Folate-Linked Lipid-Based Nanoparticles Deliver a NF κ B Decoy into Activated Murine Macrophage-Like RAW264.7 Cells

Yoshiyuki HATTORI,^a Makoto SAKAGUCHI,^b and Yoshie MAITANI*,^a

^a Institute of Medicinal Chemistry, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan; and ^b AnGes MG, Inc.; 4F, Saito Bio-Incubator; 7–7–15 Saito-asagi, Ibaraki, Osaka 567–0085, Japan.

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Activated macrophages are the key effector cells in rheumatoid arthritis (RA) and secrete multiple mediators of inflammation including proinflammatory cytokines. We investigated delivery of a nuclear factor kappa B (NF κ B) decoy by folate-linked lipid-based nanoparticles (NP-F) into murine macrophages. The expression of folate receptor (FR) in RAW264.7 cells activated by lipopolysaccharide was confirmed by strong expression of FR mRNA, and association of FITC-labeled folate-BSA conjugate. When transfected *via* NP-F, the NF κ B decoy was strongly detected in the cytoplasm, and an inhibitory effect on the translocation of NF κ B into the nucleus was observed at 0.03 μ M of the decoy, suggesting that NP-F effectively delivered the NF κ B decoy into the cytoplasm. This information is of value for the design of NF κ B decoy carrier systems targeting FR in activated macrophages in gene therapy for autoimmune diseases such as RA.

Key words nuclear factor kappa B (NF κ B) decoy; folate receptor; folate-linked nanoparticle; macrophage; rheumatoid arthritis

Activated synovial macrophages are present in large numbers in arthritic joints in autoimmune diseases such as rheumatoid arthritis (RA),¹⁾ and release many proinflammatory cytokines such as tumor necrosis factor- α (TNF- α).^{1,2)} Nuclear factor kappa B (NF κ B) is a transcription factor which normally resides in the cytoplasm, complexed with an inhibitor, I κ B, in an inactive form.³⁾ Proinflammatory cytokines induce a rapid nuclear translocation of NF κ B through degradation of I κ B, and NF κ B interacts with response-associated genes such as TNF- α , and activates their transcription.²⁾ The decoy technique can block intracellular signaling pathways using a double-stranded oligonucleotide directed at a cognate sequence of NF κ B.³⁾

Although mannoseylated cationic liposomes can deliver genes to macrophages,^{4,5)} the target of the NF κ B decoy is activated macrophages; therefore, activated macrophage-selective gene targeting is of great importance. A functionally active folate receptor (FR)- β was detected in activated synovial macrophages,⁶⁾ and was utilized a folate-linked imaging agent⁷⁾ and FR- β antibody conjugates.⁸⁾ However, gene delivery with folate-linked vectors into activated macrophages has not been reported.

Previously, we reported that folate-linked lipid-based nanoparticles (NP-F) delivered DNA with selectively high transfection efficiency into the FR expressed cells.⁹⁾ In the present study, we examined the efficiency of the transfection of NP-F into activated murine macrophage-like RAW264.7 cells treated with lipopolysaccharide (LPS) by investigating the intracellular distribution of a NF κ B decoy using confocal microscopy. Furthermore, to examine the ability of the decoy to inhibit the translocation of NF κ B into the nucleus, the amount of NF κ B in the nucleus was determined by ELISA after transfection with the NF κ B decoy *via* NP-F.

MATERIALS AND METHODS

Materials The NF κ B decoy oligonucleotide used in this study is a 20-mer phosphorothioate double-stranded oligonucleotide, 5'-CCTTGAAGGGATTTCCTCC-3'. LPS from

E. coli 111:B4 was purchased from Wako Pure Chemicals (Osaka, Japan). 3([N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tween 80 was supplied by NOF Co. Ltd. (Tokyo, Japan). All reagents were of analytical grade. DMRIE-C reagent, folate deficient-RPMI1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Carlsbad, CA, U.S.A.).

