Antitumor effect of nuclear factor-κB decoy transfer by mannose-modified bubble lipoplex into macrophages in mouse malignant ascites

Yusuke Kono, Shigeru Kawakami, Yuriko Higuchi, Kazuo Maruyama, Fumiyoshi Yamashita and Mitsuru Hashida

1Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto; 2Division of Analytical Research for Pharmacoinformatics, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki; 3Department of Biopharmaceutics, School of Pharmaceutical Science, Teikyo University, Tokyo; 4Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan

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Correspondence
Shigeru Kawakami, 1-14, Bunkyo-mahci, Nagasaki 606-8501, Japan.
Tel: +81-95-819-2450; Fax: +81-95-819-2450; E-mail: skawakam@nagasaki-u.ac.jp

Mitsuru Hashida, 46-29 Yoshida-shimoadachi-cho, Sakyoku, Kyoto 606-8501, Japan.
Tel: +81-75-753-4545; Fax: +81-75-753-4575; E-mail: hashidam@pharm.kyoto-u.ac.jp

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A variety of abdominal tumors, such as ovarian, pancreatic, gastric, and colorectal malignancies, are often accompanied by MAs. Patients with MAs display several symptoms, including abdominal pain, respiratory distress, nausea, and anorexia, resulting in a significant reduction of their quality of life. In addition, MAs are associated with poor prognoses.

Malignant ascites are known to contain an abundance of leukocytes. In particular, macrophages are major components of the MA microenvironment, and these TAMs are critically involved in MA progression. It has been reported that either depletion or functional regulation of TAMs suppresses the accumulation of ascites and proliferation of cancer cells.

Macrophages are generally classified into two phenotypes, M1 and M2, according to their characteristics of humoral factor production and gene expression. M1 (classically activated) macrophages have an antitumor potency based on the expression of Th1 cytokines (e.g. IL-12, TNF-α, and IL-6) and nitric oxide production. In contrast, M2 (alternatively activated) macrophages show tumor-promoting effects through expression of several cytokines and pro-tumor factors, such as IL-10, VEGF, and MMPs. Because a large proportion of TAMs are known to show the M2 phenotype, phenotypic conversion of TAMs from M2 to M1 would be a promising approach for MA treatment.

Recently, it has been reported that inhibition of NF-kB expression and activation can suppress the phenotypic conversion of TAMs to M2. We have previously reported that NF-kB inhibition using oligonucleotides, such as siRNA and NF-kB decoy oligonucleotides, show the potential to convert the phenotype of TAMs from M2 toward M1. Recently, we have developed US-responsive, mannose-modified liposome /NF-kB decoy complexes (Man-PEG bubble lipoplexes) that may introduce a NF-kB decoy efficiently into TAMs existing in ascitic volume and number of Ehrlich ascites carcinoma cells in ascites, and prolonged mouse survival. In conclusion, we transferred a NF-kB decoy efficiently by Man-PEG bubble lipoplexes with US exposure into TAMs, which may be a novel approach for MA treatment.

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In the present study, we determined the efficiency of NF-κB decay transfection by i.p. injection of Man-PEG bubble lipoplexes combined with transdermal US exposure in the abdominal area of EAC-bearing mice. Furthermore, we investigated the effects of NF-κB decay transfection by this method on the tumor-promoting phenotype of TAMs as well as MA progression.

Materials and Methods

**Animals and cell lines.** Female ddY mice (4–5 weeks old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (Bethesda, MD, USA) and the Guidelines for Animal Experiments of Kyoto University (Kyoto, Japan). The protocol was approved by the Kyoto University Animal Experimentation Committee (approval no. 2013-44). All surgery was carried out under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The EAC cells were obtained from the Riken Bioresource Center (Osaka, Japan). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO₂.

**Nuclear factor-κB decay oligonucleotides.** Nuclear factor-κB decay oligonucleotides, FAM-labeled NF-κB decay oligonucleotides, and random decoy oligonucleotides were phosphorothioated and kindly provided by AnGes MG (Osaka, Japan). The sequences of the decoy were as follows. Nuclear factor-κB decoy: sense, GGAGGAAAATCCCTTCAAGG and antisense, CCTCCCCCTTAGGGAAGTTCC; random decoy: sense, TTGCGGTACCTGACTTAGGC and antisense, AACGGCATG GACTGAATCGG.

