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細胞特異的・高効率なsiRNA送達法の開発と難治性肝疾患治療への展開

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厚生労働科学研究費補助金（医療機器開発　研究事業）
（総括）研究報告書

細胞特異的・高効率なsiRNA送達法の開発と難治性肝疾患治療への展開

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研究要旨：本研究では、糖修飾リポソームによる細胞特異的・高効率なsiRNA送達法を開発し、難治性肝疾患治療への応用を目指す。1年目は、肝類洞血管内皮、2年目は肝星（伊東）細胞を標的とした高効率DDSキャリア構築、3年目は、肝硬変モデルマウスを用いてsiRNA DDSによる新規治療法開発が目標である。昨年度までに、肝類洞血管内皮細胞及び肝星（伊東）細胞を標的とした糖修飾リポソームの開発に成功した。最終年度となる本年度は、siRNA (gp-46) を搭載させたマンノース6リン酸（M6P）修飾リポソーム製剤のin-vivoでの評価を行った。まず、蛍光標識siRNA (gp-46)/M6P修飾リポソームをマウス尾静脈内投与し、肝臓を分離したところ、肝臓非実質細胞への集積が認められた。四塩化炭素投与による肝硬変モデルマウスにおいて、gp-46の有意なノックダウン効果が認められた。さら肝硬変マーカーとして肝臓内のgp-46, α-平滑筋アクチン (α-smooth muscle actin; α-SMA), procollagen-1ならびに組織メタロプロテアーゼ阻害物質-1 (Tissue Inhibitor of Metalloproteinase-1; TIMP-1) mRNAの有意な抑制効果が認められ、肝硬変治療へ応用できる可能性が示された。また、バブルリポプレックス製剤の医療応用を目的としたアニオン性ナノバブルリポポリプレックスの新規調製法の開発に成功した。

A．研究目的

核酸の高効率なin vivo送達は、小動物を用いて原発臓器・細胞を対象とした遺伝子機能解析並びに病態モデル動物作成による創薬標的遺伝子/タンパク質および治療薬探索や直接作用を発揮させる遺伝子治療を通じて、創薬のためのライフサイエンス研究の進展に貢献する。その実現のためには、標的細胞に核酸医薬を送達する技術の開発が不可欠であり、現在、世界中でベクター開発が活発に行われている。とりわけ近年、siRNAを用いた標的指向DDS送達による治療効果に関する報告がなされ、今後の医療展開が期待されている。

リポソームや高分子など非ウイルスベクターによる送達は、低免疫原性、安全性、調製の容易さから広範囲な研究施設での使用やヒト適用において利点を有する。申請者らは、肝臓を構成する各細胞が固有に有し、厳密な基質認識性を有する糖鎖認識機構に認識されるように分子設計した糖修飾カチオン性リポソームを開発した。その後、全身・臓器・細胞レベルでの定量的な動態解析・最適化を行い、細胞選択的な核酸医薬送達並びに治療展開に成功した。また、糖修飾リポプレックスとバブルリポソームを別々に投与し、体外からの超音波(ultrasound; US)照射を行うことで、糖修飾リポソームの標的細胞でのキャビテーションエネルギーによる細胞穿孔に基づいた細胞質内への遺伝子導入に基づく遺伝子発現の増強に成功した。

このような背景を基盤として本研究では、申請者らが開発を進めている糖鎖による生体分子の特異的認識に基づくin vivo誘導と超音波による細胞穿孔を組み合わせた新規DDS技術を、創薬シーズが多く生体での多様な作用が期待できるsiRNA送達法に応用し開発を進める。さらに、肝類洞血管内皮並びに肝星（伊東）細胞を標的とした高効率送達による難治性肝疾患に対する新規治療法開発を目指す。

本計画は3年間であり、1年目は、マンノース修飾バブルリポソームと超音波を用いた肝類洞血管内皮への送達法開発、2年目はマンノース6リン酸（M6P）修飾リポソームを用いた肝星（伊東）細胞を標的とし、かつ、標的細胞内へ高効率にsiRNAを送達させるためのDDSキャリア開発、3年目は肝硬変モデルマウスを用い、2年目までに開発したM6P修飾リポソームによるsiRNA送達に基づく肝硬変モデルマウスにおける治療効果の確認が目標である。

昨年度までに、肝類洞血管内皮細胞及び肝星（伊東）細胞を標的とした糖修飾リポソームの開発
に成功した。最終年度となる本年度は、siRNA (gp46) を搭載させたM6P修飾リポソーム製剤のin-vivoでの評価を行った。また、1年目の研究成果を発展させるため、超音波造影ガスを封入したバブルリポソームの脂質組成の最適化を行った。

まず、siRNA (gp46)/放射標識M6P修飾リポ

ソームをマウス尾静脈内投与し、肝臓を分離したところ、肝臓非実質細胞への集積が認められた。また、四塩化炭素（CCl_4）投与による肝硬変モデルマウスにおいて、gp-46の有意なノックダウン効果が認められた。さら肝硬変マーカーとして肝臓内のgp46, α -SMA), procollagen-1 ならびにTIMP-1 mRNAの有意な抑制効果が認められた。また、新たに赤血球と相互作用しないアニオン性バブルリポポリプレックス製剤の調製に成功した。

以上、M6P修飾リポソームが肝硬変モデルマウスにおいて肝星（伊東）細胞へ細胞選択的に移行し、siRNA複合体を用いることで肝硬変治療へ応用できる可能性が示された。また、安全な超音波応答性リポソーム製剤の調製法構築に成功した。

B．研究方法

siRNA (gp-46):

gp46 siRNA 並びに scrambled siRNA は以下の配列のものを用いた（A:アデノシン、G:具アノシン、C:シチジン、U:ウリジン、T:チミジンであり、またX：リボヌクレオチド、dX：デオキシリボヌクレオチド（Xは各略号）とする)。

gp46 siRNA：センス鎖：
GUUCCACCAUAAGAUGGUAGACAACA GdTdT
アンチセンス鎖：
GUUGUCUACCAUCUUAUGGUGGAACA UdTdT
scrambled siRNA：センス鎖：
CGAUUCGCUAGACCGGCUUCAUUGCAG dTdT
アンチセンス鎖：
GCAAUGAAGCCGGUCUAGCGAAUCGA UdTdT

