a result, TNF-α production was not observed in all of US exposure timing including 10, 20, and 30 min (Fig. 5C–F), and suggests the involvement of endocytosis on TNF-α production. Taking these into considerations, the high level of gene expression with low TNF-α production would be achieved when US is exposed at 5 min by both unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure. These results suggested that US exposure timing is important for gene transfection using both unmodified and Man-PEG_{2000} bubble lipoplexes.

Since cytotoxicity is important for carrier development, we investigated cytotoxicity using both unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure in cultured mouse macrophages. As shown in Fig. 5B and C, the cytotoxicity was observed when US was exposed at 10, 20, and 30 min in the gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure. Since TNF-α is possibly involved in the cytotoxicity *via* the interaction with death receptors, such as TNF receptors and TNF-related apoptosis-inducing ligand (TRAIL) receptors [47,48], we investigated cytotoxicity on the TLR-9 signal inhibition experiments. In the inhibitory condition of TNF-α production, since the cytotoxicity was observed when US was exposed at 10, 20, and 30 min (Fig. 5B and C); the produced TNF-α is not involved in the cytotoxicity in gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes and US exposure. Next, it is assumed that the cytotoxicity followed by the destruction of both bubble lipoplexes in the endosomes would be higher than that on the cell surface. Since both bubble lipoplexes are considered to be taken up into the cells *via* endocytosis at over 10 min after the addition of lipoplexes (Fig. 1A), both bubble lipoplexes are assumed to be destructed in the endosomes followed by US exposure, when US was exposed at 10, 20, and 30 min after the addition of lipoplexes. In the endocytosis inhibitory experiments, the cytotoxicity was not observed whenever US was exposed while endocytosis was inhibited (Fig. 5B and C). Therefore, it is suggested that both bubble lipoplexes are destructed in the endosomes followed by US exposure when US was exposed at over 10 min, and the destruction of bubble lipoplexes in the endosomes may affect the lethal effects to the cells.

5. Conclusions

In the present study, we showed that a large amount of pDNA was transferred into the cytoplasm followed by US-mediated destruction of bubble lipoplexes in the gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with optimized US exposure. Moreover, the effective gene expression was obtained without the production of TNF-α in gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure, when US was exposed until 5 min after the addition of bubble lipoplexes. The findings obtained from this study suggest that gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure enables to transfer a large amount of pDNA into the cytoplasm, and optimized US exposure timing is important to achieve the high levels of gene expression and the low levels of pro-inflammatory cytokine production in this gene transfection method.

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Efficient Suppression of Murine Intracellular Adhesion Molecule-1 Using Ultrasound-Responsive and Mannose-Modified Lipoplexes Inhibits Acute Hepatic Inflammation

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Hepatitis is often associated with the overexpression of various adhesion molecules. In particular, intracellular adhesion molecule-1 (ICAM-1), which is expressed on hepatic endothelial cells (HECs) in the early stage of inflammation, is involved in serious illnesses. Therefore, ICAM-1 suppression in HECs enables the suppression of inflammatory responses. Here, we developed an ICAM-1 small interfering RNA (siRNA) transfer method using ultrasound (US)-responsive and mannose-modified liposome/ICAM-1 siRNA complexes (Man-PEG_{2000} bubble lipoplexes [Man-PEG_{2000} BLs]), and achieved efficient HEC-selective ICAM-1 siRNA delivery in combination with US exposure. Moreover, the sufficient ICAM-1 suppression effects were obtained via this ICAM-1 siRNA transfer *in vitro* and *in vivo*, and potent anti-inflammatory effects were observed in various types of inflammation, such as lipopolysaccharide, dimethylnitrosamine, carbon tetrachloride, and ischemia/reperfusion-induced inflammatory mouse models. *Conclusion:* HEC-selective and efficient ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure enables suppression of various types of acute hepatic inflammation. This novel siRNA delivery method may offer a valuable system for medical treatment where the targeted cells are HECs. (HEPATOLOGY 2012;56:259-269)

Hepatitis resulting from conditions such as drug-induced hepatic inflammation and ischemia/reperfusion (IR)-induced liver injury followed by surgery is a major obstacle for medical treatment.[1,2] Moreover, it was reported that chronic hepatitis progresses to cirrhosis and liver cancer[3,4]; therefore, the prevention and early treatment of hepatitis are important for patients and medical professionals. Most drug-induced hepatitis is caused by nuclear factor-κB activation and proinflammatory cytokine production followed by various stimulations in medical treatments.[5] In IR-induced liver injury, a large amount of reactive oxygen species produced by IR stimulation is involved in the induction of inflammatory responses.[6] Although the mechanism for each inflammatory response is different, various adhesion molecules, such as vascular cell adhesion molecule (VCAM) and intracellular adhesion molecule (ICAM), are abundantly expressed on hepatic endothelial cells (HECs) in the early stage of inflammatory responses followed by various types of stimulation.[7] Among these, ICAM-1 is known as a major molecule that is highly involved in the adhesion, diapedesis, and tissue infiltration of leukocytes contributing to the deterioration in inflammatory responses.[8] During alcohol-

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BL, bubble lipoplex; CCl_4, carbon tetrachloride; DAPI, 4′,6-diamidino-2-phenylindole; DMN, dimethylnitrosamine; FITC, fluorescein isothiocyanate; H&E, hematoxylin and eosin; HEC, hepatic endothelial cell; ICAM, intracellular adhesion molecule; IFN-γ, interferon-γ; IL, interleukin; IR, ischemia/reperfusion; iv, intravenous; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; MDA-5, melanoma differentiation-associated gene 5; mRNA, messenger RNA; RIG-1, retinoic acid-inducible gene 1; siRNA, small interfering ribonucleic acid; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α; US, ultrasound.

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induced liver injury, it was reported that ICAM-1 expression and the resultant leukocyte infiltration are involved in the deterioration of alcohol-induced liver injury.[9] Therefore, the suppression of inflammatory responses may be achieved by selective knockdown of ICAM-1 in HECs.

RNA interference is an important endogenous mechanism for gene regulation by cleaving specific messenger RNA (mRNA) possessing the complementary sequence using small interfering RNA (siRNA).[10,11] Although siRNA is a promising candidate for molecular therapy, an effective method for siRNA transfer into the cytoplasm of targeted cells *in vivo* is still being developed. The effective methods for *in vivo* siRNA delivery involve nonviral carriers, including liposomes, emulsions, micelles, and polymers.[12-18] However, because the nonviral carriers are taken up into the cells via endocytosis, degradation within endosomes and escape from endosomes are major obstacles for the improvement of siRNA therapeutics. Moreover, efficient and selective siRNA delivery into HECs is essential to achieve the potent anti-inflammatory effects produced by ICAM-1 siRNA.

Recently, the benefits have become appreciated of delivery of nucleic acids into cells using microbubbles and ultrasound (US) (also known as "sonoporation methods"), due to the high transfer efficiency into the cytoplasm.[19-22] Our group has developed US-responsive and mannose-modified liposomes/plasmid DNA complexes for *in vivo* gene transfer and successfully obtained efficient gene expression in mannose receptor-expressing cells, such as HECs and splenic dendritic cells.[23-25] Moreover, we demonstrated that a large amount of plasmid DNA could be directly transferred into the cytoplasm through a mechanism involving transient pores created on the cell membrane by the destruction of microbubbles after US exposure.[26] Therefore, the efficient transfer of ICAM-1 siRNA into HECs might be achieved by applying this method to siRNA delivery.

In the present study, we developed an ICAM-1 siRNA transfer system based on US-responsive and mannose-modified liposome/siRNA complexes (Man-PEG_{2000} bubble lipoplexes [Man-PEG_{2000} BLs]) for anti-inflammatory therapy. ICAM-1 siRNA delivered by Man-PEG_{2000} BLs and US exposure was selectively and efficiently transferred into HECs *in vitro* and *in vivo*. Furthermore, sufficient ICAM-1 suppression and potent anti-inflammatory effects were achieved by ICAM-1 siRNA transfer against various types of inflammation induced by lipopolysaccharide (LPS), dimethylnitrosamine (DMN), carbon tetrachloride (CCl_4), and IR. To our knowledge, this is the first report of a gene transfer method using Man-PEG_{2000} BLs and US exposure for the selective and efficient transfer of siRNA to HECs. This novel siRNA transfer method could be valuable for medical treatments that target HECs.

Materials and Methods

In vitro *siRNA Delivery*. After incubation of HECs for 72 hours, the culture medium was replaced with Opti-MEM I (Invitrogen, Carlsbad, CA) containing lipoplexes/BLs (1 μg siRNA). At 5 minutes after siRNA transfer, HECs were exposed to US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity, 4.0 W/cm^2) for 20 seconds. In the siRNA delivery using naked siRNA and conventional nanobubbles, at 5 minutes after addition of naked siRNA (1 μg) and conventional nanobubbles (60 μg total lipids), cells were immediately exposed to US. US was generated using a Sonopore-4000 sonicator (Nepa Gene, Chiba, Japan). At 1 hour after US exposure, the medium was replaced with RPMI-1640 and incubated for an additional 23 hours. Lipofectamine 2000 (Invitrogen) was used according to the recommended procedures with an exposure time of 1 hour, which is the same exposure time in other experiments using lipoplexes.

In Vivo *siRNA Delivery*. Six-week-old C57BL/6 female mice were intravenously injected with BLs containing 10 μg siRNA via the tail vein. At 5 minutes after the injection of the bubble lipoplexes, US (frequency, 1.045 MHz; duty, 50%; burst rate, 10 Hz; intensity 1.0 W/cm^2; time, 2 minutes) was applied transdermally to the abdominal area using a Sonopore-4000 sonicator. In the siRNA delivery using naked siRNA and conventional nanobubbles, at 4 minutes after intravenous injection of conventional nanobubbles (500 μg total lipid), naked siRNA (10 μg) was intravenously injected and US was exposed at 1 minute after naked siRNA injection.