**Construction of Man-PEG bubble lipoplexes.** Man-PEG bubble lipoplexes were constructed according to our previous reports. Briefly, to produce the liposomes for bubble lipoplexes, DSDAP, DSPC, and NH₂-PEG-DSP (Avanti Polar Lipids Inc., Alabaster, AL, USA) or mannose-modified PEGDSP (Man-PEG-DSP) were mixed in chloroform at a molar ratio of 7:2:1. The liposome construction mixture was dried by evaporation, vacuum desiccated, and the resultant lipid film was resuspended in a sterile 5% glucose solution. After hydration for 30 min at 65°C, the dispersion was sonicated for 10 min in a bath-type sonicator and then in a tip-type sonicator for 3 min to produce liposomes. The liposomes were sterilized by passing through a 0.45-μm membrane filter (Nihon-Millipore, Tokyo, Japan). Lipoplexes were prepared by gently mixing with equal volumes of NF-κB decoy and liposome solution at a charge ratio of 1.0:2.3 (−/−). To enclose US imaging gas in lipoplexes, perfluoropropane gas (Takachiho Chemical Industries, Tokyo, Japan) was applied to prepare lipoplexes under pressure, which were sonicated using a bath-type sonicator (AS ONE, Osaka, Japan) for 5 min. The particle sizes and ζ-potentials of liposomes/lipoplexes were determined by a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK).

**Separation of F4/80⁺ cells (TAMs) from tumor ascites.** Ehrlich ascites carcinoma cells (1 × 10⁶ cells) were inoculated i.p. into mice. The ascites were then harvested at predetermined times post-inoculation. F4/80⁺ cells were separated by magnetic cell sorting with a phycoerythrin-labeled anti-mouse F4 sol:80 antibody (Bay Bioscience, Hyogo, Japan), phycoerythrin-positive selection kit, and EasySep (Veritas, Tokyo, Japan) following the manufacturer’s instructions.

**In vivo internalization study.** At 4 days post-i.p. inoculation of EAC cells into mice, 200 μL of bubble lipoplexes constructed with the FAM-labeled NF-κB decay (10 μg NF-κB decoy) was injected i.p. At 5 min post-injection, US (frequency, 1.056 MHz; duty, 50%; burst rate, 10 Hz; intensity, 1.0 W/cm²; time, 2 min) was exposed transdermally to the abdominal area using a Sonopore-4000 sonicator (NEPA GENE, Chiba, Japan) with a probe of 20 mm in diameter. At 1 h post-injection, the ascites were harvested to separate the TAMs. The cell-associated fluorescence in 10 000 cells was measured using a BD FACSCanto II Flow Cytometer (Becton Dickinson, Tokyo, Japan).

**Measurement of intranuclear NF-κB.** At 4 days after inoculation of EAC cells into mice, we carried out in vivo NF-κB decay transfection. After 12 h of in vivo NF-κB decay transfection, the ascites were harvested, and TAMs were separated from ascites. Nuclear extracts of the TAMs were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Nuclear proteins were stored at −80°C until use. The protein concentration was measured with a Protein Quantification Kit (Dojindo Molecular Technologies, Tokyo, Japan). The amounts of p50 and p65, which are the components of NF-κB, in the nuclear extracts were measured using a TransAM NFkB Family Kit (Active Motif) according to the recommended procedures.

**Quantitative RT-PCR.** At 4 days after inoculation of EAC cells into mice, we carried out in vivo NF-κB decay transfection. After 24 h of in vivo NF-κB decay transfection, the ascites were harvested, and TAMs were separated from ascites. Total RNA was isolated from TAMs using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of mRNA was carried out using a PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). Detection of cDNAs (IL-10, IL-12p70, TNF-α, IL-6, VEGF-A, and GAPDH) was carried out by real-time PCR using SYBR Premix Ex Taq (Takara Bio) and a LightCycler Quick System 3500 (Roche Diagnostics, Indianapolis, IN, USA). The primers for IL-10, IL-12, TNF-α, IL-6, and GAPDH cDNAs were as follows: IL-10, 5’-GCT CTT ACT GAC TGG CAT GAG-3’ (forward) and 5’-CGC AGT CCT AGG AGC ATG TG-3’ (reverse); IL-12, 5’-ACT CTG CGC CAA CCT C-3’ (forward) and 5’-CAC CCT GGT GAT GGC CAC GAC-3’ (reverse); TNF-α, 5’-CCT CCC TCT CAT CAG TTC TA-3’ (forward) and 5’-ACT TGG TGG TTT GCT ACG AC-3’ (reverse); IL-6, 5’-TAG TCC TTC CTT CTC CCA GTA TTT CC-3’ (forward) and 5’-TTG GCT TTC AGC GAC TTC TCC-3’ (reverse); VEGF-A, 5’- AGC ACA GCA GAT GTG AAT GC-3’ (forward) and 5’-AAT GCT TCC TCC GCT CTG AA-3’ (reverse); and GAPDH, 5’-TCT CCT GCC AGC ACT ACA-3’ (forward) and 5’-GCT GTA GCC GTA ATT GT-3’ (reverse).