M6P修飾リポソームの調製：

siRNAとの複合体形成可能なカチオン性を有するM6P修飾コレステロール誘導体含有リポソームを作製するために、各種構成脂質を下記構成比率（図6）でクロロホルム中に溶解し、ナス型フラスコに分取後、ロータリーエバポレーターを用いて溶媒を減圧留去して脂質薄膜とし、減圧下で 3 時間以上乾燥した。これに5%グルコース溶液を加え、振盪機を用いた攪拌後、バス型ソニケーターにより 10 分間超音波処理後、窒素置換下チップ型ソニケーターを用いた 3 分間の超音波処理を行い、0.45 μm の孔径を有するポリカーボネート膜を用いて滅菌濾過を行った。リポソーム濃度はリン脂質またはコレステロール量を基準に測定した。

その後、M6P修飾リポソーム/siRNA（gp46）複合体を形成するため、siRNA（gp46）とM6P修飾コレステロール誘導体含有カチオン性リポソームを電荷比1.0:3.1 (-:+) として5%デキストロース中で混合して調製した。M6P修飾リポソーム/siRNA（gp46）複合体の物理化学的性質としては、ゼータサイザーナノにより平均粒子径およびζ電位を測定することで評価し、平成23年度　厚生労働省科学研究費補助金　医療機器開発推進　研究事業　総括研究報告書とほぼ同じ物理化学的性質であることを確認した。

四塩化炭素（CCl_4）誘導肝硬変モデルマウスの作製：

肝硬変モデルマウスの肝星（伊東）細胞ではM6P受容体が発現誘導されることが知られている。そこで、四塩化炭素（CCl_4）溶液（2% in olive oil, 10 mL/kg）をC57BL/6マウス腹腔内に週2回/4週間頻回投与して四塩化炭素誘導肝硬変モデルマウスを作製した。

肝臓内分布の評価：

肝硬変モデルマウスの肝星細胞においてはM6P受容体が発現誘導されることが知られているため、四塩化炭素（CCl_4）溶液（2% in olive oil, 10 mL/kg）をC57BL/6マウス腹腔内に週2回/4週間頻回投与して四塩化炭素（CCl_4）誘導肝硬変モデルマウスを作製し、当該肝硬変モデルマウスに対して[^{3}H]標識リポソーム製剤を静脈内投与し、6 時間後に肝臓をコラゲナーゼ灌流により実質細胞（PCs）及び非実質細胞（NPCs）に分画し、各画分の放射活性を細胞数で標準化して評価した。この際、リポソームの脂質組成としては、DSPC:Chol:M6P-Cholのモル比を5：35：5 (M6P (5.0)-liposome), 5：25：15 (M6P (15)-liposome), 5 : 0 : 40 (M6P (40)-liposome)

とし、リポソームにおけるM6P修飾率の割合の評価を行った。

M6P酸修飾コレステロール誘導体含有リポソーム/gp46 siRNA複合体による四塩化炭素誘導肝硬変モデルマウスにおけるgp46発現抑制効果、並びに肝硬変治療効果の評価：

M6P修飾リポソーム/gp46 siRNA複合体を電荷比1.0:3.1(-:+)として作製した。リポソームの脂質組成としては、1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) :cholesterol (Chol) :M6P-Cholのモル比を5 : 35 : 5 (M6P (5.0)-liposome), 5 : 30 : 10 (M6P (10)-liposome), 5 : 25 : 15 (M6P (15)-liposome), 5 : 20 : 20 (M6P (20)-liposome)とし、リポソームにおけるM6P修飾率の割合の評価を行った。M6P修飾コレステロール誘導体含有リポソーム/gp46 siRNA複合体 (50 μg/マウス) を頻回静脈内投与（週2回/3週間、この間、四塩化炭素（CCl_4）は週2回腹腔内投与を継続）による、肝硬変モデルマウスにおける肝臓内gp46発現抑制効果をリアルタイムPCR法ならびにウエスタンブロティング法により評価した。

四塩化炭素（CCl_4）誘導肝硬変における各種マーカーに及ぼす影響に関しては、肝硬変病態においてコラーゲン産生に関与する活性化肝星細胞のマーカー分子であるα-平滑筋アクチン(α-smooth muscle actin; α-SMA)は、肝繊維化・肝硬変に繋がるコラーゲンの前駆体であるプロコラーゲン-1(procollagen-1)、肝硬変病態において発現誘導され、コラーゲン分解等に関与する組織メタロプロテアーゼの阻害因子である組織メタロプロテアーゼ阻害物質-1(Tissue Inhibitor of Metalloproteinase-1; TIMP-1)の肝臓中でのmRNA量をリアルタイムPCR法により評価した。

ナノバブルリポポリプレックスの調製と評価：

カチオン性の高分子としてプロタミン硫酸やポリリジン、ポリアルギニンとポリリジンデンドリマーを用いてポリプレックスを調製し、DSPG (1,2-Distearoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)]), polyethylene glycol $(PEG)_{2,000}$-DSPCから構成されるアニオン性リポソームと3元複合体を調製し、その後、平成22年度総括報告書と同様の方法でパーフルオロプロパンガスを封入しナノバブルリポポリプレックスを調製した。その後、マウス赤血球との相互作用を評価した。

（倫理面への配慮）

動物実験を行うにあたり、京都大学で策定された動物実験倫理規定に従ってプロトコルを作成し、倫理委員会によって承認を受けるとともに、実験遂行時にはプロトコルを遵守した。また、環境問題および実験者の安全に十分配慮して行った。

