跡した研究である．追跡期間中に 787 例の大腸癌と 1,012 例の大腸腺腫が診断されたが，食事調査で把握した食物繊維の摂取量と大腸癌，大腸腺腫の発生に明らかな関係はみられなかった．

さらに，この集団と47,325人の男性医療専門家を対象に，野菜や果物に注目しても解析した報告[4]や，カナダの女性45,491人を追跡した研究[5]でも，野菜や果物を多く摂取しても大腸癌の発生を予防する傾向はみられなかった．これらのコホート研究の報告や，後に紹介する介入試験の結果から，食物繊維は大腸癌を予防することはできないと考えられるようになった．しかし，2003年に別の大規模なコホート研究により食物繊維が大腸癌を予防することを見出した結果が報告され，再び混乱が始まった．この研究は，ヨーロッパ8カ国による519,978人を最長10年間追跡するきわめて大規模なコホート研究であり[6]，食物繊維摂取量別に5分位に分け，最も摂取量の少ない群に比して最も多い群では大腸癌罹患が0.75と有意に減少していた．

最近，厚労省研究班のコホート研究（JPHC研究）において，食物繊維と大腸癌罹患について報告された[7]．この報告では食物繊維の摂取量と大腸癌リスクに関連は認めなかったが，女性においてきわめて食物繊維の摂取量の少ない群でのみ大腸癌のリスクが上昇する傾向が認められた．このことより食物繊維の摂取量がきわめて少ない場合のみ大腸癌は促進されるが，通常に食事をしている集団では，さらに食物繊維を多く摂取しても大腸癌は予防できないとしている．

しかし，2007年に文科省研究班のコホート研究（JACC研究）においても食物繊維と大腸癌についての結果が報告[8]されたが，それでは食物繊維を多く摂取するほど結腸癌の罹患が減少する傾向を示した．このように，日本で行われた大規模なコホート研究でも結果が一致せず，大腸癌と食物繊維の関係については未だ混乱している．

2. 食物繊維による大腸癌予防介入試験

食物繊維が大腸癌を予防するかどうかを明らかにするもっとも確実な方法は，対象を無作為に2群に分けて，一つの群に食物繊維を投与し，もう一つの群に食物繊維を投与せず，大腸癌の発生の差を確認することである．しかし，大腸癌の発生頻度は低いため，多数を長期間介入する必要があり，また，倫理的にも問題も大きいため，大腸癌の前癌病変と考えられている腺腫の発生を観察する研究が多く行われている．

大腸腺腫や早期大腸癌などの大腸腫瘍を持つ者の大腸は，それらを持たない者の大腸よりも大腸癌の発生率が高いため，大腸癌の予防試験では，大腸癌の高危険度群である大腸腺腫保有既往者が対象にされている．また，食物繊維を多く摂取させる方法には，食事指導により食事内容を変更させる方法と，サプリメント（強化食品）により食物繊維を投与する方法が用いられている．

食物繊維の大腸癌予防の効果を評価するために行われた大規模な無作為割付臨床試験は6つある．McKeown-Eyssenらの研究[9]はカナダで行われた研究であり，対象者は165人である．参加者を，1日50 g以下またはエネルギー比率20%以下の低脂肪食及び1日50 g以上の高食物繊維食の食事指導を行うグループと，通常の西欧食を摂取させるグループの2群に分けた．平均2年間の間隔で大腸内視鏡検査を行い，新たな大腸腺腫の発生を調べたが，2群間に差は見られなかった．

MacLennanらの研究[10]はオーストラリアで行われた研究であり，対象者は390人である．参加者を，小麦ふすま25 g投与の有無，βカロテン20 mg投与の有無，脂肪摂取量を総エネルギーの25%にする指導の有無により8群に分けた．2年目と4年目に大腸内視鏡検査を行い，新たな大腸腺腫の発生を調べた．主エンドポイントである腺腫の発生の有無に差はなく，低脂肪食で小麦ふすまを投与したグループでのみ1 cm以上の腺腫の発生が，わずかではあるが有意に減少した．