**Measurement of VEGF concentrations in ascites.** At 2 days after inoculation of EAC cells into mice, in vivo NF-κB decay transfection was carried out three times every other day (days 2, 4, and 6 after inoculation of EAC cells). Ascites were har-
vested at 10 days after EAC cell inoculation. The ascites were centrifuged at 10,000 g for 10 min at 4°C and the resultant supernatant was applied to a commercial ELISA kit (Pepro-Tech, Rocky Hill, NJ, USA) to measure the concentration of VEGF.

**Determination of angiogenesis.** At 2 days after inoculation of EAC cells, in vivo NF-kB decoy transfection was carried out three times every other day (days 2, 4, and 6 after inoculation of EAC cells). At 10 days after EAC cell inoculation, the mice were photographed, their peritoneum was cut open, and then the inner lining of the peritoneal cavity was photographed.

**Measurement of ascitic fluid.** At 2 days after inoculation of EAC cells, in vivo NF-kB decoy transfection was carried out three times every other day (days 2, 4, and 6 after inoculation of EAC cells). At 10 days after EAC cell inoculation, the volume of the ascitic fluid was determined by aspiration with a needle and syringe.

**Measurement of the number of EAC cells in ascites.** To measure EAC cell numbers, EAC cells with stable expression of the firefly luciferase gene (EAC/Luc) were established as reported previously. At 2 days after inoculation of EAC/Luc cells into mice, in vivo NF-kB decoy transfection was carried out three times every other day (days 2, 4, and 6 after inoculation of EAC cells). After 10 days of EAC/Luc cell inoculation, 500 μl ascites were harvested and mixed with an equal volume of lysis buffer (0.05% Triton X-100, 2 mM EDTA, and 0.1 M Tris, pH 7.8). Then the mixed solution was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was mixed with luciferase assay buffer (Pigacene; Toyobo, Tokyo, Japan) and the luciferase activity was measured in a luminometer (Lumat LB 9507; EG&G Berthold, Bad Wildbad, Germany). The luciferase activity in the ascites was converted to the number of EAC/Luc cells using a regression line.

**Measurement of the body weight and survival rates of EAC-bearing mice.** The body weight of EAC-bearing mice was measured every day until the death of the first mouse. The survival of mice was monitored up to 60 days after EAC cell inoculation.

**Statistical analysis.** Results are presented as the mean ± SD of more than three experiments; ANOVA was used to test the statistical significance of differences among groups. Two-group comparisons were carried out using Student’s t-test. Multiple comparisons between control groups and other groups were carried out by Dunnett’s test, and multiple comparisons between all groups used the Tukey-Kramer test. Survival curves were compared using the log-rank test.

**Results**

**Physicochemical properties of bubble lipoplexes.** The physicochemical properties of bubble lipoplexes were evaluated by measuring the particle sizes and ζ-potentials. Mean particle sizes and ζ-potentials of Bare-PEG and Man-PEG bubble lipoplexes were approximately 550 nm and ±20 mV, respectively (Table 1). These results are comparable with our previous report. In vivo NF-kB decoy transfer into TAMs. We carried out in vivo TAM-targeted NF-kB decoy transfer by Man-PEG bubble lipoplexes constructed with a FAM-labeled NF-kB decoy combined with US exposure. The amount of FAM-labeled NF-kB decoy introduced into TAMs by Man-PEG bubble lipoplexes and US exposure was eightfold higher than that by Man-PEG bubble lipoplexes without US exposure (Fig. 1). Moreover, this level of FAM-labeled NF-kB decoy transfer by Man-PEG bubble lipoplexes and US exposure was much higher than that by Bare-PEG bubble lipoplexes and US exposure. Similarly, intranuclear p50 and p65 levels in TAMs were significantly decreased by NF-kB decoy transfer using Man-PEG bubble lipoplexes and US exposure. In addition, the concentration of VEGF in the ascites was significantly decreased by NF-kB decoy transfer using Man-PEG bubble lipoplexes and US exposure (Fig. 2).