C．研究結果　&　D．考察

1-a　M6P 修飾リポソームの肝臓内分布の評価

肝星（伊東）細胞は、コラゲナーゼ灌流法において、肝臓非実質細胞区分に回収されると考えられる。そこで、各種リポソームの肝硬変マウスにおける肝臓内分布の評価をおこなった。未修飾リポソーム(Bare-liposomes) は、肝臓実質細胞(PCs)と非実質細胞(NPCs)にほぼ同程度分布したのに対し、M6P 修飾リポソームでは、肝非実質細胞選択的なリポソームの分布が認められ、また、肝非実質細胞への移行割合は、M6P 修飾率の増大に従い増加することが示された (図１)。

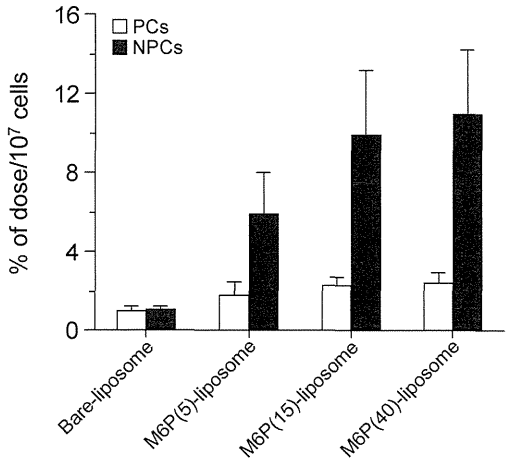

図１M6P 修飾リポソームの肝硬変病態時の肝臓内移行特性

1-b　M6P 酸修飾コレステロール誘導体含有リポソーム/gp46 siRNA 複合体による四塩化炭素誘導肝硬変モデルマウスにおける gp46 発現抑制効果、並びに肝硬変治療効果の評価：

gp46はコラーゲン産生に関与するシャペロンタンパク(ヒトにおいてはHSP47)であり、肝硬変病態時において発現誘導されることが報告されており（Niitsu et. al., Nature Biotech.. 26, 431-442, 2008）、当該遺伝子の抑制によりコラーゲン産生が抑制され、肝硬変進行の抑制並びに治療が達成されると考えられる。そこで、gp46に対するsiRNAを設計し、肝星（伊東）細胞へ送達後のgp46遺伝子ノックダウン効果と肝硬変に関連する各種マーカー遺伝子の発現変動の評価を行った。

糖修飾リポソーム/siRNA複合体の物理化学的性質は、細胞選択的ターゲティングを実現する上で重要な因子である。そこで、M6P修飾リポソーム/gp46 siRNA複合体での細胞選択的ターゲティングを行う為、細胞選択的核酸ターゲティングに適している範囲の電荷比である1.0:3.1(-:+)で調製した(S. Kawakami et al., Pharm Res, 17 (3), 306-313, 2000; S. Kawakami et al., Pharmazie, 59 (5), 405-408, 2004, Y. Kuramoto et al., J Gene Med. 10 (4), 392-399, 2008 他)。

実験の結果、四塩化炭素（CCl_4）誘導肝硬変モデルマウス(control)においてgp46の発現誘導が認められた。一方、M6P修飾コレステロール誘導体含有リポソーム/gp46 siRNA静脈内投与により、mRNA並びにタンパク質レベルで抑制された(**図2**)。また、このgp46発現抑制効果はM6P修飾コレステロール誘導体を方法に記載しているように、5, 10, 15, 20%と増加させ、M6P修飾コレステロール誘導体の含有量の影響を検討したところ、M6P含量に依存してgp46遺伝子ノックダウン効果は増大し、M6P修飾コレステロール誘導体含有量15-20%（M6P (15)-liposome, M6P (20)-liposome）で最大となった。一方、gp46 siRNA　scrambled siRNAでは、gp46の発現抑制効果は認められず、この遺伝子ノックダウン効果は、siRNAの配列に依存したものであることが示された。

gp46遺伝子ノックダウンの結果を基に、M6Pの修飾割合を15%に固定し(M6P (15)-liposome)、肝硬変モデルマウスに対し、M6Pリポソーム/gp46 siRNA複合体を頻回静脈内投与後の、肝臓内gp46、α-SMA、procollagen-1並びにTIMP-1発現量を評価した結果、M6P修飾リポソーム/gp46 siRNA静脈内投与に伴うgp46の発現抑制に伴い、いずれの因子も顕著抑制された(図3)。一方、gp46 siRNA scrambled siRNAでは、gp46の発現抑制効果は認められ、これらの遺伝子

ノックダウン効果は、siRNAの配列に依存したものであることが示唆された。

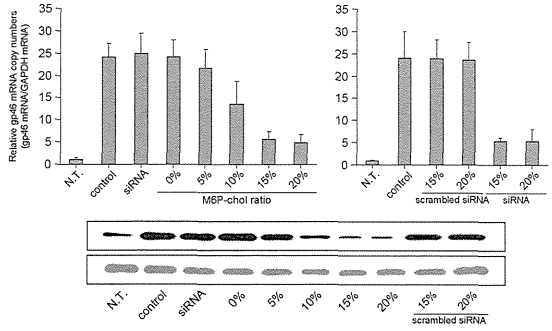

図2 M6P修飾リポソーム/gp46 siRNA複合体による肝臓内gp46発現抑制効果

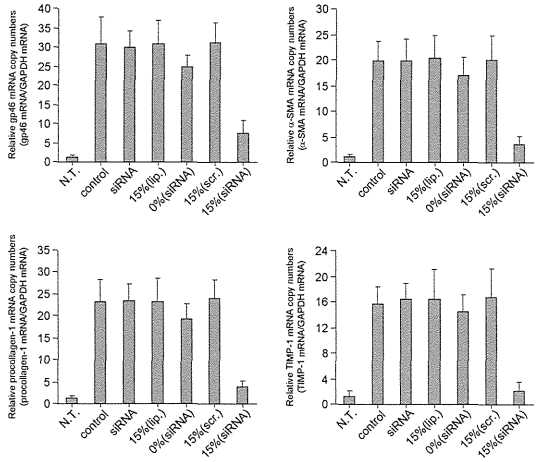

図3 M6P修飾リポソーム/gp46 siRNA複合体による各種肝硬変マーカー抑制効果

2-a　生体適合性材料を用いたナノバブルリポポリプレックス調製法の開発：

2010年度に肝類洞血管内皮細胞を標的として核酸の高効率なin vivo送達を実現できる超音波応答性マンノース修飾バブルリポプレックス調製法を開発に成功した (K. Un, S. Kawakami et al., Hepatology, 56(1), 259-269, 2012)。しかしながら、本製剤はカチオン性を示すことから赤血球と静電的な相互作用により毒性を示すことが考えられ、物理化学的性質に関して改善する余地が残されている。