Cell Culture RAW 264.7 cells were supplied by Riken Cell Bank (Ibaraki, Japan). The cells were grown in a folate-deficient RPMI-1640 medium supplemented with 10% heat-inactivated FBS and kanamycin (100 μ g/ml) at 37 °C in a 5% CO₂ humidified atmosphere.

RT-PCR Analysis of FR Expression in RAW 264.7 Cells Cells were incubated with or without 100 ng/ml of LPS in medium for 4 h. Total RNA was isolated from the cells and RT-PCR was performed as described previously.⁹⁾ For the amplification of mouse FR- α , the primers FR- α -FW, 5'-AG-GACTGAAGTTCTCAATGTCTGCATGG-3', and FR- α -RW, 5'-GCTTGTAGGAGTGACTCCAGATTTCTC-3', were used. For the amplification of mouse FR- β , the primers FR- β -FW, 5'-CAAGCTGCATGACCAGTGTAGTCCATGG-3', and FR- β -RW, 5'-ATCTGGATGCAGCGGCCACTCC-CTCTGC-3', were used. For the amplification of mouse β -actin, the primers β -actin-FW, 5'-ACCCACACTGTGCC-CATCTA-3', and β -actin-RW, 5'-CTGCTTGCTGATCCA-CATCT-3', were used. The PCR products for FRs and β -actin were analyzed by 1.5% agarose gel electrophoresis in a Tris-Borate-EDTA (TBE) buffer. The products were visualized by ethidium bromide staining.

Real time PCR was performed on the corresponding cDNA synthesized from each sample described above. The optimized settings were transferred to real time PCR protocols on iCycler MyiQ detection systems (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and SYBR Green I assay (iQTM SYBER Green Supermix, Bio-Rad Laboratories) was used for quantification. Samples were run in triplicate and the expression level of FR- α mRNA was normalized for the amount of β -actin in the same sample. Difference of 1 cycle

* To whom correspondence should be addressed. e-mail: yoshie@hoshi.ac.jp

was calculated as a 2 fold-change in the gene expression.

Association of a FITC-Labeled Folate-Bovine Serum Albumin Conjugate Synthesis of a FITC-labeled folate-BSA conjugate (FITC-f-BSA) was carried out as previously described.⁹⁾ Cells were plated into 35-mm culture dishes, and were treated with 100 ng/ml of LPS for 4 h before the experiment. FITC-f-BSA was diluted to 10 μ M in 1 ml of folate-deficient RPMI medium containing 10% serum and then incubated with the cells in the presence or absence of 1 mM folic acid. After 30 min of incubation, the cells were washed twice with PBS (pH 7.4) to remove the unbound FITC-f-BSA and fixed with 10% formaldehyde in PBS for 15 min at room temperature. For staining the nucleus, the fixed cells were washed and incubated with 0.5 mg/ml of RNase in PBS for 10 min at 37 °C. Subsequently, the cells were washed and incubated with propidium iodide (PI) for 10 min at room temperature. FITC-f-BSA and PI were visualized with a Radiance 2100 confocal laser scanning microscope (Bio-Rad Laboratories) as previously described.⁹⁾

Preparation of Folate-Linked Nanoparticles Folate-polyethyleneglycol-distearoylphosphatidylethanolamine (f-PEG₂₀₀₀-DSPE, mean molecular weight of PEG: 2000 Da) was synthesized as previously described.¹⁰⁾ NP-F was prepared with lipids (DC-Chol:Tween 80:f-PEG₂₀₀₀-DSPE=93:5:2, molar ratio=10:1.3:1.3 mg) in 10 ml of water by a modified ethanol injection method as described previously.⁹⁾ NP-F formed nanoplex of about 200 nm, and even in the presence of serum, maintained its size (about 400 nm).⁹⁾