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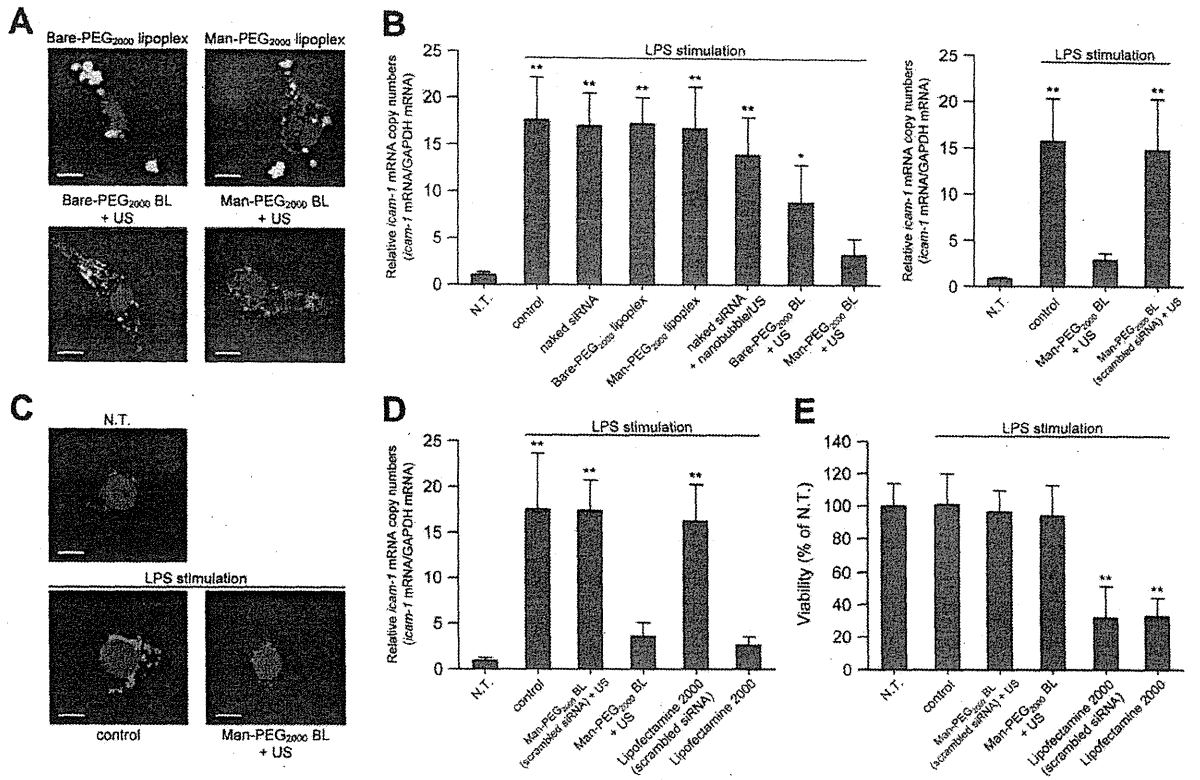

Fig. 1. Suppression effects of *icam-1* mRNA expression and cytotoxicity followed by ICAM-1 siRNA delivery in LPS-stimulated primary mouse HECs. (A) *In vitro* confocal images of cellular associated ICAM-1 siRNA (1 μg siRNA) transferred by various methods 1 hour after treatment in primary mouse HECs. US was directly exposed to HECs at 5 minutes after addition of BLs. The lipoplexes were constructed with AlexaFluor-594–labeled ICAM-1 siRNA (red), and the endosomes were labeled with AlexaFluor-488 transferrin conjugates (green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bars, 10 μm. (B,C) The level of *icam-1* mRNA expression (B) and *in vitro* confocal images of ICAM-1 expression (C) obtained by ICAM-1 siRNA transfer (1 μg siRNA) using various types of methods 24 hours after LPS stimulation in primary mouse HECs. US was directly exposed to HECs at 5 minutes after addition of BLs, and cells were exposed to LPS (100 ng/mL) at 24 hours after the addition of siRNA or lipoplexes/BLs. ICAM-1 was labeled with anti-mouse ICAM-1 antibody and fluorescein isothiocyanate (FITC)-conjugated secondary antibody (green), and nuclei were counterstained by DAPI (blue). Scale bars, 10 μm. (D,E) Comparison of the suppression of *icam-1* mRNA expression (D) and cell viability (E) obtained by siRNA transfer using Man-PEG_{2000} BLs (1 μg siRNA) and US exposure with that by Lipofectamine 2000. $*P < 0.05$, $**P < 0.01$ versus no treatment. Each value represents the mean + SD ($n = 5$). N.T., no treatment.

Statistical Analyses. Results are presented as the mean ± SD of more than three experiments. Analysis of variance was used to test the statistical significance of differences among groups. Two-group comparisons were performed using the Student *t* test and multiple comparisons between control and other groups were performed using the Dunnett's test.

Results

Suppression Effects of ICAM-1 siRNA. The suppression of LPS-induced ICAM-1 expression by ICAM-1 siRNAs (Supporting Fig. 1A) was investigated in primary mouse HECs. As shown in Supporting Fig. 1B, the suppression of ICAM-1 was the highest in ICAM-1 siRNA with sequence 1, and not observed in scrambled siRNA. Therefore, ICAM-1 siRNA containing sequence 1 and scrambled siRNA were used in the following examinations.

Physicochemical Properties of Man-PEG_{2000} BLs. Following enclosure of US imaging gas into Man-PEG_{2000} BLs, lipoplexes became cloudy (data not shown) and the average particle size increased (Supporting Fig. 2A). Following gel electrophoresis experiments, the formation of siRNA complexes in BLs was confirmed (Supporting Fig. 2B). Moreover, ζ-potentials of BLs were lower than that of liposomes (Supporting Fig. 2A), suggesting that siRNA was attached to the surface of cationic bubble liposomes. These physicochemical properties are consistent with our previous reports using plasmid DNA.[23-26]

Intracellular Transport Characteristics of ICAM-1 siRNA. The siRNA transfer efficiency was investigated in primary mouse HECs expressing mannose receptors (Supporting Fig. 4). The amount of siRNA delivered by BLs and US exposure was significantly higher than that by lipoplexes only (Supporting Fig. 3A).

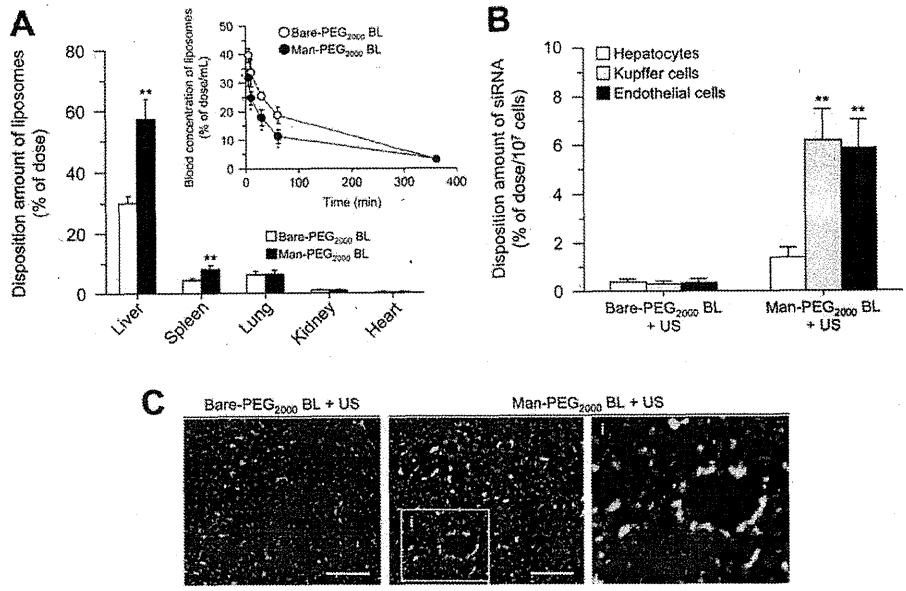

Fig. 2. *In vivo* distribution of ICAM-1 siRNA delivered by Man-PEG_{2000} BLs and US exposure. (A) Tissue distribution and pharmacokinetics of radiolabeled bare- and Man-PEG_{2000} BLs complexed with 10 μg ICAM-1 siRNA after intravenous (iv) administration into mice. Tissue distribution of lipoplexes was measured at 6 hours after iv administration of lipoplexes. Inset shows blood concentration of lipoplexes at predetermined times after iv administration. $^*P < 0.05$, $^{**}P < 0.01$ versus the corresponding group of bare-PEG_{2000} lipoplexes. Each value represents the mean $\pm$ SD (n = 5). (B) Hepatic cellular localization of AlexaFluor-594 labeled ICAM-1 siRNA delivered by bare- and Man-PEG_{2000} BLs (10 μg siRNA) and US exposure at 6 hours after iv administration of lipoplexes into mice. Liver was separated to hepatocytes, Kupffer cells, and endothelial cells by collagenase perfusion, one-step density gradient centrifugation, and magnetic cell sorting as described in the Supporting Materials and Methods. $^{**}P < 0.01$ versus the corresponding group of hepatocytes. Each value represents the mean + SD (n = 5). (C) Fluorescent images of hepatic localization of AlexaFluor-594–labeled ICAM-1 siRNA (red) delivered by bare- and Man-PEG_{2000} BLs (10 μg siRNA) and US exposure. HECs were labeled with anti-mouse CD146 antibody and FITC-conjugated secondary antibody (green), and nuclei were counterstained with DAPI (blue). Livers were harvested at 6 hours after iv administration of lipoplexes into mice, and magnified images corresponding to the areas enclosed in boxes are shown in the inset (i). Scale bars, 100 μm.

Moreover, the amount of siRNA delivered by Man-PEG_{2000} BLs and US exposure was higher than unmodified BLs. However, the amount of siRNA was significantly suppressed in the presence of mannan but not suppressed in the presence of chlorpromazine, an endocytosis inhibitor (Supporting Fig. 3B,C). Confocal microscopy analysis of cells after siRNA transfer by bubble lipoplexes with US exposure revealed that siRNA was not colocalized in endosomes (Fig. 1A). These observations suggest that siRNA is directly transferred into the cytoplasm of targeted cells and is not mediated by endocytosis in this siRNA transfer method.

***Suppression Effects of LPS-Induced ICAM-1 Expression* In Vitro.** As shown in Fig. 1B,C, ICAM-1 expression induced by LPS stimulation was suppressed by approximately 80% in siRNA transfer using Man-PEG_{2000} BLs and US exposure. The suppression effect of ICAM-1 expression was not observed for scrambled siRNA. Moreover, this suppression effect was comparable to that by Lipofectamine 2000 (Fig. 1D) but with decreased cytotoxicity (Fig. 1E).

In Vivo *distribution of ICAM-1 siRNA*. We investigated the pharmacokinetic profiles and the tissue distribution of BLs after intravenous administration into mice. Compared with nonmodified BLs, the retention time of Man-PEG_{2000} BLs in the blood was reduced, and localization in both the liver and spleen were increased (Fig. 2A). Moreover, a large amount of ICAM-1 siRNA was distributed in HECs that abundantly express mannose receptors when delivered using Man-PEG_{2000} BLs and US exposure (Fig. 2B,C).