そこで本研究成果の臨床応用に向けて本年度は、生体適合性材料を用いたアニオン性のナノバブルリポポリプレックス調製法の開発を行った。

ナノバブルリポポリプレックスの最適な組成をプロタミン硫酸、DSPGを用いたアニオン性リポソームを用いて調製することで、安定な超音波造影ガス封入が可能であることを見出した。また本製剤は、赤血球との相互作用も無く、安全な核酸デリバリーが行えることが示唆された。今後は、本研究成果の臨床応用に向け、アニオン性バブル製剤の調製に成功した。

今後、このアニオン性のナノバブルリポポリプレックスをプラットホーム製剤として臨床応用に向けたDDSの開発を進める予定である。

E．結論

開発したM6P修飾リポソームが肝硬変モデルマウスにおいて肝星（伊東）細胞へ細胞選択的に移行し、siRNA複合体を用いることで肝硬変治療へ応用できる可能性が示された。また、平成22年度の研究成果の臨床応用への展開を目的に、アニオン性バブルリポソーム製剤の新規調製法に成功した。

本製剤は、生体内に分布するM6P受容体発現細胞に対して効率的に低分子化合物やタンパク質、核酸化合物を送達することができると考えられる。また、これらの方法論は超音波造影ガス封入バブルリポソーム製剤化により、外部刺激の利用によるターゲティングの精度を大きく向上させることができる。今後、得られた知見を基に、M6P受容体を標的とした細胞選択的ターゲティングシステムに基づく新たな肝疾患治療法を開発していく予定である。

F．健康危険情報　なし

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H．知的財産権の出願・登録状況
（予定を含む。）

1. 特許出願　　2件

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2. 実用新案登録　　なし

3. その他　　なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
該当無し							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Keita Un, Shigeru Kawakami†, Mitsuru Yoshida, Yuriko Higuchi, Ryo Suzuki, Kazuo Maruyama, Fumiyoshi Yamashita, Mitsuru Hashida† (†: corresponding authors)	Efficient suppression of ICAM-1 using ultrasound-responsive and mannose-modified lipoplexes inhibits acute hepatic inflammation	**Hepatology**	56 (1)	259-269	2012
Tomoaki Kurosaki, Shigeru Kawakami, Ryo Suzuki, Kazuo Maruyama, Hitoshi Sasaki, Mitsuru Hashida	Novel ultrasound-responsive gene carrier with ternary structure	**Human Gene Therapy**	23 (10)	A141-A142	2012
運 敬太、川上 茂、橋田 充	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	実験医学増刊	30 (7)	135-141	2012
川上 茂† (†: corresponding author)	外部刺激を利用したin vivo核酸デリバリー法の開発と評価	薬剤学	73 (3)	153-160	2013

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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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Additional Supporting Information may be found in the online version of this article.