Transfection Based on a preliminary assay of the inhibition of NF κ B activation, the optimized total concentration of lipid in NP-F and DMRIE-C was determined as 60 μ g/ml. For the transfection with NP-F, the NF κ B double-stranded decoy was diluted to 20, 60, 200 and 2000 μ M, and a single-stranded decoy was diluted to 400 and 4000 μ M with medium. A complex (NP-F nanoplex) was formed by adding the decoy (3.6 μ l of 20, 60, 200, 2000 μ M double-stranded decoy and 400 and 4000 μ M single-stranded decoy, respectively) to the NP-F solution (17.1 μ l), mixing with gentle shaking, and leaving the mixture at room temperature for 15 min. The mixture was added to 3.6 ml of medium, and 1 ml was then added to 1 ml of cell culture in a 35-mm culture dish. The transfection with DMRIE-C was performed according to the manufacturer's instructions.

Intracellular Distribution of the NF κ B Decoy Cells were plated into 35-mm culture dishes, and treated with or without 100 ng/ml of LPS for 2 h before the experiment. The NP-F nanoplex or DMRIE-C lipoplex was formed with the FITC-labeled NF κ B decoy as described above and then diluted in 3.6 ml of medium. The mixtures (1 ml) were added to 1 ml of cell culture and incubated with the cells for 4 h in the presence of 100 ng/ml of LPS. After the incubation, the cells were fixed with 10% formaldehyde and incubated with PI for staining of the nucleus as described above. Examinations were performed with the confocal laser scanning microscope.

Measurement of the Amount of NF κ B in the Nucleus Cells were transfected with either the double- or single-stranded decoy using NP-F or the DMRIE-C reagent, and incubated for 4 h in the medium without LPS and then treated with 100 ng/ml of LPS for 1 h. The activation of p65, a transcription factor of the NF κ B family, was analyzed using a

TransAM assay kit according to the manufacturer's directions (Active Motif Europe, Rixensart, Belgium). Briefly, the LPS-treated cells were scraped off and centrifuged for 10 min at 1000 rpm. The pellet was suspended in 100 μ l of hypotonic buffer and 5 μ l of detergent, and was centrifuged for 30 s at 14000 g. The pellet was resuspended in 20 μ l of lysis buffer, incubated for 30 min at 4 °C, and centrifuged for 10 min at 14000 g. The supernatant constitutes the cell extract. Cell extract from each sample was diluted 5 times and incubated in 96-well plates coated with the NF κ B consensus double-stranded oligonucleotide sequence (5'-AGTTGAGGGGAC-TTTCCAGGC-3') for 1 h. Bound p65 was detected with rabbit anti-mouse p65 antibody and peroxidase-conjugated anti-rabbit IgG antibody. After a colorimetric reaction, the optical density was read at 450 nm.

Cytotoxicity The cytotoxicity upon transfection using NP-F or DMRIE-C was evaluated with a cell proliferation assay kit (Dojindo, Kumamoto, Japan). Cells were placed in a 24-well plate in the medium containing serum, and were transfected with the double-stranded NF κ B decoy at 0.1 and 1 μ M or single-stranded NF κ B decoy at 2 μ M via NP-F or DMRIE-C. After 24 h of incubation, the medium was removed, and the cells were treated with a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt) solution (20 μ l) in medium containing 10% FBS (200 μ l) for 1 h. Cell viability was expressed relative to the absorbance at 450 nm of untreated cells.

Statistical Analysis The statistical significance of the data was evaluated with the Dunnett test. A *p* value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

One major problem in gene therapy is how to transfect target cells efficiently and specifically. First, to investigate the possibility of a FR-based macrophage-targeting therapy, we examined the expression of FR in RAW264.7 cells. RT-PCR analysis of FR- α and β mRNA was done using total RNA from RAW264.7 cells with or without LPS treatment. The unstimulated cells with LPS expressed FR- α mRNA weakly, and FR- β mRNA not at all (Fig. 1A). However, RAW264.7 cells activated by LPS strongly expressed the FR- α mRNA, but did not express the FR- β mRNA (Fig. 1A). FR- α mRNA was increased about 4-fold in the cells by LPS-treatment (Fig. 1B). This suggested that folic acid could be utilized to target activated RAW264.7 macrophages via FR- α . Paulos *et al.* reported that in human macrophages from healthy individuals, FR- β was detected immunologically on the surface, but was found to be functionally inactive.⁷⁾ They also showed that the FR- β on activated cells can bind folate-linked compounds with high affinity.^{6,7)} In our study, we found a difference in the expression of the FR isoforms between human RA macrophages and murine RAW264.7 cells. The differences between human primary and murine macrophage-like cell lines can not be compared directly, but it is possible that FR has important roles in the activation of macrophages.