***Suppression Effects of Drug-Induced Hepatic ICAM-1 Expression* In Vivo.** The suppression of ICAM-1 expression by siRNA delivery was investigated in an LPS/D-galactosamine–induced acute hepatitis mouse model (Fig. 3A). As shown in Fig. 3B-D, ICAM-1 mRNA and protein levels in HECs induced by LPS/D-galactosamine stimulation were suppressed by approximately 80% using Man-PEG_{2000} BLs and US exposure. Moreover, ICAM-1 expression induced by CCl_4 and DMN stimulation was also significantly suppressed by the same ICAM-1 siRNA delivery system (Supporting Figs. 6B and 7B). The effects of siRNA dose on ICAM-1 suppression and the duration of ICAM-1 suppression were examined in an LPS/D-galactosamine-induced inflammatory mouse model. Following siRNA delivery using Man-

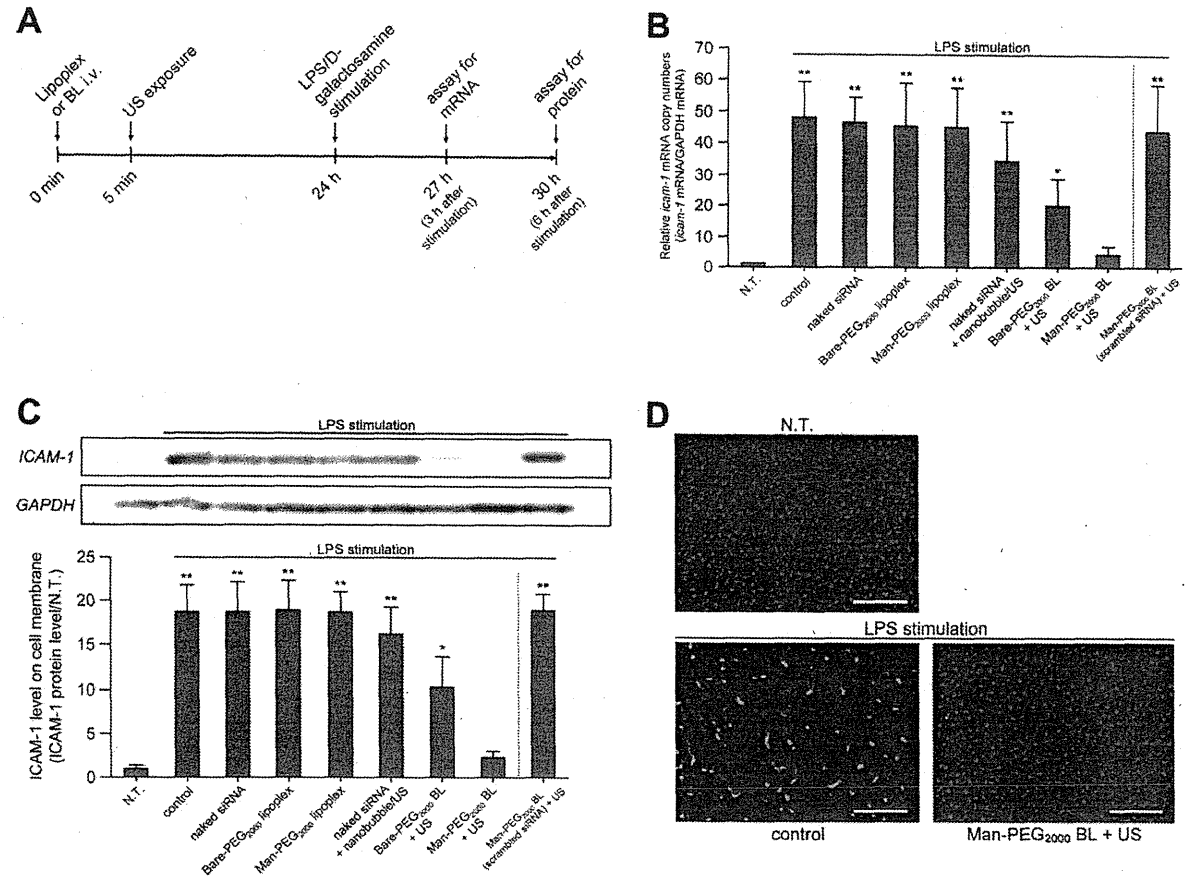

Fig. 3. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on *icam-1* mRNA and protein expression in HECs of an LPS/D-galactosamine-induced inflammatory mouse model. (A) Evaluation schedule of ICAM-1 expression in LPS/D-galactosamine-stimulated mice. (B-D) The expression level of *icam-1* mRNA in cells (B) and protein on the cell membrane (C, D) obtained by siRNA delivery (10 μg siRNA) using various methods in HECs. At 24 hours after siRNA delivery, LPS/D-galactosamine (1 μg/100 mg/kg) was intraperitoneally administered into mice to induce the acute inflammatory responses. HECs were isolated by collagenase perfusion, one-step density gradient centrifugation, and magnetic cell sorting as described in the Supporting Materials and Methods. The *icam-1* mRNA and protein expression in HECs was determined via quantitative reverse-transcription polymerase chain reaction (B), western blotting/enzyme-linked immunosorbent assay (C), and confocal images (D). The expression levels of mRNA and protein were detected at 3 and 6 hours after LPS/D-galactosamine stimulation, respectively. $^*P < 0.05$, $^{**}P < 0.01$ versus no treatment. Each value represents the mean + SD (n = 5). ICAM-1 was labeled with anti-mouse ICAM-1 antibody and FITC-conjugated secondary antibody (green), and nuclei were counterstained with DAPI (blue). Scale bars, 100 μm. N.T., no treatment.

PEG_{2000} BLs and US exposure, suppression was obtained at 10 μg of ICAM-1 siRNA (Supporting Fig. 5A), and was sustained for at least 3 days (Supporting Fig. 5B).

Anti-inflammatory Effects Against Drug-Induced Hepatitis. First, the suppression of leukocyte infiltration by ICAM-1 siRNA delivery was evaluated in an LPS/D-galactosamine–induced inflammatory mouse model (Fig. 4A). As shown in Fig. 4B,D, the expression of interleukin (IL)-8 and monocyte chemotactic protein 1 (MCP-1) was suppressed, and a significantly decreased number of infiltrated leukocytes were detected after siRNA delivery using Man-PEG_{2000} BLs and US exposure. Moreover, the production of proinflammatory cytokines (tumor necrosis factor α [TNF-α], interferon-γ [IFN-γ], and IL-6) were also suppressed by this siRNA delivery (Fig. 4C).

The anti-inflammatory effects obtained by ICAM-1 siRNA delivery were investigated next. As shown in Fig. 5A, alanine aminotransferase (ALT)/aspartate aminotransferase (AST) activities in the serum were markedly suppressed by siRNA delivery using Man-PEG_{2000} BLs and US exposure (Fig. 5A). As shown in Fig. 5B, hepatic apoptosis induced by LPS/D-galactosamine stimulation was significantly suppressed by this ICAM-1 siRNA delivery. Moreover, we performed hematoxylin and eosin (H&E) staining of liver sections to evaluate the effects on hepatic structural features. Although the circular and tube formations of the hepatic central vein were observed in normal liver section (Fig. 5C, left), they were crushed in the LPS-stimulated liver section (Fig. 5C, middle). On the other hand, destruction of the hepatic central vein induced by

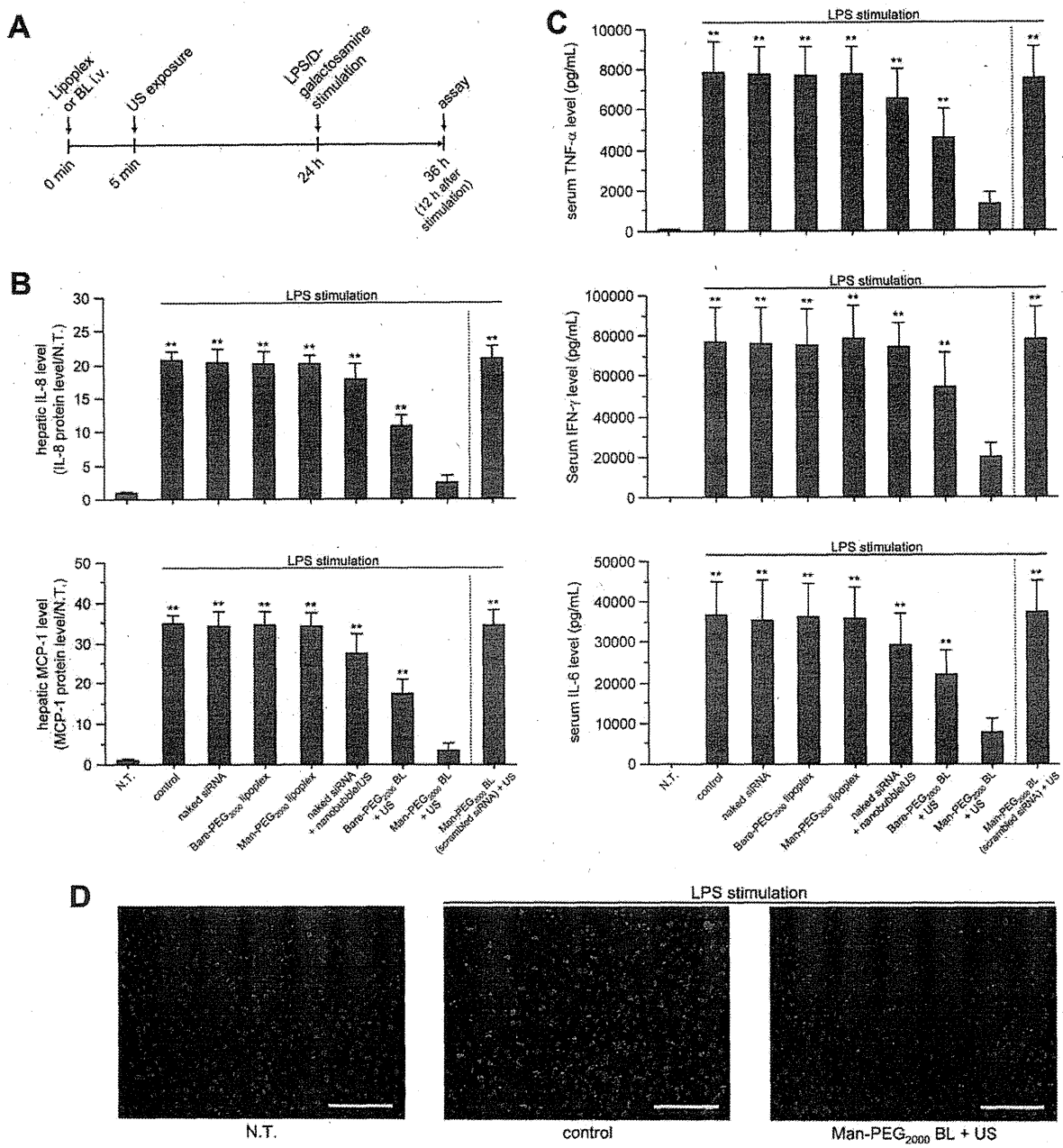

Fig. 4. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on leukocyte infiltration and proinflammatory cytokine production in an LPS/D-galactosamine-induced inflammatory mouse model. (A) Evaluation schedule of leukocyte infiltration and proinflammatory cytokine production in LPS/D-galactosamine-stimulated mice. (B,C) Levels of IL-8 and MCP-1 expression in the liver (B) and the levels of TNF-α, IFN-γ, and IL-6 secretion in the serum (C) after siRNA delivery (10 μg siRNA) using various delivery methods 12 hours after LPS/D-galactosamine stimulation. $^{**}P < 0.01$ versus no treatment. Each value represents the mean + SD (n = 5). N.T., no treatment. (D) Photomicrographs of infiltrated leukocytes after siRNA delivery using Man-PEG_{2000} BLs (10 μg siRNA) and US exposure in LPS/D-galactosamine-stimulated mouse liver. Leukocytes were labeled with anti-mouse Gr-1 (Ly-6G) antibody and rhodamine isothiocyanate-conjugated secondary antibody (red), and nuclei were counterstained with DAPI (blue). Scale bars, 100 μm. $^{**}P < 0.01$ versus no treatment. Each value represents the mean + SD (n = 5). N.T., no treatment.