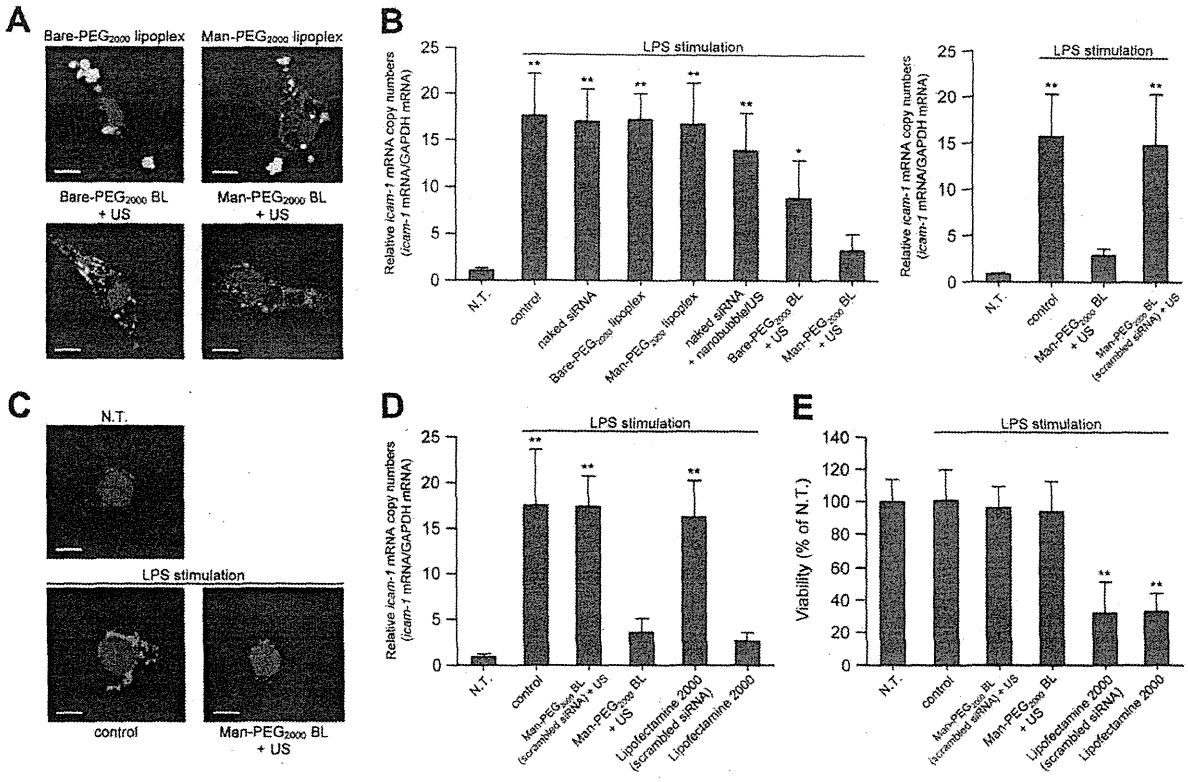

Fig. 1. Suppression effects of *icam-1* mRNA expression and cytotoxicity followed by ICAM-1 siRNA delivery in LPS-stimulated primary mouse HECs. (A) *In vitro* confocal images of cellular associated ICAM-1 siRNA (1 μg siRNA) transferred by various methods 1 hour after treatment in primary mouse HECs. 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).

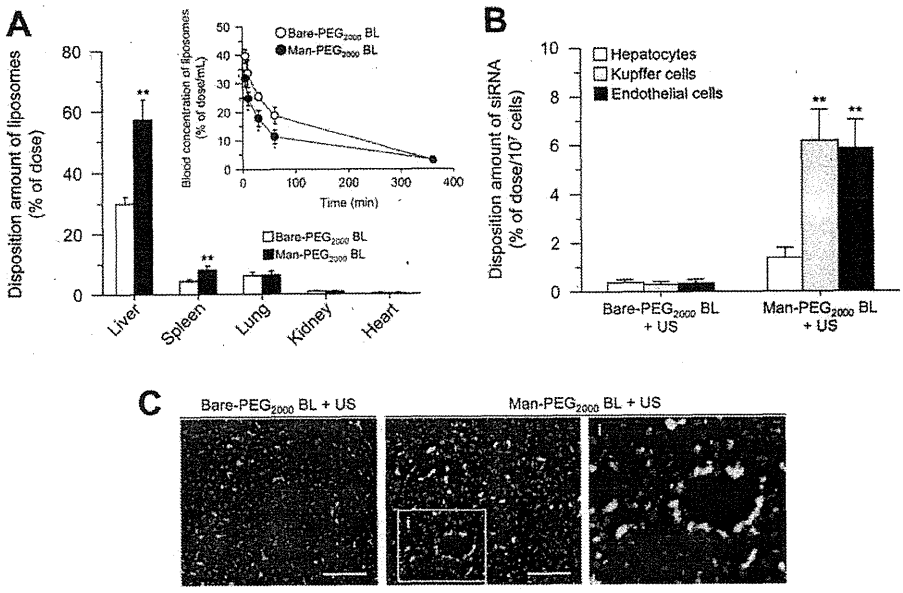

Fig. 2. *In vivo* distribution of ICAM-1 siRNA delivered by Man-PEG_{2000} BLs and US exposure. (A) Tissue distribution and pharmacokinetics of radiolabeled bare- and Man-PEG_{2000} BLs complexed with 10 μg ICAM-1 siRNA after intravenous (iv) administration into mice. Tissue distribution of lipoplexes 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 ± 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-