Schatzkinらの研究[11]は米国で行われた研究であり，対象者は1,905人である．参加者を，脂肪の減少（全摂取カロリーの20%），高食物繊維（1000 kcalあたり18 g），果物と野菜の摂取（1000 kcalあたり3.5杯）の食事摂取するように強力に指導する介入グループと，健康的な食事に関する標準的な小冊子を与えたコントロールグループの2群に分けた．1年目と4年目に大腸内視鏡検査を行った．両群間で新たな腺腫の発生の割合に差は認められなかった．

Albertsらの研究[12]も米国で行われた研究であり，対象者は1,303人である．参加者を，1日13.5 gの小麦ふすまを含むシリアルを投与するグループと，1日2 gの小麦ふすまを含むシリアルを投与するグループの2群に分けた．1年目と3年目に大腸内視鏡検査を行い，新たな腺腫の発生を調べた．主エンドポイントである腺腫の発生の有無に差はなかったが，3個以上の腺腫を発生した者は高食物繊維投与群で有意に多かった．

Bonithon-Koppらの研究[13]は欧州で行われた研究であり，対象者は552人である．参加者を，1日2 gのカルシウムを投与するグループと，1日3.5 gのサイリウムを投与するグループと，それらのプラシーボのみを投与するグループの3群に分けた．尚，この論文に書かれているサイリウム(Ispaghula husk)とは，Plantago ovataのハスクの英国における薬局方名であり，インドではIsapol Hask，米国ではPsyllium Huskと呼ばれている．日本で類似のものとして，オオバコPlantago asiaticaの種子がシャゼンシとして収載されている．オオバコの一種の種子から採った天然植物ガムで，インドでは便秘によく用いられてい

る．とても保水性が高く，フィチン酸が多く含まれ，鉄の吸収障害が懸念される．実際，ボランティアを用いた投与試験で，貧血を認めた報告がある．日本でも便秘薬として比較的容易に入手でき，一般にはサイリウムと呼ばれている．3 年目に大腸内視鏡検査を行い，新たな腺腫の発生を調べた．プラシーボ群に比べ，カルシウム投与群では有意ではないものの腺腫の発生は減少した (OR=0.66) が，サイリウム投与群では有意に腺腫の発生が増加した (OR=1.67).

著者らの研究[14] は日本で行われた研究であり，対象者は 380 人である．参加者を，全員に総摂取エネルギーに対して脂肪由来のエネルギーが 18 から 22%になることを主目的とした食事指導を行い，1 日 7.5 g の小麦ふすまを投与する群，1 日 3 g の乳酸菌製剤を投与する群，小麦ふすまと乳酸菌製剤の両方を投与する群，食事指導のみの群の 4 群に分け，4 年間，介入を行った．4 年目の大腸内視鏡検査では，小麦ふすまを摂取している者は，摂取していない者に比して，3 mm 以上や 10 mm 以上腺腫の発生が有意に増えた．乳酸菌製剤を摂取している者は，摂取していない者に比して，中等度異型以上の腺腫の発生が有意に抑制された．

3. 介入試験の成績に関する考察

上述の欧米諸国や日本で行われた無作為割付臨床試験の成績からは，食物繊維が大腸癌を予防する可能性を示唆する知見は少なく，逆に腺腫の発生を促進している可能性が強く示唆する結果になっている．

これらの試験の結果から，考えられる可能性を以下に記す．

1) 食物繊維の摂取で大腸癌の発生を予防することはできない

食物繊維を摂取しても大腸癌の発生を予防することができないのであれば，これらの試験の結果は，おおむね納得できる．食物繊維には，腸管粘膜の増殖を亢進させる作用があり，それが腫瘍の増大にも関与していることも考えられる．