**Change of cytokine mRNA expression in TAMs.** Because the phenotype of macrophages is generally classified according to the profile of cytokine expression, we assessed the expression levels of cytokine mRNA in TAMs transfected with the NF-kB decoy. Expression of IL-10 mRNA in TAMs transfected with the NF-kB decoy by Man-PEG bubble lipoplexes and US exposure was the lowest among all groups (Fig. 3a). In contrast, mRNA expression levels of IL-12, TNF-α, and IL-6 in TAMs transfected with the NF-kB decoy by Man-PEG bubble lipoplexes and US exposure were significantly higher compared with those in other groups (Fig. 3b-d).

**Suppressive effect of NF-kB decoy transfer on tumor angiogenesis.** Angiogenesis is a critical event for the progression of MA (30-32). Because VEGF predominantly regulates angiogenesis (33,34), the level of VEGF in tumor ascites and the expression levels of VEGF mRNA in TAMs were measured after NF-kB decoy transfer into TAMs. As shown in Figure 4(a), the concentration of VEGF in the ascites was significantly low in mice transfected with the NF-kB decoy by Man-PEG bubble lipoplexes and US exposure. In addition, expression of VEGF mRNA in TAMs transfected with the NF-kB decoy by Man-PEG bubble lipoplexes and US exposure was the lowest among all groups (Fig. 4b). Moreover, we evaluated the degree of angiogenesis in the peritoneum after NF-kB decoy transfer into TAMs. As shown in Figure 4(c), suppressed angiogenesis in the peritoneum was observed in mice transfected with the NF-kB decoy by Man-PEG bubble lipoplexes and US exposure.

**Antitumor effects of NF-kB decoy transfer into TAMs on EAC-bearing mice.** Finally, we examined the antitumor effects of NF-kB decoy transfer into TAMs using Man-PEG bubble lipoplexes and US exposure against MA. The strongest inhibitory effect on EAC cell proliferation in ascites and ascitic fluid was observed by NF-kB decoy transfer into TAMs using Man-PEG bubble lipoplexes and US exposure (Fig. 5a,b). In addition, the increase of body weight was significantly suppressed and the survival of EAC-bearing mice was significantly prolonged by NF-kB decoy transfer into TAMs.
TAMs using Man-PEG bubble lipoplexes and US exposure (Fig. 5c,d).

Discussion

In this study, we applied the combined use of Man-PEG bubble lipoplexes and US exposure for TAM-targeted delivery of a NF-κB decoy in EAC-bearing mice. Man-PEG bubble lipoplexes possess the following two hallmarks: (i) mannose receptor-mediated cell-selective targeting; and (ii) direct delivery of nucleic acids into the cytoplasm by “sonoporation.”(28,35) Because TAMs are known to express high levels of mannose receptor on their surface,(9,38) we hypothesized that Man-PEG bubble lipoplexes would achieve efficient delivery of a NF-κB decoy into TAMs in EAC-bearing mice. We showed that the combined use of Man-PEG bubble lipoplexes with US exposure was able to introduce the NF-κB decoy into TAMs. In contrast, p50/p50 homodimers inhibit transcriptional activation.(40–43) Saccani et al. and Porta et al. have reported that abundant nucleic accumulation of the p50 NF-κB subunit in TAMs inhibits M1 polarization, and ablation of the p50 subunit prevents TAM polarization to the M2 phenotype.(24,25) The NF-κB decoy used in this study disrupted NF-κB activity by binding to NF-κB p50, and we showed a reduction of nuclear p50 in TAMs after NF-κB decoy transfection (Fig. 2a). These results suggest that phenotypic conversion of TAMs is induced by efficient NF-κB decoy transfection into TAMs.