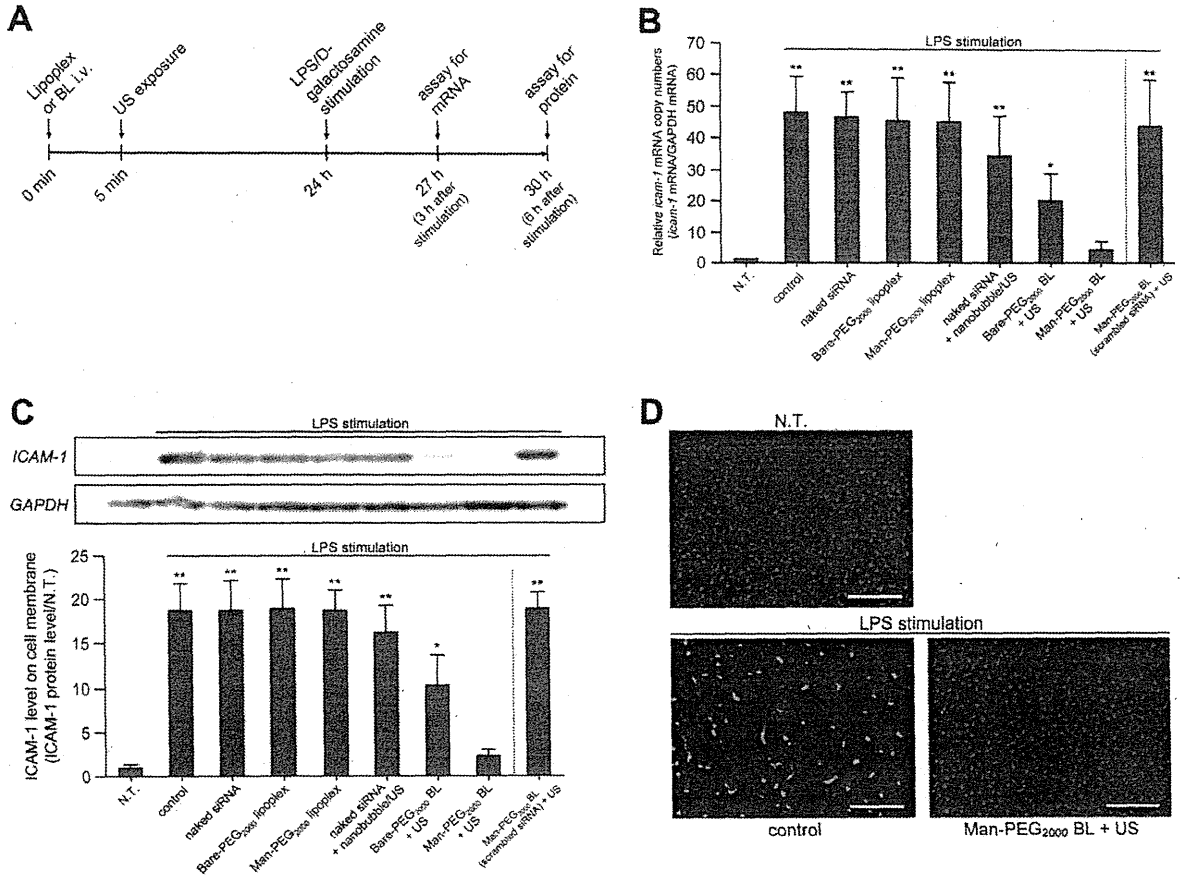

Fig. 3. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on *icam-1* mRNA and protein expression in HECs of an LPS/D-galactosamine-induced inflammatory mouse model. (A) Evaluation schedule of ICAM-1 expression in LPS/D-galactosamine-stimulated mice. (B-D) The expression 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

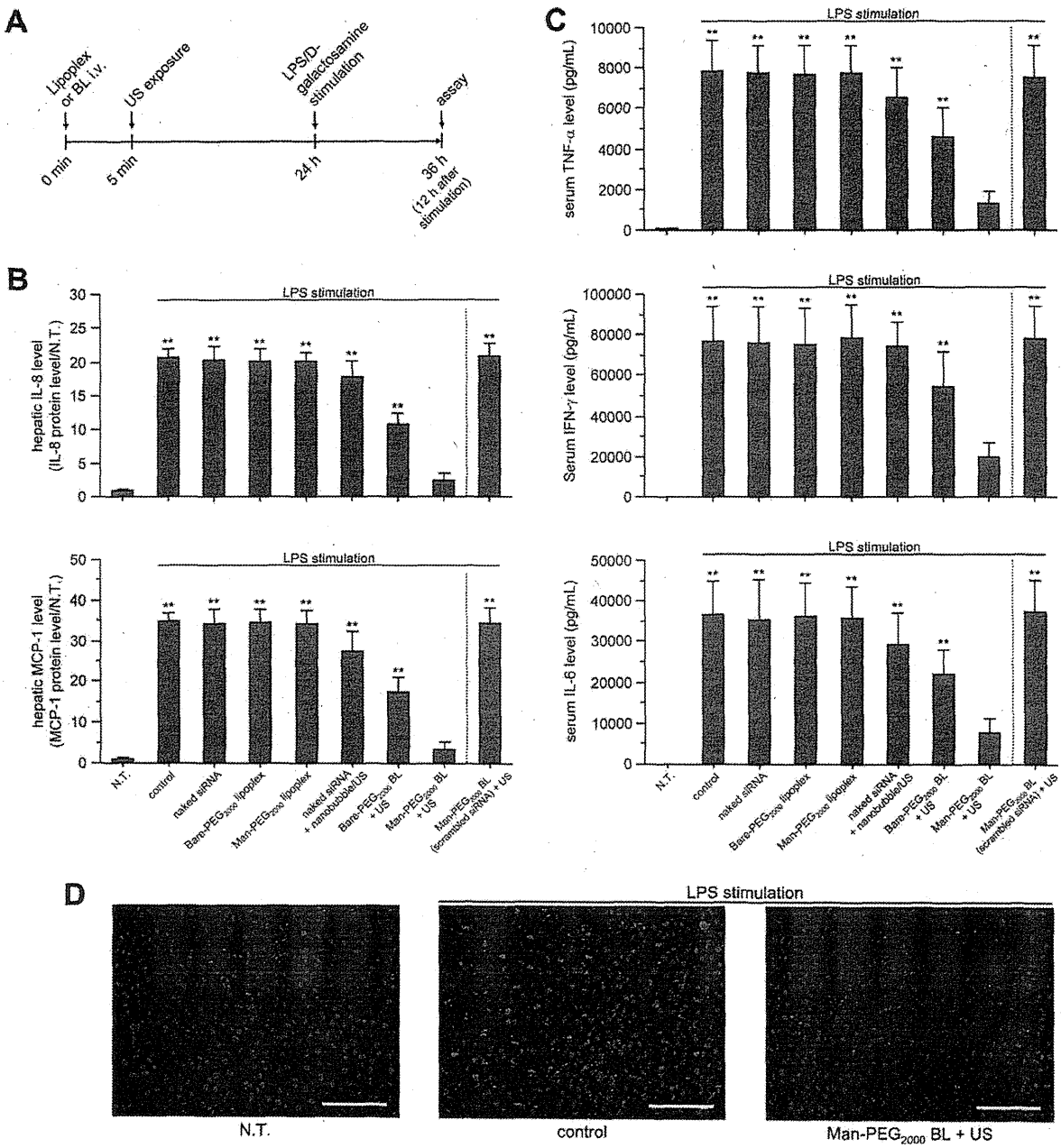

Fig. 4. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on leukocyte infiltration and proinflammatory cytokine production in an LPS/D-galactosamine-induced inflammatory mouse model. (A) Evaluation schedule of leukocyte infiltration and proinflammatory cytokine production 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