しかし，臨床試験の問題点として以下のようなことも考えられる．

2) 腺腫の発生を大腸癌の中間代理指標とする事の問題

これらの無作為割付臨床試験のエンドポイントは，大腸腺腫の発生の有無であり，大腸癌の発生ではない．しかし，腺腫内癌の存在，経過観察により腺腫が大腸癌になった症例報告，腺腫と大腸癌の組織学的，分子生物学的特徴の共通点，大腸癌患者の大腸に腺腫を併せ持つ者が多いこと，疫学的に腺腫と大腸癌のリスク要因が類似すること，腺腫保有者の大腸癌罹患リスクが高いこと，家族性大腸腺腫症の大腸癌罹患リスクがきわめて高いこと，などの理由より，腺腫は大腸癌の中間代理指標となり得る可能性は高いと考えられる．しかし，腺腫の増減が大腸癌罹患の増減と相関したという証明はされていない．

また，家族性大腸腺腫症を対象とした発癌予防試験において，スリンダクの投与で大腸腺腫が顕著に縮小したにも関わらず，大腸癌が発生したとする報告がいくつもされており，大腸腺腫が大腸癌の中間代理指標となり得るかどうかは，慎重に判断すべきである．

この問題点を解決するためには，臨床試験の参加者は試験終了後も追跡し，大腸癌の発生や死亡まで確認し，それと試験期間に把握した腺腫の発生程度の関係を調べる必要がある．中間代理指標を用いた介入試験では，このような追跡調査を行うことを当初から計画すべきであろう．

また，中間代理指標を用いる場合，一つだけを用いるのではなく，機序の異なる複数の生物学的指標を用いることも重要である．例えば，大腸粘膜の細胞増殖能やアポトーシス，細胞分化程度なども測定し，それらが仮説通りに変化するかどうかを併せて調査すべきだと考える．今後の分子生物学的技術を用いた生物学的指標の開発が期待される．

大腸腺腫をエンドポイントとした場合の問題点として，大腸内視鏡検査の診断能力もある．欧米の報告は，私たちの試験に比べて，発見される腺腫の頻度は低く，直径が大きい傾向があり，大腸内視鏡検査における見落としがある可能性が考えられる．

3) 試験期間・時期の問題

これらの介入試験の試験期間は最長でも 4 年である．動物実験などでは，生存期間の大半を試験期間にした研究が多いが，80 年以上生存するヒトにおいて，その 20 分の 1 程度の期間だけの介入では，効果を判断するのは困難かもしれない．

また，成人になってからの介入より，若年時期の介入の方が効果的であるのかもしれない．しかし，実際には 80 年もかかる臨床試験は不可能であるし，80 年も結果が出るのを待っていたら，社会状況も激変し，結果の得られた時には役に立たない知見になっているであろう．臨床試験の限界の一つであろう．

4) 食物繊維の種類・投与量に関する問題

前述の試験ではサプリメントとして小麦ふすまとサイリウムが用いられている．これらの食品由来の食物繊維では大腸癌の予防はできなかったが，その他の食品由来の食物繊維なら大腸癌が予防できた可能性を否定したのではない．これが食物繊維の研究が難しい原因の一つで

ある.

食物繊維は五訂日本食品標準成分表において『ヒトの消化酵素で消化されない食品中の難消化性成分の総体』と定義されている．その多くは植物由来の炭水化物であるが，一部は動物由来や非炭水化物のものも含まれる．この定義が示すように，食物繊維はいろいろな成分の総称であり，生体に与える影響も一定ではない．従って，食物繊維による効果を研究する場合には，食物繊維のなかのどの成分の作用を検討しているのかを念頭に入れておく必要がある．ただし，食物繊維の成分別の生体に与える影響の研究はあまり進んでいない．