We determined the changes of both Th1 and Th2 cytokine expression in TAMs with or without NF-κB decoy transfection. As shown in Figure 3(a), mRNA expression of the Th2 cytokine IL-10 in TAMs transfected with the NF-κB decoy by Man-PEG bubble lipoplexes and US exposure was significantly lower compared with that in other groups. In contrast, mRNA levels of Th1 cytokines IL-12, TNF-α, and IL-6 in TAMs transfected with the NF-κB decoy by Man-PEG bubble lipoplexes and US exposure were the highest (Fig. 3b–d). These alterations of cytokine expression levels are in line with our previous report that showed the conversion of cytokine production profiles in TAMs by NF-κB decoy transfection into solid tumor (colon26)-bearing mice.(27) In addition, NF-κB decoy transfection by Man-PEG bubble lipoplexes and US exposure did not affect the viability of TAMs (data not shown). These results suggest that TAMs transfected with a NF-κB decoy by Man-PEG bubble lipoplexes and US exposure convert their phenotype from M2 toward M1.
To investigate the therapeutic potential of NF-κB decoy transfer by mannose-modified bubble lipoplexes into TAMs against EAC-bearing mice, we measured ascitic volumes and the number of EAC cells in ascites, and monitored body weight changes and survival of EAC-bearing mice. Saccani et al. (24) have reported that TAMs cultured in standard conditions for 24-72 h showed altered characteristics, such as cytokine expression. Taking this into consideration, we designed a protocol in which EAC-bearing mice were i.p. injected with NF-κB decoys three times every other day. As shown in Figure 5(a,b), the strongest inhibitory effects on ascites and EAC cell accumulation in the peritoneal cavity were observed in mice with NF-κB decoy transfer by Man-PEG bubble lipoplexes and US exposure. As we have confirmed that NF-κB decoy transfer had no direct cytotoxic effect on EAC cells (Fig. S1), this therapeutic effect may be attributed to the regulation of TAM function. Furthermore, we observed a significant prolongation of survival and suppression of the increase of body weight (Fig. 5c,d). Taken together, our results indicate that NF-κB decoy transfer by Man-PEG bubble lipoplexes and US exposure into TAMs is an effective method to inhibit MA progression in a mouse peritoneal dissemination model of EAC.
Angiogenesis is strongly linked to tumor growth and metastasis.\(^{30-32}\) It is also known that ascitic tumor growth including EAC is dependent on angiogenesis, and inhibition of angiogenesis exerts a significant antitumor effect in EAC-bearing mice.\(^{30,31,44,45}\) Because TAMs are reported to promote tumor angiogenesis through expression of VEGF,\(^{17-19}\) phenotypic regulation of TAMs is expected to suppress angiogenesis in EAC-bearing mice. As shown in Figure 4, VEGF expression in ascites and in TAMs were significantly reduced and peritoneal angiogenesis was more suppressed in the mouse peritoneal dissemination model of EAC transfected with the NF-κB decoy by Man-PEG bubble lipoplexes and US exposure. In addition, we have observed that NF-κB decoy transfer had no direct cytotoxic effect on vascular endothelial cells (normal HUVECs) (Fig. S1). Taking these factors into consideration, suppression of angiogenesis by NF-κB decoy transfer into TAMs might be supported by phenotypic conversion of TAMs and their antitumor effect in the mouse peritoneal dissemination model of EAC.

In conclusion, we succeeded in efficiently delivering a NF-κB decoy into TAMs by Man-PEG bubble lipoplexes and US exposure in a mouse peritoneal dissemination model of EAC. Moreover, we observed potent antitumor effects against MA by efficient transfer of the NF-κB decoy into TAMs. Although further studies are needed to clarify the mechanism, efficient NF-κB decoy transfer by Man-PEG bubble lipoplexes with US exposure into TAMs may be a novel approach for MA treatment.

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**Disclosure Statement**

The authors have no conflict of interest.

**Abbreviations**

- Bare-PEG: mannose-unmodified NH₂-PEG-DSPE
- DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine
- EAC: Ehrlich ascites carcinoma
- FAM: 6-carboxyfluorescein
- IL: interleukin
- MA: malignant ascite
- Man-PEG bubble lipoplexes: ultrasound-responsive and mannose-modified liposome/nuclear factor-κB decoy complexes
- NF-κB: nuclear factor-κB
- PEG: polyethylene glycol-2000
- TAM: tumor-associated macrophage
- TNF-α: tumor necrosis factor-α
- US: ultrasound
- VEGF: vascular endothelial growth factor

**Fig. 5.** Effect of in vivo nuclear factor-κB (NF-κB) decoy transfection by ultrasound (US)-responsive and mannose-unmodified liposome/NF-κB decoy complexes (Bare-PEG bubble lipoplexes) or ultrasound (US)-responsive and mannose-modified liposome/NF-κB decoy complexes (Man-PEG bubble lipoplexes) (10 μg NF-κB decoy) with or without US exposure on tumor growth and mouse survival. The number of Ehrlich ascites carcinoma (EAC) cells in ascites (a) and the volume of ascites (b) 10 days after EAC cell inoculation into mice. Data are represented as the mean ± SD (n = 4). *P < 0.05, **P < 0.01 compared with non-treatment (N.T.). The body weight (c) and survival (d) of EAC-bearing mice post-transfection. Data are represented as the mean ± SD (n = 8). Survival was monitored up to 60 days after EAC cell inoculation into mice. *P < 0.05, **P < 0.01 compared with N.T.

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Fig. S1. Effect of in vitro NF-κB decoy transfection by Man-PEG bubble lipoplexes with US exposure on cell viability.

Data S1. Materials & Methods.