LPS stimulation was significantly suppressed by ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure (Fig. 5C, right), suggesting that the liver injury induced by LPS-stimulation is suppressed by this siRNA delivery. Similar effects by this ICAM-1 siRNA delivery were also observed for CCl_4- and DMN-induced inflammatory mouse models (Supporting Figs. 6C,D and 7C,D).

Anti-inflammatory Effects Against IR-Induced Liver Injury. The effects of ICAM-1 suppression by delivery of siRNA was evaluated for IR-induced liver

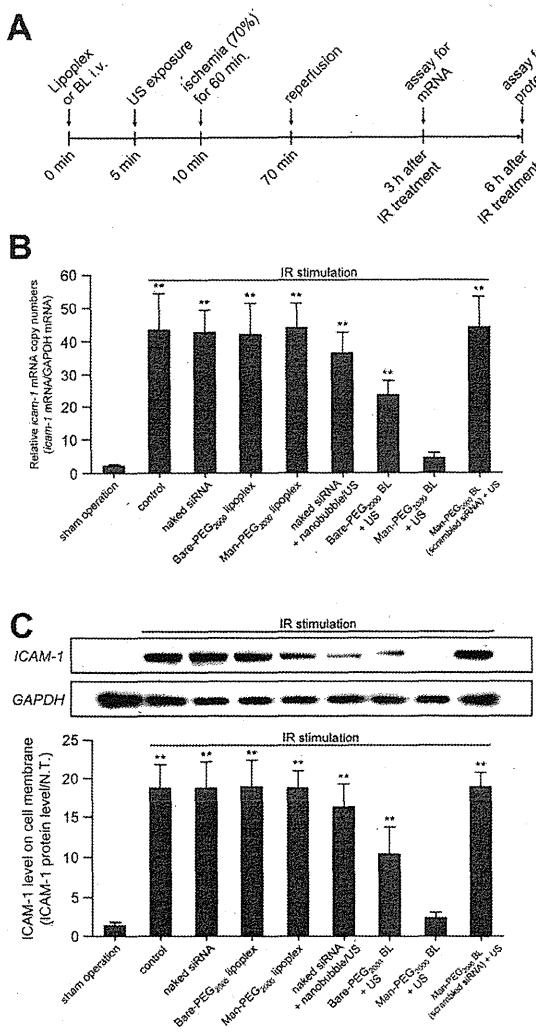

Fig. 6. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on *icam-1* mRNA and protein expression in HECs of an IR-induced hepatic inflammatory mouse model. (A) Evaluation schedule of ICAM-1 expression in hepatic IR-stimulated mice. (B,C) Expression level of *icam-1* mRNA in cells (B) and protein on the cell membrane (C) obtained by siRNA delivery (10 μg siRNA) using various delivery methods in HECs. HECs were isolated via collagenase perfusion, one-step density gradient centrifugation, and magnetic cell sorting as described in the Supporting Materials and Methods. The *icam-1* mRNA and protein expression in HECs was determined via quantitative reverse-transcription polymerase chain reaction (B) and western blotting/enzyme-linked immunosorbent assay (C). Expression levels of mRNA and protein were detected at 3 and 6 hours after IR stimulation, respectively. $*P < 0.05$, $**P < 0.01$ versus sham operation. Each value represents the mean + SD (n = 5).

transfer using Man-PEG_{2000} BLs and US exposure[23-26] would be also suitable for siRNA delivery. In the present study, we applied this gene transfer method for the selective and efficient delivery of siRNA to HECs *in vivo* and investigated the anti-inflammatory effects in various types of inflammatory responses.

The innate inflammatory responses based on the interaction with siRNA and Toll-like receptor (TLR)-3, -7, and -8 should be excluded for evaluating the gene suppression effects of siRNA, but should be considered for clinical applications of siRNA.[28,29] The proinflammatory cytokines (such as TNF-α, IFN-γ, and IL-6) can be induced by siRNA interaction with endosomal TLR-3, -7, and -8 in siRNA transfer using conventional nonviral carriers.[28,29] Transfer of siRNA using Man-PEG_{2000} BLs and US exposure results in the direct deposition into the cytoplasm and is not mediated by endocytosis (Fig. 1A and Supporting Fig. 3C).[26] Therefore, the inflammatory responses followed by the interaction with TLRs are expected to be low, but siRNA is also recognized by cytoplasmic retinoic acid-inducible gene 1 (RIG-1)/melanoma differentiation-associated gene 5 (MDA-5) involved in inflammatory responses.[28,30] Because the modification of 3′-overhang sequences is suppressed by the activation of interferon-responsive factors 3/7, transcriptional factors that exist downstream of the RIG-1/MDA-5 pathway,[31,32] we used siRNAs with 3′-dTdT overhang sequences (Supporting Fig. 1A).

As shown in Figs. 1B-D and 3, ICAM-1 expression in LPS-stimulated HECs was significantly suppressed by ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure, both *in vitro* and *in vivo*. Similarly, tissue infiltration of leukocytes and proinflammatory cytokine production were both suppressed after ICAM-1 suppression by siRNA delivery using this method (Fig. 4). Furthermore, potent anti-inflammatory effects were obtained by this ICAM-1 siRNA delivery in an LPS-stimulated inflammatory mouse model (Fig. 5). The delivery of siRNA to HECs, which express mannose receptors (Supporting Fig. 4),[33] was selective and efficient using Man-PEG_{2000} BLs with US exposure (Fig. 2B,C). Moreover, because a large amount of siRNA was directly transferred into the cytoplasm (Fig. 1A and Supporting Fig. 3C),[26] endosomal escape and degradation within endosomes could be evaded. These data may indicate that nucleic acid transfer using Man-PEG_{2000} BLs and US exposure can be applied for siRNA delivery.

Although LPS is widely used to evaluate the induction of acute inflammatory responses, they are induced by not only various medicines but also surgical operations.[34] Aiming for the clinical application of anti-inflammatory therapy using our siRNA delivery method, the anti-inflammatory effects against various inflammatory models in mice were investigated. After evaluation of the anti-inflammatory effects against CCl_4-, DMN-, and IR-stimulated inflammation, ICAM-1 expression in HECs and the inflammatory responses was significantly suppressed by ICAM-1

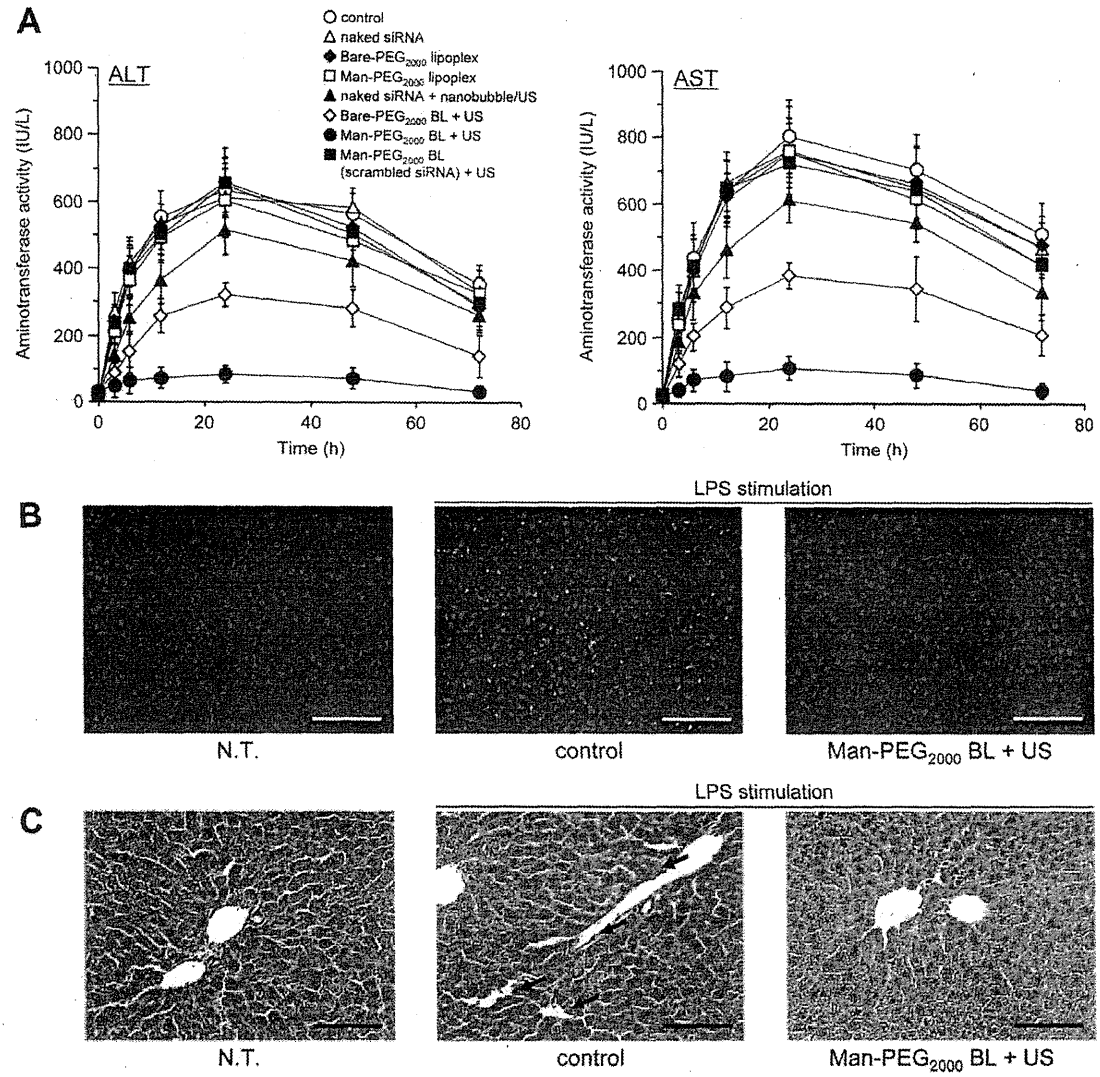

Fig. 5. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on liver toxicity in an LPS/D-galactosamine-induced inflammatory mouse model. (A) The level of serum ALT/AST activities after siRNA delivery (10 μg siRNA) using various methods at predetermined times after LPS/D-galactosamine stimulation. Each value represents the mean ± SD (n = 5). (B) Fluorescent images of apoptosis after siRNA delivery using Man-PEG_{2000} BLs (10 μg siRNA) and US exposure in LPS/D-galactosamine-stimulated mice. Apoptosis (green) was detected via terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling, and nuclei were counterstained with DAPI (blue). Scale bars, 100 μm. (C) Liver histology with H&E staining 24 hours after siRNA delivery using Man-PEG_{2000} BLs (10 μg siRNA) and US exposure in LPS/D-galactosamine-induced inflammatory mouse model. Black arrows: destruction of tube formation in hepatic central vein. Scale bars, 100 μm.