To examine the selectivity of the folate moiety in the uptake into RAW264.7 cells, the intracellular distribution of FITC-f-BSA was visualized by confocal microscopy. The NP-F nanoplex had a positive ζ -potential (about 30—

Fig. 1. (A) FR- α , FR- β and β -Actin mRNA Expression was Detected in RAW264.7 Cells with or without LPS Treatment by RT-PCR and (B) Relative Amount of FR- α mRNA in the Cells with or without LPS Treatment was Compared by an SYBR Green I-Based Quantitative PCR Analysis

The y-axis indicated the fold induction of gene expression. The expression level of FR- α mRNA was normalized for the amount of β -actin in the same sample. Each result represents the mean $\pm$ S.D. ($n=3$). ** $p<0.01$, compared with LPS(-).

(C and D) Association of FITC-f-BSA with RAW264.7 Cells

Cells were treated with 100 ng/ml of LPS for 4 h. After the treatment, FITC-f-BSA was incubated with the cells for 30 min in the absence (C) or presence (D) of 1 mM free folic acid. The FITC-f-BSA was visualized by confocal microscopy (magnification $\times 600$). The red signals show the location of the nucleus, and the green signals, that of the FITC-f-BSA. Scale bar = 50 μ m.

40 mV).⁹⁾ It was reported that a negatively charged folic acid formed a charge-mediated complex with positively charged particles through its carboxyl groups and increased non-specifically the efficiency of transfection.¹¹⁾ In NP-F, we observed that the addition of folic acid into culture medium decreased non-specifically the efficiency of transfection by interaction between positively charged NP-F and folic acid (data not shown). Therefore, we prepared FITC-f-BSA and incubated it with LPS-stimulated or unstimulated RAW264.7 cells in the absence or presence of 1 mM folic acid. The amount of FITC-f-BSA associated with the cells increased on LPS treatment (data not shown). The presence of 1 mM folic acid caused a significant decrease in the amount of FITC-f-BSA associated with the LPS-stimulated cells (Figs. 1C, D). These results indicated that the folate moiety promoted the association of FITC-f-BSA with LPS-stimulated RAW264.7 cells, suggesting that FR can be utilized for targeting activated macrophages.

Next, we examined the localization of the FITC-labeled NF κ B decoy after transfection *via* NP-F into RAW264.7 cells. In the unstimulated cells, the FITC-signal was moderately strong in the cytoplasm of a few cells (Fig. 2A). In the LPS-stimulated cells, it was detected throughout the cytoplasm strongly and diffusively in most of the cells (Fig. 2B). This might suggest that the FITC-labeled NF κ B decoy

Fig. 2. Comparison of the Intracellular Distribution of the FITC-Labeled NF κ B Decoy between NP-F and DMRIE-C Transfection

In (A), RAW264.7 cells were transfected with NP-F nanoplex of 0.1 μ M NF κ B decoy and incubated for 4 h without LPS. In (B)–(D), the cells were pretreated with 100 ng/ml of LPS for 2 h. After the pretreatment, the cells were transfected with NP-F nanoplex of 0.1 μ M NF κ B decoy (B), DMRIE-C lipoplex of 0.1 μ M NF κ B decoy (C), and 1 μ M naked NF κ B decoy (D), respectively, and incubated for 4 h in the presence of LPS. The FITC-labeled NF κ B decoy was visualized by confocal microscopy (magnification $\times 1200$). The red signals show the location of the nucleus, and the green signals, that of the FITC-labeled NF κ B decoy. Scale bar = 50 μ m.