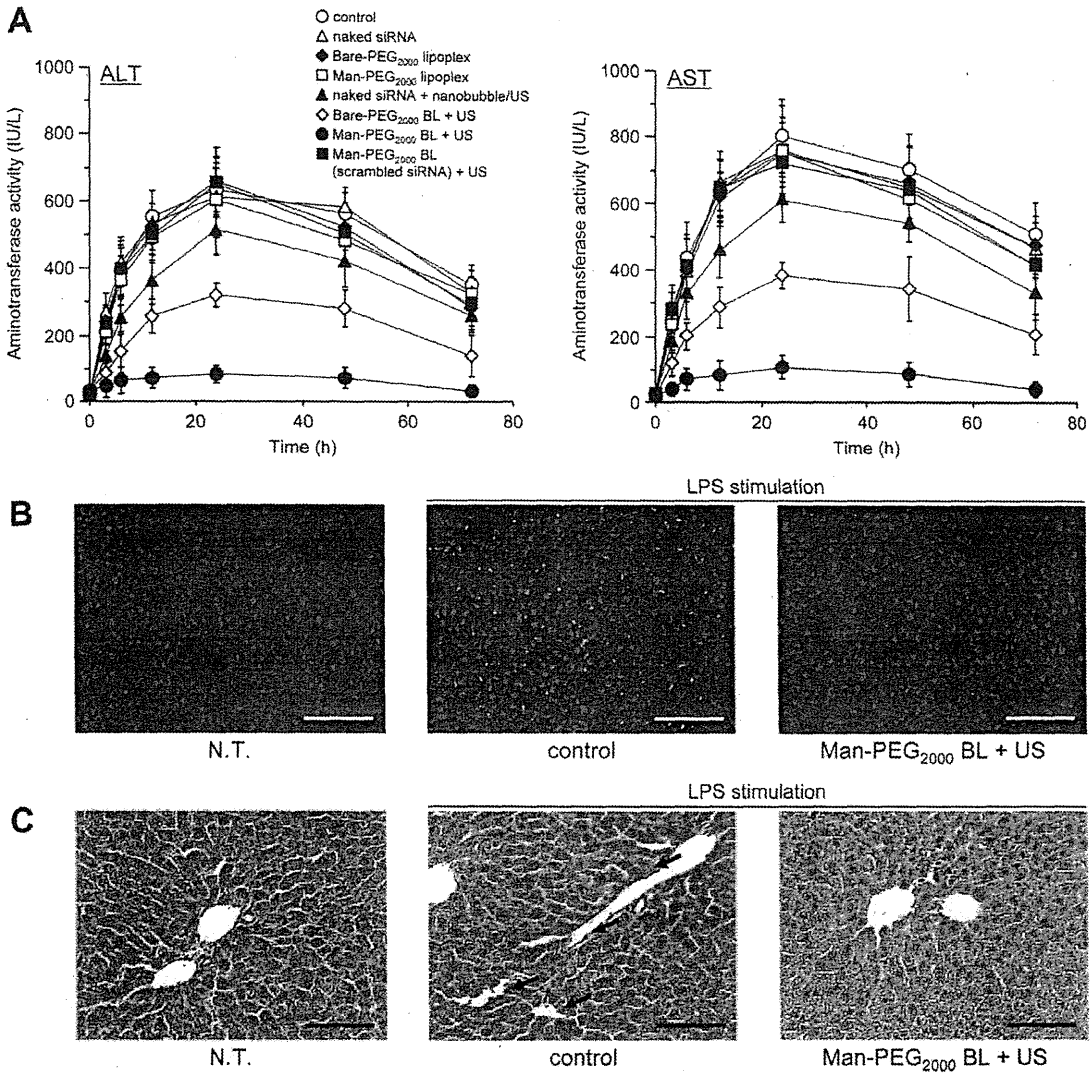

Fig. 5. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on liver toxicity in an LPS/D-galactosamine-induced inflammatory mouse model. (A) The level of serum ALT/AST activities after siRNA delivery (10 μg siRNA) using various methods at predetermined times after LPS/D-galactosamine 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

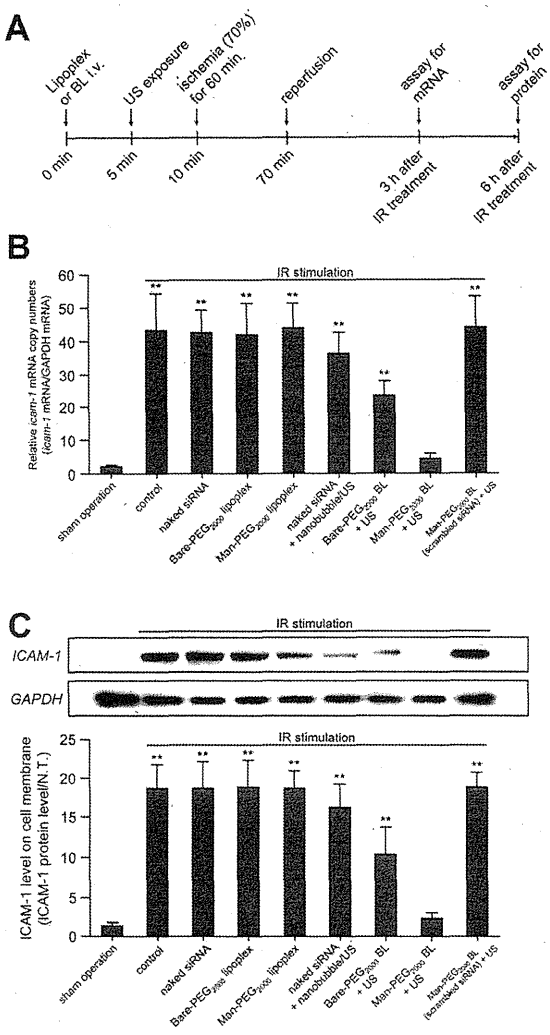

Fig. 6. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on *icam-1* mRNA and protein expression in HECs of an IR-induced hepatic inflammatory mouse model. (A) Evaluation schedule of ICAM-1 expression in hepatic IR-stimulated mice. (B,C) Expression level of *icam-1* 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