injury (Fig. 6A). As shown in Fig. 6B,C, ICAM-1 expression induced by IR stimulation was suppressed by siRNA delivery using Man-PEG_{2000} BLs and US exposure. Moreover, IL-8/MCP-1 expression and proinflammatory cytokine production were also suppressed (Fig. 7B,C). Following the examination of liver toxicity, ALT/AST activities in the serum and hepatic apoptosis were significantly suppressed (Fig. 8A,B). Moreover, after H&E staining of liver sections, the circular and tube formations of hepatic central vein in the normal liver (Fig. 8C, left) section is destructed by IR stimulation (Fig. 8C, middle), on the other hand, IR-derived destruction of hepatic central vein was suppressed by this ICAM-1 siRNA delivery (Fig. 5C, right).

Discussion

In the sonoporation method, transient pores are created on the cell membrane followed by the destruction of microbubbles, and a large amount of nucleic acids can be directly transferred into the cytoplasm.[21,26,27] Because siRNA is functionalized in the cytoplasm, gene

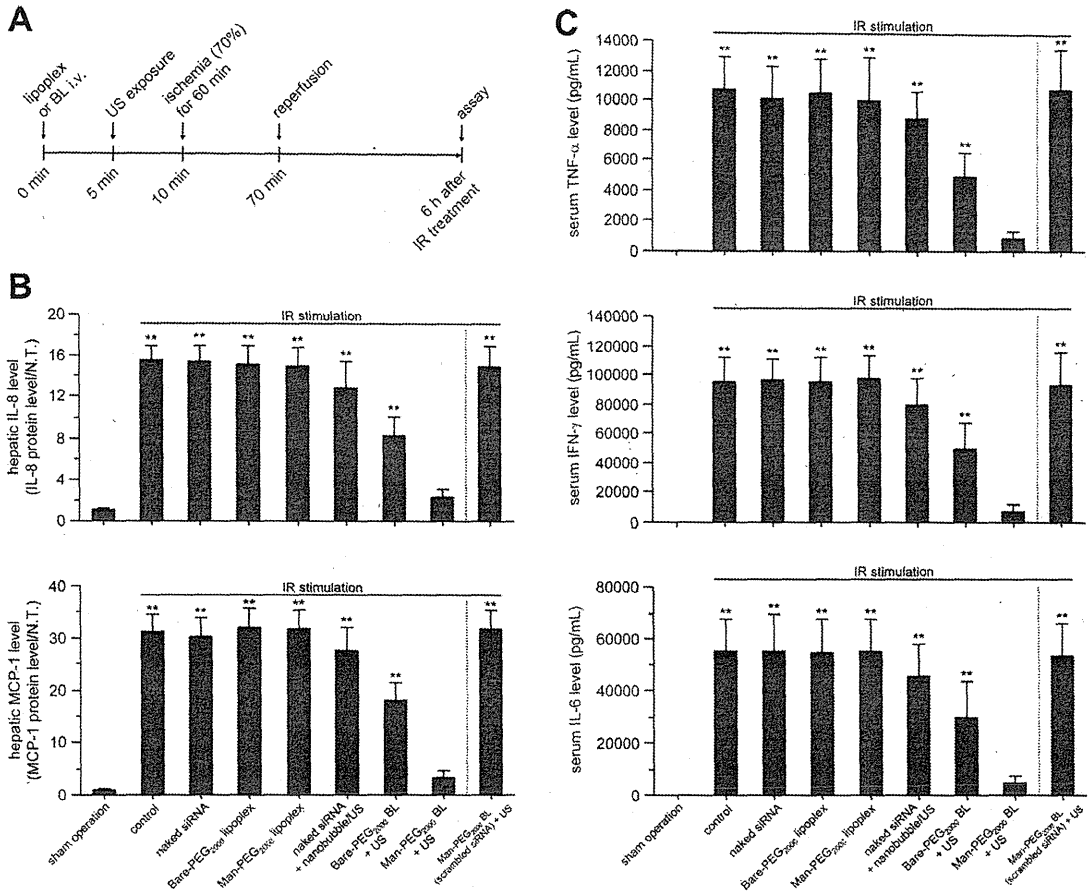

Fig. 7. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on leukocyte infiltration and proinflammatory cytokine production in IR-induced hepatic inflammatory mouse model. (A) Evaluation schedule of leukocyte infiltration and proinflammatory cytokine production in hepatic IR-stimulated mice. (B,C) Levels of IL-8 and MCP-1 expression in the liver (B) and TNF-α, IFN-γ, IL-6 secretion in the serum (C) after siRNA delivery (10 μg siRNA) using various delivery methods 6 hours after IR stimulation. $^{**}P < 0.01$ versus sham operation. Each value represents the mean + SD (n = 5).

siRNA delivery using Man-PEG_{2000} BLs and US exposure in these inflammatory mouse models (Figs. 6-8 and Supporting Figs. 6 and 7). Although the mechanisms of inflammatory responses as a result of LPS, CCl_4, DMN, and IR stimulation are different,[5,6,35,36] ICAM-1 expression in HECs is reported in various types of inflammation, including drug-induced hepatic inflammation and IR-induced liver injury.[7] These data suggest that anti-inflammatory effects obtained by ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure may be beneficial for acute hepatitis and liver injury.

In the present study, efficient ICAM-1 suppression was obtained at a dose of 1 μg siRNA/mouse (0.05 mg/kg) for siRNA delivery using Man-PEG_{2000} BLs and US exposure *in vivo* (Supporting Fig. 5A). This dose of siRNA is lower than those reported for other studies evaluating the therapeutic effects using siRNA, although the therapeutic mechanism and delivery methods of each siRNA are likely to be different.[37-39] These findings suggest that the increased distribution of siRNA into HECs by mannose modification (Fig. 2) and the enhancement of intracytoplasmic siRNA transfer by sonoporation (Fig. 1A and Supporting Fig. 3) could contribute to the potent anti-inflammatory effects observed at a low dose of siRNA in our siRNA delivery method.

ICAM-1 suppression effects were only sustained for 72 hours by siRNA delivery using Man-PEG_{2000} BLs and US exposure (Supporting Fig. 5B). However, because the disease target of this study was acute inflammation, the potent therapeutic effects might be obtained in short duration and single administration of siRNA. Recently, it has been reported that ICAM-1 is involved in various diseases not only for acute/chronic hepatic failure, but also Crohn's disease, ulcerative colitis, and ileus.[40-42] In addition, antisense oligonucleotides against ICAM-1 (ISIS-2302; Alicaforsen)

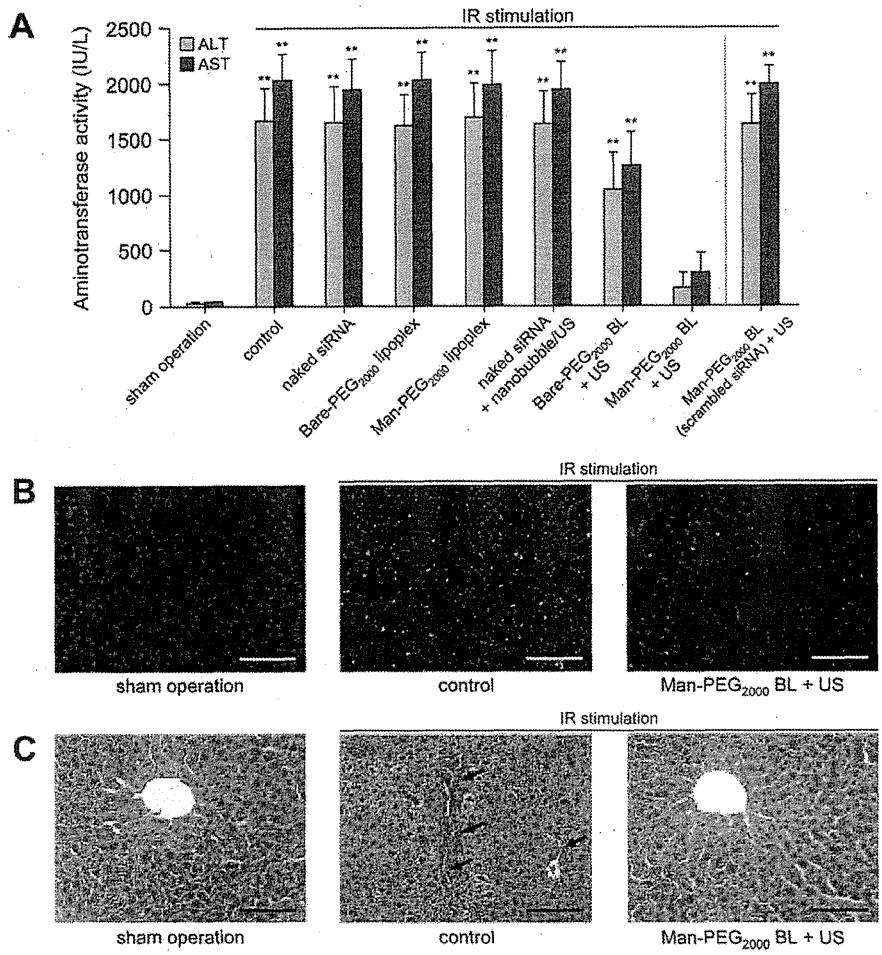

Fig. 8. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on liver toxicity in an IR-induced hepatic inflammatory mouse model. (A) The level of serum ALT/AST activities after siRNA delivery (10 μg siRNA) using various delivery methods 24 hours after hepatic IR stimulation. $^{**}P <$ 0.01versus the corresponding sham operation group. Each value represents the mean + SD ($n = 5$). (B) Fluorescent images of apoptosis followed by siRNA delivery using Man-PEG_{2000} BLs (10 μg siRNA) and US exposure in IR-induced hepatic inflammatory mouse model. Apoptosis (green) was detected via terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling, and nuclei were counterstained with DAPI (blue). Scale bars, 100 μm. (C) Liver histology at 24 hours after siRNA delivery using Man-PEG_{2000} BLs (10 μg siRNA) and US exposure in IR-induced hepatic inflammatory mouse model. Arrows indicate the destruction of tube formation in the hepatic central vein. Scale bars, 100 μm.

are currently under development for the treatment of Crohn's disease and ulcerative colitis.[43,44] However, most of these inflammatory diseases are based on chronic inflammation. In the present study, it is strongly suggested that transfer of ICAM-1 siRNA using Man-PEG_{2000} BLs and US exposure enables a large amount of siRNA to be delivered the cytoplasm of targeted cells (Fig. 1A and Supporting Fig. 3). Therefore, to prolong the duration of gene suppression using this siRNA delivery system, future studies using cholesterol-modified siRNA[45] or locked nucleic acid,[46] which are forms of stable siRNA resistant to enzymatic degradation, might be necessary for application to a variety of chronic inflammatory diseases.