食物繊維のその成分を同定して検討することは困難なため，大まかに水溶性と非水溶性に分けて検討することが多い．しかし，生体に与える影響からは，保水性の程度，腸内細菌叢による発酵の有無，イオン交換能の有無などから分ける方が適切な場合もあると考えるが，そのように分けた成分表もなく，今後の検討が必要である．

サプリメントとしては小麦ふすまを用いることが多い．しかし，小麦ふすまも原産地により，そこに含まれる組成も異なることが考えられる．また，サイリウムなど，生体に与える影響が異なると考えられる食品を用いた場合には，それらのことも留意する必要があろう．

また，用いた小麦ふすまも，脱脂しているのか，どのくらい細粒にしているのかも問題になる．動物実験では，用いる小麦ふすまの細かさにより発癌予防効果に差があるとの報告もあり，小麦ふすまの抽出分画によっても発癌予防効果に差があるとの報告もされている．また，抗酸化作用を持つフィチン酸の含有量や残留農薬の有無も把握しておく必要があろう．

次に，投与量であるが，欧米の報告では，食物繊維の投与量はかなり多い．私たちは，ボランティアに小麦ふすまビスケットをサプリメントとして強制的に投与した場合，食事由来の食物繊維の摂取量が減少することを確認した[15]．欧米の研究では大量の小麦ふすまを投与しており，食事の変化は無視できないのではないかと考えている．Albertsらの研究では高食物繊維投与群で脱落例が多かったが，参加者には，かなりの負担があったことが考えられる．

5) 食事調査，食事指導の問題

食事指導に関する問題点として，食事指導内容が適切であったかどうか，食事調査が正確であったかどうか，の2点があげられる．

食事指導内容であるが，生野菜と果物の摂取を主体とした食事指導より穀類や海草類や煮た野菜を勧めるべきだったのかもしれない．

食事調査に関しては，Schatzkinらの報告では，半定量食事摂取頻度調査では脂肪摂取も総摂取カロリーも食物繊維摂取量も，理想的に変化しているが，血清コレステロール値，体重，血清カロテノイド値の変化は微小であり，食事調査が正確に行われていなかった可能性が示唆される．

これらの問題点に対しては，半定量食事摂取頻度調査だけではなく，3日間連続記録式食事調査や，頻回の1日思いだし調査を併用すべきであったと考える．Schatzkinらの報告では，一部参加者に対しては，そのような調査も併用したとの記載があるので，それらの成績の詳細の公表が待たれる．

6) 参加者集団の問題

前述の試験に参加した集団が適切でなかった可能性がある．大腸腺腫を持たない低リスク集団であれば食物繊維の投与は有効であったかもしれないし，参加を拒否した人ならば有効であったかもしれない．

しかし，私たちの試験では，拒否例はきわめて少なく，また，欧米や日本で同様の結果が出たため，この可能性は少ないと考える．

4. 最後に

得られた結果について信頼性の高いコホート研究においては，食物繊維が大腸癌の予防に有用かどうかは，一定した結論は出ていない．食物繊維を投与する無作為割付臨床試験では，食物繊維をサプリメントとして投与することにより大腸癌の発生を促進する可能性も示されている．しかし，食事から食物繊維を多く摂取する試験ではそのような促進作用は認められなかった．野菜の摂取量が少ないと大腸癌を促進するとする数多くの観察的疫学研究も報告されている．また，食物繊維は糖尿病や心疾患に対して予防的働きがあることが多く示されている．

これらのことより，現時点では，大腸癌予防に関しては，食物繊維を多く含む野菜などの摂取は勧められるが，サプリメントとして食物繊維強化食品の摂取は推奨できないと考えている．

このように，機能性食品の有効性の評価は，膨大な手間と長時間を必要とする．しかし，国民の健康のためには，妥協することなく，研究を続けていかなくてはならない．

研究者は，機能性食品の有効性は慎重に，有害事象は積極的に，情報を学術誌に公開することが必要と考える．

参考文献

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