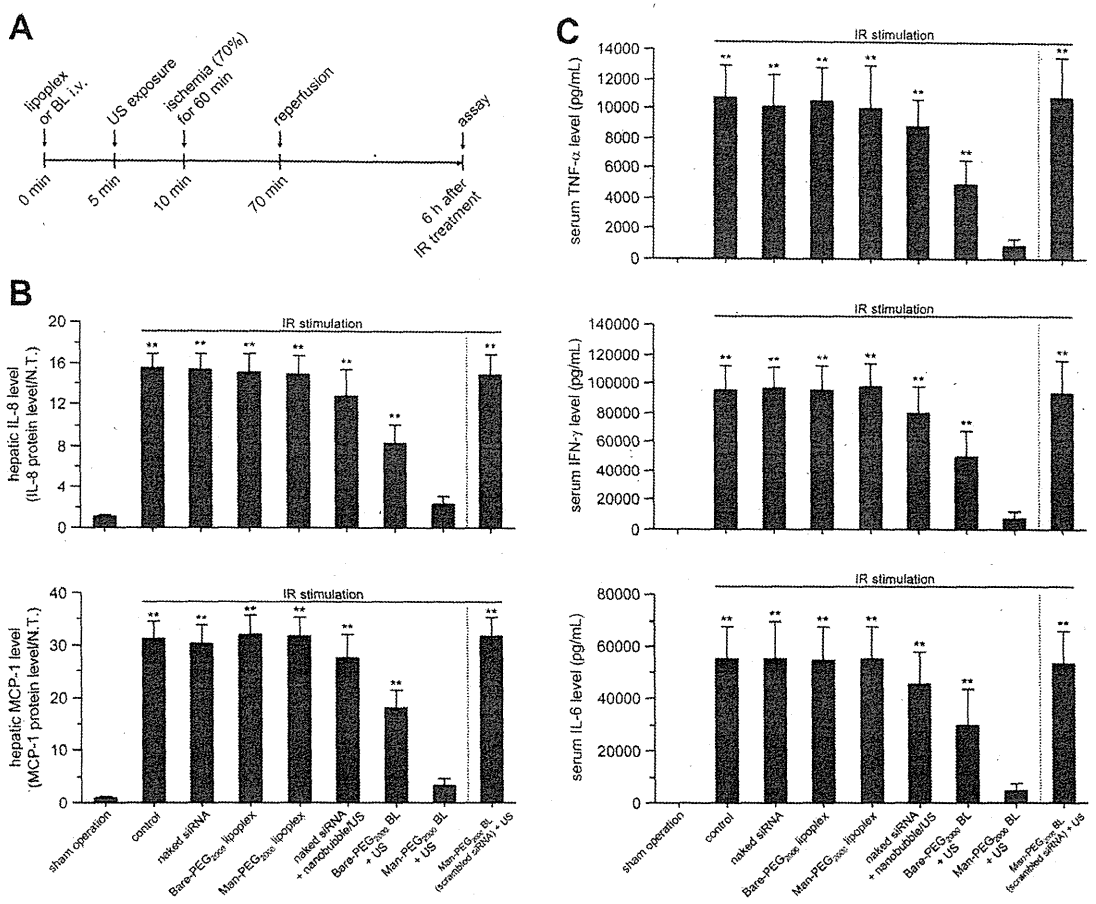

Fig. 7. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on leukocyte infiltration and proinflammatory cytokine production in IR-induced hepatic inflammatory mouse model. (A) Evaluation schedule of leukocyte infiltration and proinflammatory cytokine production in 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)

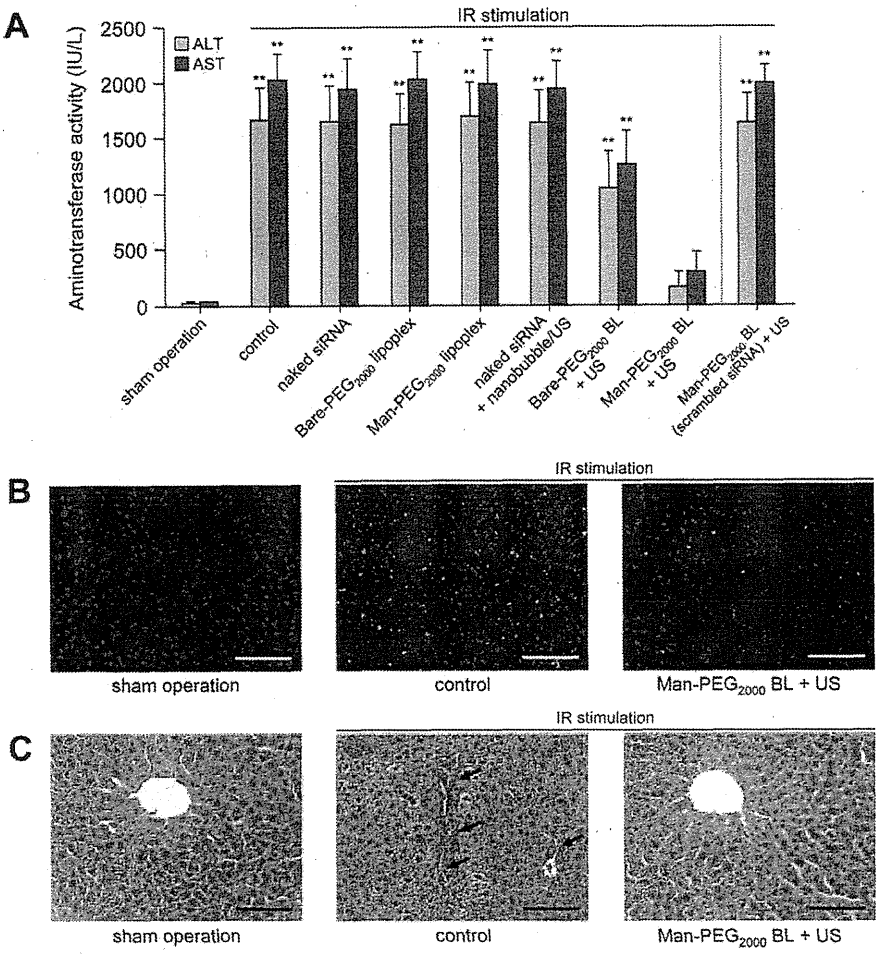

Fig. 8. Suppression effects of ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure on liver toxicity in an IR-induced hepatic inflammatory mouse model. (A) The level of serum ALT/AST activities after siRNA delivery (10 μg siRNA) using various delivery methods 24 hours after hepatic IR stimulation. $^{**}P <$ 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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