In conclusion, ICAM-1 siRNA was transferred into HECs selectively and efficiently, and sufficient ICAM-1 suppression effects were obtained by ICAM-1 siRNA transfer using Man-PEG_{2000} BLs and US exposure, both *in vitro* and *in vivo*. Moreover, potent anti-inflammatory effects were achieved against various types of inflammation by this ICAM-1 siRNA transfer. These findings contribute to overcoming the poor efficiency of siRNA transfer into the cytoplasm of the targeted cells using nonviral carriers, and this novel siRNA delivery method using Man-PEG_{2000} BLs and US exposure may offer a valuable system for medical treatment where the cellular targets are HECs.

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P245

Galactosylated liposomes with proton sponge capacity: a novel hepatocyte-specific gene transfer system

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Hepatocyte-directed liposomal gene delivery has received much attention due to the lack of suitable treatment for several liver-associated disorders. While targeting of liposomes to the asialoglycoprotein receptor (ASGP-R), nearly-exclusive to hepatocytes, is a well-documented means of achieving cell-specificity, endo/lysosomal degradation of the internalised DNA is one of several factors which hinder successful gene transfer. This study has attempted to address this concern by modifying hepatotropic liposomes with an endosomal escape-inducing proton sponge moiety.

Novel galactosylated (SH02) and imidazolylated (SH04) cholesterol derivatives were successfully synthesised with the aim of conferring the respective functions of ASGP-R-specificity and proton sponge capability upon cationic liposome formulations. These formed unilamellar vesicles with the cytofectin, 3β[*N*-(*N'*,*N'*-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and co-lipid, dioleoylphosphatidylethanolamine (DOPE), when incorporated at 10 mol%. Liposomes effectively bound pCMV-*luc* plasmid DNA, provided protection against serum nucleases; and were well tolerated by both hepatocytes and kidney cells in culture. Competitive inhibition assays showed that liposomes containing SH02 were internalised predominantly via the ASGP-R. Acid titration experiments highlighted the endosomal pH-buffering capacity of SH04. SH04 improved the transfection activity of the Chol-T/DOPE system, but not that of its targeted counterpart, in kidney cells only. Both SH02 and SH04 individually exhibited transfection-enhancing properties and the transgene expression levels using both novel lipids were promising. With further optimisation of the proton sponge and targeting abilities, the liposomes may achieve desired transgene expression levels for use *in vivo*.

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Evaluation of skin angiogenesis stimulated by ointment preparations containing angiogenic genes

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From a point of view of classic pharmacotherapy genes should be treated as active substances that condition the biological activity of a medicinal product that is used. In the case of angiogenic genes, a gene therapy product exerts angiogenic properties - and after having been introduced into appropriate cells it stimulates processes leading to the formation of new blood vessels. In this work we performed a series of experiments aimed to select a group of vehicles, ointment ingredients that could be useful in the systems that could introduce genes into the skin of laboratory animals. Experiments were conducted on plasmids encoding VEGF, FGF, SDF proteins. Appropriate ointment formulas were prepared for experiments, and they were applied on the skin of laboratory mice; after pre-determined time mice were sacrificed, transfected skin specimens were collected and the presence of a pDNA sequence in samples was analysed with qPCR. The analysis of angiogenesis stimulation was also performed. The sequences of apllied pDNAs were found in the mouse skin. Selected vehicles make it possible to introduce pDNA into skin cells; however, the *in vivo* transfection capacity is not high. Based on estimations 10–30% of pDNA molecules applied in ointment pass into the animal skin cells. Experiments also indicate that plasmid pVEGF, pSHH, pSDF stimulate angiogenesis in animal skin and proangiogenic properties depend on a plasmid dose which is used. This work was supported by a grant from Polish Ministry of Science and Higher Education (N N 405 456039).

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Muscle spontaneous regeneration in dwarf mice treated with a bicistronic vector followed by electrotransfer

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Gene therapy combines the correction of defective or missing gene with low risk to the patient. Our group has developed an *in vivo* gene therapy model for the treatment of growth hormone (GH) deficiency based on injection of naked DNA followed by electrotransfer. This strategy provided the presence of human growth hormone (hGH) for at least 60 days in the circulation of immunodeficient/dwarf (lit/scid) mice, that presented a weight gain of up to 33%. The aim of the present work is to verify the safety of our method, evaluating the presence of inflammatory infiltrate and the pattern of muscle regeneration at the electroporation site. A bicistronic vector containing the murine GH (mGH) and the GFP genes under the control of the CMV promoter was utilized. Lit/lit mice were treated with 50 μg of DNA or saline (control group), injected into the quadriceps muscle, followed by electrotransfer using eight 50-V pulses of 20 ms at a 0.5s interval. Histological analysis was performed on day 0, 1, 3, 6 and 12. Muscle damage was verified on the initial days after treatment, but appeared regenerated on the 12th day. GFP maximum expression was observed on the third day. Since increased circulating mGH levels were not observed, GH mediator, i.e. mouse insulin-like growth factor-I (mIGF-I), will be determined to evaluate electroporation efficiency. The results indicate that muscle spontaneously regenerates after this treatment.

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Novel ultrasound-responsive gene carrier with ternary structure.

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Backgrounds: Recently, ternary complex constructed with pDNA, cationic compounds, and anionic compounds were reported to show high gene expressions and low toxicities *in vivo*. In this experiment, we newly constructed novel ultrasound-responsive gene carrier with ternary structure for effective and secure transfection.

Methods: pDNA was mixed with some cationic polymers and cationic complexes were formed. The cationic complexes and the anionic liposomes were mixed for formations of ternary complexes. Then, perfluoropropane gas was entrapped into the ternary complex and ultrasound-responsive gene carriers were constructed.

Results: The stabilities of the gene delivery vectors were determined by gel electrophoresis and the stable complex formations were clarified. Furthermore, physicochemical properties of the gene delivery vectors were determined. Before entrapment of perfluoropropane gas, the gene carrier showed approximately 150 to 250 nm particle size and −20 to −40 mV ζ-potential. Entrapment of perfluoropropane gas increased particle size and approximately 550 to 600 nm particles were formed. Intravenous administration of the ultrasound-responsive gene carrier with ultrasound exposure from abdominal area significantly improved gene expressions in the mouse liver, kidney, and spleen.

Conclusion: This biocompatible ultrasound-responsive gene carrier with ternary structure would be novel formulation for effective and secure gene delivery.

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Combining MAR elements and transposon systems for improved gene expression and integration

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Safety, integration and long-term expression of a transgene constitute a major challenge in gene therapy applications. In this study, we combined the efficiency of transgene integration of the transposon system and the anti-silencing properties of a genetic element called matrix attachment region (MAR). We observed that the addition of the MAR 1–68 in the *PiggyBac* transposon does not interfere with transposition, by maintaining high frequency of transgene integration in CHO cells. Moreover, it seems that this association leads to higher transgene expression from few transposon integration events. This property would be particularly interesting to be tested in muscle progenitor mesoangioblast cells. These cells are important candidates for future stem cell therapy for myopathic patients and known to be difficult to transfect. Encouragingly, our first experiments show that *PiggyBac* and *Sleeping Beauty 100X* systems are greatly efficient in these hard-to-transfect cells. Since *in vivo* electroporation is a possible strategy for the local treatment of muscle disorders, we are currently testing the combination of transposon and MAR using this method in mice muscle to see if transposon systems may promote sustained gene expression over time and/or increase transgene integration. Assessing efficiency and the advantages of this new association may lead to the discovery of a novel system possessing interesting properties for gene or cell-based therapy application.

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Novel carotenoid lipid vectors for ocular gene therapy

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The eye has several advantages for gene therapy: small size, low immune and inflammatory responses and minimal diffusion of drug to the systemic circulation. Cationic lipids, one of the most studied non-viral vectors, possess either rigid or non-rigid hydrophobic chains, leaving a gap in chain rigidity to be investigated. Our objective is to evaluate the efficiency of DNA delivery to human Retinal Pigmented epithelium (RPE) cells by novel cationic lipid vectors. These novel vectors, designated as C30-20 and C20-20, both possess a highly unsaturated, conjugated, rigid polyene chain, one of C30:9 and the other C20:5, respectively, plus a non-rigid saturated alkyl C20:0 chain.

Lipoplexes, formulated by solvent evaporation of ethanolic mixtures of the new polyene compound with a co-lipid, such as DOPE or cholesterol, and incubated with DNA, were characterized by gel retardation assays, and biocompatibility and transfection assays using RPE cells.

The different lipid formulations encapsulated DNA, were biocompatible with RPE cells, with better results for those with DOPE. The C20-20/DOPE formulation had transfection efficiencies above a commercial transfection agent (GeneJuice). These results show this new polyene vectors to be promising for ocular gene therapy.

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Mitochondrial Gene Targeting in Mammalian Systems using Novel 'Mitochondriotropic' Liposomes

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2. 糖修飾超音波応答性リポソームによるがん免疫治療・抗炎症治療戦略

運　敬太，川上　茂，橋田　充

個々の疾患に応じた細胞に核酸医薬品を選択的かつ高効率に導入する技術開発は，遺伝子治療のみならず*in vivo*遺伝子機能解析や疾患モデル動物作製に不可欠である．特定の細胞表面には各種糖鎖認識機構が発達しており，細胞選択的かつ高効率核酸送達の標的として期待される．また近年，細胞内への核酸導入効率を増強させる方法として，超音波などの外部刺激の利用が積極的に試みられている．本稿では，単一製剤に超音波応答性と細胞指向性を付与した超音波応答性マンノース修飾リポソーム/核酸複合体に関するわれわれの研究を中心に概説する．

はじめに

遺伝子治療はがんや嚢胞性線維症などの遺伝子変異に起因した疾患治療に有望な方法である．一方，効果的な遺伝子治療効果の達成のためには疾患に応じた標的細胞への遺伝子送達が不可欠であり，標的細胞への選択的かつ高効率な遺伝子送達を可能にする方法の開発が求められる．がん免疫療法や抗炎症療法の標的細胞となり得る抗原提示細胞※1や血管内皮細胞の細胞表面には，マンノースやガラクトース，フコース受容体などの糖鎖認識機構が発達しており，糖修飾キャリアの標的細胞として知られている．現在，糖鎖受容体介在性エンドサイトーシスに基づき，標的細胞への核酸送達を可能にする糖修飾リポソーム/核酸複合体が開発されているが，臨床において優れた治療効果を得るためには，細胞選択的かつより効率的な核酸送達を達

[キーワード&略語]
遺伝子導入，糖修飾，ソノポレーション，DNAワクチン，抗炎症治療

CTLs：cytotoxic T-lymphocytes（細胞傷害性T細胞）
ERK：extracellular signal-regulated kinase（細胞外シグナル制御キナーゼ）
ICAM-1：intercellular adhesion molecule-1（細胞間接着分子-1）
IFN-γ：interferon-gamma（インターフェロン-γ）
JNK：Jun-N-terminal kinase（Jun-N末端キナーゼ）
LPS：lipopolysaccharide（リポ多糖）
PEG：polyethylene glycol（ポリエチレングリコール）
TLR-9：Toll-like receptor-9（Toll様受容体-9）
TNF-α：tumor necrosis factor-alpha（腫瘍壊死因子-α）
VCAM：vascular cell adhesion molecule（血管細胞接着分子）

Cancer immunotherapy and anti-inflammatory therapy by sugar-modified and ultrasound-responsive liposomes

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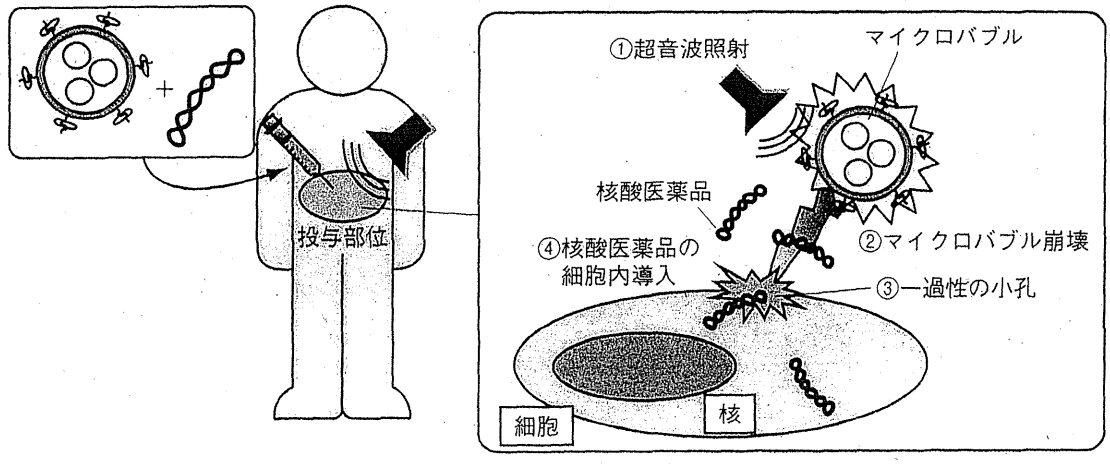

図1　ソノポレーションによる遺伝子導入法
ソノポレーションによる遺伝子導入では，超音波造影剤であるマイクロバブル製剤と超音波照射を併用する．最適強度の超音波照射によってマイクロバブル製剤が崩壊し，同時に発生するキャビテーションエネルギーによって細胞膜上に一過性の小孔が生じ，それを介して多量の核酸医薬品が細胞質内に導入される

成し得る方法論の開発が課題としてあげられる．

細胞内への遺伝子/薬物導入効率を増強するために，電気刺激[1]，物理的圧力[2]，高水圧[3]や超音波照射[4]などの外部刺激の利用が積極的に試みられている．中でも，超音波造影剤であるマイクロバブル製剤と超音波照射を併用するソノポレーション※2法では，最適強度の超音波照射によって誘導される製剤の崩壊とともに発生するキャビテーションエネルギーにより細胞膜上に一過性の小孔が生じ，それを介して多量の核酸医薬品を細胞質内に直接導入可能であることが報告されている（**図1**）[5]．さらにマイクロバブル製剤はLevovist®やSonazoid®に代表される血管造影剤として，また超音波照射機器は超音波検査や結石治療などの分野ですでに利用されているため，臨床応用の可能性が高い遺伝子導入法としても注目されている．一方で，核酸医薬品とマイクロバブル製剤を別個投与する従来のソノポレーション法では，標的臓器または標的細胞選択的に高い遺伝子発現効率を得ることは難しい．

近年，われわれはソノポレーションを利用した効率的核酸導入と糖鎖認識機構に基づく細胞選択的核酸送達の融合を図り，単一製剤に超音波応答性と標的細胞指向性を付与した超音波応答性マンノース修飾リポソーム/核酸複合体の開発研究を進めてきた（**図2**）．さらにがんに対するDNAワクチン※3や抗炎症治療への応用研究を試みてきた．本稿では，超音波応答性マンノース修飾リポソーム/核酸複合体と超音波照射併用に基づく核酸導入法および疾患治療への応用に関する最近の知見を概説する．

1 超音波応答性マンノース修飾リポソーム/核酸複合体の開発

まず，マンノース受容体認識機構と超音波照射に伴う細胞穿孔誘導機構を単一製剤内に組込むことを考え，

※1　抗原提示細胞
体内に侵入してきた細菌やウイルス感染細胞などの断片を抗原として自己の細胞表面上に提示し，T細胞を活性化する細胞．抗原提示細胞は細胞表面上に主要組織適合抗原分子（MHC分子）をもち，これに抗原が提示される．T細胞はMHC分子上に提示された抗原を認識して活性化し，免疫反応を起こす．

※2　ソノポレーション
高周波超音波（1～3 MHz）が，生細胞へ与える効果について指す言葉．マイクロバブルの存在下で超音波を適用すると，細胞膜に一時的な孔を開けることができ，そこから薬品分子，タンパク質または外来遺伝子などが細胞内に導入される．

※3　DNAワクチン
抗原情報をコードしたDNAを接種するワクチン療法．抗原提示細胞内で導入したDNAからがん抗原タンパク質が産生され，特に細胞性免疫が活性化される免疫誘導方法．

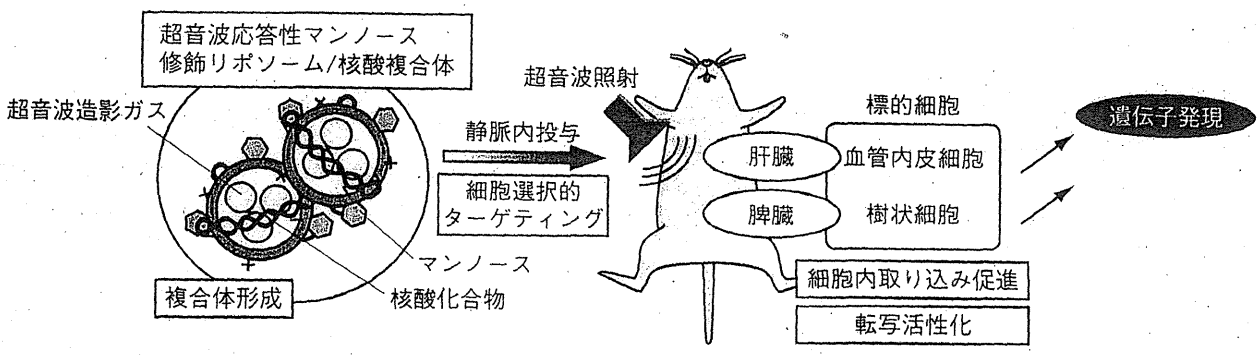

図2　超音波応答性マンノース修飾リポソーム/核酸複合体による*in vivo*遺伝子導入法
超音波応答性マンノース修飾リポソーム/核酸複合体を静脈内投与すると，マンノース受容体を豊富に発現する肝臓の血管内皮細胞や脾臓の樹状細胞に到達する．その後，対外からマウス腹部に超音波照射することで，バブル製剤の崩壊に伴い肝血管内皮細胞や脾樹状細胞の細胞膜上に一過性に生じる小孔を介して多量の核酸医薬品が細胞質内に導入される．また，超音波照射に起因した転写活性化も誘導され，これらが複合的に関与して標的細胞選択的な遺伝子発現の増強が*in vivo*において認められる

超音波応答性と標的細胞指向性を併せもつ超音波応答性マンノース修飾リポソーム/核酸複合体を構築した（**図2**）．脂質組成最適化を通じて，超音波造影ガスをリポソーム内に安定に封入するためには，ポリエチレングリコール（PEG）修飾および飽和脂肪酸が必須であり，また超音波造影ガスによる内圧に対して安定にリポソームの球形構造を維持するためには，低膜流動性脂質で製剤が構成される必要があることを見出した．次に，超音波応答性マンノース修飾リポソーム/核酸複合体と超音波照射を利用した遺伝子導入により*in vivo*遺伝子発現特性を評価した結果，マウス肝臓および脾臓において顕著に高い遺伝子発現が示され，その遺伝子発現はマンノース受容体発現細胞である肝非実質細胞や脾臓の樹状細胞において選択的に認められることを見出した（**図3**）．一方，肺や腎臓，心臓においては超音波照射に伴う遺伝子発現増強は得られなかった（**図3**）．われわれはこの原因として，肺においてはガス交換のための組織構造学的な特徴により超音波刺激が組織全体に伝播しないことに起因し，腎臓および心臓においては製剤の組織移行性が低いことに起因するものであると考えている[6]．

リポソームやエマルジョンなどを利用した従来の遺伝子導入法では，エンドサイトーシス後に遺伝子配列中のCpGモチーフがエンドソーム内のTLR-9に認識され[7]，TNF-αやIFN-γなどの炎症性サイトカイン産生に起因した組織傷害が誘導される[8]．そこでわれわれは，超音波応答性マンノース修飾リポソーム/核酸複合体と超音波照射を利用した遺伝子導入後の血清中アミノトランスフェラーゼ活性（ALT/AST）を指標とした毒性評価を行った．その結果，本遺伝子導入操作に基づく肝毒性は従来法と比較して低いことを見出した．したがって，本方法に基づく遺伝子導入は，標的細胞選択的かつ高い遺伝子発現を低毒性条件下で可能にし得る方法であると言える[6]．

2 遺伝子発現増強機構の解明

超音波応答性マンノース修飾リポソーム/核酸複合体と超音波照射を利用した*in vivo*遺伝子導入法では，従来のリポフェクション法と異なる機構で遺伝子発現が誘導されていると推察された．したがって，この機構解明は，遺伝子発現効率の改善や疾患治療への応用を考えるうえで不可欠である．そこで，*in vivo*遺伝子発現に関与する核酸医薬品の体内動態挙動，細胞内導入機構，ならびに遺伝子導入に伴う転写活性化に焦点を当て，超音波応答性マンノース修飾リポソーム/核酸複合体と超音波照射を用いた遺伝子導入機構とその支配因子について評価した．

まず，超音波応答性マンノース修飾リポソーム/核酸複合体と超音波照射による遺伝子導入時の核酸医薬品の体内動態挙動について評価した結果，製剤のマンノース修飾，ならびに超音波照射により，肝臓および脾臓への核酸送達量が増大することが示された．また

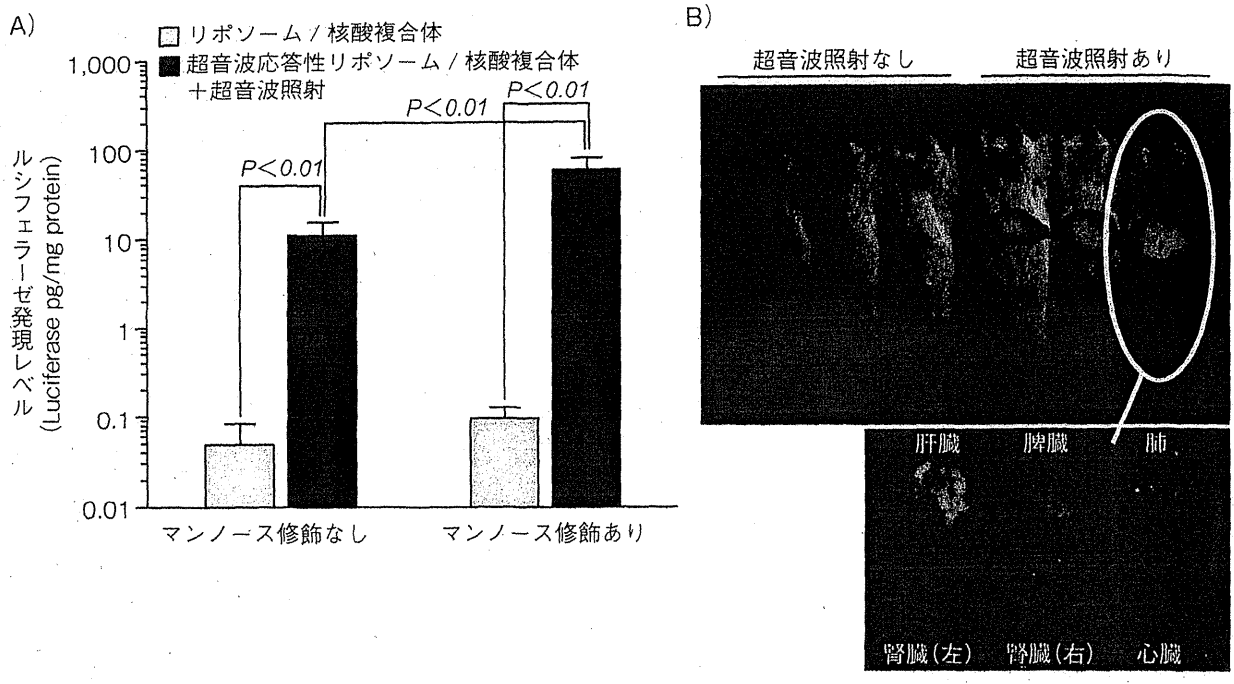

図3　マンノース修飾リポソーム / 核酸複合体と超音波照射による *in vivo* 遺伝子発現特性

A) マウス肝臓における遺伝子発現量を，ホタルルシフェラーゼをレポーター遺伝子として評価した．マンノース修飾を施した超音波応答性リポソーム / 核酸複合体静脈内投与後，超音波照射を施した群において顕著に高い遺伝子発現量が得られた

B) 超音波応答性マンノース修飾リポソーム / 核酸複合体投与後，超音波照射の有無によるホタルルシフェラーゼ活性の *in vivo* イメージング写真．超音波照射により肝臓および脾臓においてのみ高い遺伝子発現が認められ，その一方，他の臓器（肺，腎臓，心臓）では遺伝子発現の増強が認められない（文献6より転載）

ソノポレーションを利用した方法では，超音波照射によるマイクロバブル製剤の崩壊に起因して細胞膜上に生じる一過性の小孔を介して多量の核酸医薬品が細胞質内に導入される[9]．したがって，超音波照射に伴う肝臓および脾臓への核酸移行量の増大は，核酸医薬品の細胞質内導入の促進に起因するものであると推察され，超音波照射時に多量の核酸医薬品を肝臓および脾臓内に送達し得る製剤を利用した遺伝子導入において，より高い核酸医薬品の組織内移行量の増大が認められると考えられる．したがって，超音波応答性リポプレックスと超音波照射による *in vivo* 遺伝子導入においては，製剤投与後，超音波照射時における標的臓器内への核酸移行性が遺伝子発現の多寡につながることが示唆された[9]．

一方，近年物理刺激に伴うp38，ERKおよびJNK活性化に起因して転写活性化が誘導され，さらに遺伝子発現の増強が得られることが報告されている[2)3)10)11)]．そこで超音波応答性マンノース修飾リポソーム / 核酸複合体と超音波照射を利用した遺伝子発現増強に及ぼす転写活性化の関与についても評価した結果，遺伝子発現制御プロモーターにおける特定の転写因子結合部位の有無が遺伝子発現の増強に影響することが示された[12]．さらに超音波照射に伴い，上記特定の転写因子の活性化が誘導されることも示され[12]，他の物理刺激に起因した転写活性化機構と同様，本方法においても超音波照射を付与した細胞において同様の生理的変化が誘導されていることを示唆している．

このようにわれわれは，標的細胞選択的に遺伝子発現効率を増強させるために，超音波応答性マンノース修飾リポソーム / 核酸複合体と超音波照射を利用した遺伝子導入法を開発し，肝臓および脾臓のマンノース受容体発現細胞選択的かつ高効率な遺伝子発現を達成可能であることを見出した．また，この遺伝子発現増強には，①マンノース修飾による核酸医薬品の標的細胞への移行性増大，②超音波照射に起因した製剤崩壊に伴う細胞質内への核酸送達の増強，ならびに③超音

波照射に伴う転写活性化，という三因子が複合的に関与していることが示唆された．この方法は，製剤の静脈内投与と超音波照射という簡便な操作で構成され，かつ基盤技術であるマイクロバブル製剤，ならびに超音波照射機器は診断・治療などの分野ですでに臨床応用されている．したがって，がんや先天性疾患などに対する遺伝子治療への臨床応用が期待される．

3 疾患治療への応用

1）がんに対するDNAワクチンへの応用

がん抗原により付与されるがん特異免疫を利用したがん免疫療法は，標的がん細胞特異的な抗腫瘍効果が得られるとともに，治療に伴う毒性が低いという特徴を有することから，がん患者の転移・再発予防法として期待されている[13]．特にがん特異抗原発現遺伝子を利用するDNAワクチンは，液性免疫，ならびに細胞性免疫を付与できるとともに，強い殺細胞活性を示す標的がん細胞特異的な細胞傷害性T細胞（CTLs）を効率的に誘導可能である[14]．しかしながら，DNAワクチンにより優れた治療効果を得るためには，がん特異免疫誘導において重要な役割を果たす抗原提示細胞に対して選択的かつ高効率にがん抗原発現遺伝子を導入することが不可欠であるが[15]，組織内に分布する抗原提示細胞数はきわめて少なく，*in vivo*において抗原提示細胞選択的な遺伝子導入を達成することは難しい．

超音波応答性マンノース修飾リポソーム/核酸複合体を用いた遺伝子導入では，樹状細胞への選択的かつ高効率な遺伝子導入が可能となるため，DNAワクチンへの応用が期待される．そこで，がん特異抗原発現プラスミドを用いて作製した超音波応答性マンノース修飾リポソーム/核酸複合体によるDNAワクチン効果を評価した．メラノーマ関連抗原を発現するプラスミドを用いて超音波応答性マンノース修飾リポソーム/核酸複合体作製して評価した結果，製剤の静脈内投与と超音波照射併用により免疫誘導を施したマウス脾臓において，MHCクラスI分子上への抗原提示が認められ，それに伴い，メラノーマ特異的なCTL活性の誘導が認められた[16]．さらにメラノーマ特異的に高い腫瘍増殖抑制効果，ならびに生存日数の延長効果が固形腫瘍，ならびに転移性肺腫瘍に対して認められた（**図4**）[16]．メラノーマは高転移性・再発特性を有するため，患者の予後がきわめて悪い一方，さまざまな特異抗原の同定が進んでいるがん種であるため，転移・再発予防法としてのDNAワクチンの有効性が期待されている．したがって超音波応答性マンノース修飾リポソーム/核酸複合体と超音波照射を利用したDNAワクチンが，メラノーマの転移・再発に対する予防・治療法へ発展することが期待される．

2）細胞間接着分子の発現抑制に基づく抗炎症治療への応用

薬剤性肝炎や虚血再灌流性肝傷害は，臨床における薬物治療や生体肝移植などの外科手術時における有害事象として認識され，さまざまな薬物投与に伴うNF-κB活性化や活性酸素誘導，およびそれに起因した炎症性サイトカイン産生によって惹起される[17]．これらの炎症反応発生初期には，血管細胞接着分子※4（vascular cell adhesion molecule：VCAM）や細胞間接着分子（intercellular adhesion molecule：ICAM）が肝臓の血管内皮細胞上に発現誘導され，好中球などの血管内皮細胞との接着，回転，ならびに組織内浸潤に関与することが報告されている（**図5**）[18]．特にICAM-1は，炎症反応の重症化につながる好中球などの組織内浸潤に強く関与する分子であり[18]，アンチセンスDNAによるICAM-1発現抑制や抗ICAM-1抗体によるICAM-1と好中球との相互作用の阻害による炎症反応の抑制に関する研究が近年進められている．

この肝血管内皮細胞には，マンノース受容体に基づく糖鎖認識機構が発達しており，マンノース修飾キャリアの標的細胞となり得る．超音波応答性マンノース修飾リポソーム/核酸複合体を用いた遺伝子導入法は肝血管内皮細胞への選択的かつ高効率な遺伝子導入を可能にするため，ICAM-1に対するsiRNA送達に応用し，肝血管内皮細胞内にICAM-1 siRNAを効率的に送達することによる抗炎症効果を評価した．超音波応答性マンノース修飾リポソーム/核酸複合体と超音波照射を利用したICAM-1 siRNA送達では，ICAM-1発現を惹起するLPS誘導急性肝炎モデルマウスにおいて，

※4 細胞接着分子

血管細胞接着分子（VCAM）や細胞間接着分子（ICAM）などが含まれ，好中球などの細胞接着，回転，ならびに組織内浸潤に関与する．特に炎症反応発生初期に発現誘導され，炎症の重症化に関与する．