nanoplex was efficiently delivered into the cells, was released from the nanoplex, and diffused into the cytoplasm. These results suggested that the association of the FITC-labeled NF κ B decoy with the activated macrophages occurred *via* FR and that NP-F was able to deliver the decoy into the cytoplasm of activated macrophages. This *in vitro* distribution of the NF κ B decoy observed in RAW 264.7 cells agreed with that reported by Higuchi *et al.*, showing that most of the fluorescent-labeled NF κ B decoy was observed in the cytoplasm when transfected by cationic liposomes into RAW264.7 cells stimulated with LPS.¹²⁾ DMRIE-C reagent is a liposomal formulation of the cationic lipid DMRIE composed of 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol at 1 : 1 (M/M). It is known to be suitable for the transfection of DNA into suspended cells and other lymphoid-derived cell lines. When the ability to carry genes into LPS-stimulated RAW264.7 cells was compared between NP-F and the DMRIE-C reagent, the intracellular distribution of the FITC-labeled NF κ B decoy transfected with DMRIE-C was much weaker than that obtained with NP-F in the cytoplasm (Fig. 2C). In the case of the naked NF κ B decoy, little intracellular localization was observed in the cells (Fig. 2D).

To examine the ability of the decoy to inhibit the translocation of NF κ B into the nucleus after the stimulation by LPS, the amount of NF κ B in the nucleus was determined by ELISA after transfection with the NF κ B decoy *via* either NP-F or the DMRIE-C reagent. Following LPS treatment, the amount of NF κ B in the nucleus was greater when the single-stranded decoy as a control was transfected by either NP-F or DMRIE-C into the cells (Fig. 3A), suggesting that this

Fig. 3. (A) Inhibition of the Translocation of NFκB in LPS-Treated RAW264.7 Cells by the NFκB Decoy Transfected by NP-F or DMRIE-C Reagent. Cells were incubated with NP-F nanoplex or DMRIE-C lipoplex of either single- or double-stranded decoy for 4 h and then treated with 100 ng/ml of LPS for 1 h.

(B) Cytotoxicity of NP-F Nanoplex and DMRIE-C Lipoplex of NFκB Decoy

Each result represents the mean $\pm$ S.D. ($n=3$). Statistically significant difference (* $p<0.05$) from untransfected cells.

decoy could not inhibit the translocation of NFκB. The double-stranded NFκB decoy transfected by DMRIE-C did not inhibit the translocation of NFκB even at 0.1 μM, however, it well inhibited it at 1 μM. The double-stranded NFκB decoy transfected by NP-F, inhibited the translocation of NFκB at 0.03 and 0.1 μM, indicating that NP-F could achieve the same degree of inhibition at a 10 times lower concentration of decoy. The cellular uptake of the NFκB decoy was much greater with NP-F than the DMRIE-C reagent (Fig. 2); therefore, the inhibition of NFκB's translocation into the nucleus may be more effective than that obtained with DMRIE-C.

Finally, we investigated the cytotoxicity of the transfection by NP-F or the DMRIE-C reagent. Either the NP-F nanoplex or DMRIE-C lipoplex was formed at various concentrations of the NFκB decoy (0.1 and 1 μM), and the cytotoxicity was assessed by the WST-8 assay after incubation of the cells with the nanoplex or lipoplex in the medium with serum for 24 h. No cytotoxicity was observed without the NFκB decoy using NP-F. However, significant toxicity was observed using DMRIE-C compared with untransfected cells (Fig. 3B). When the cells were transfected with the NP-F nanoplex containing 0.1 and 1 μM double-stranded NFκB decoy or 2 μM single-stranded NFκB, no cytotoxicity was observed (Fig. 3B). A liposomal formulation containing DC-Chol has been administered to patients in clinical trials for treating cancer because of its strong transfection activity and low toxicity.¹³⁾ The DC-Chol-based folate-linked nanoparticle used in this study showed little cytotoxicity in RAW264.7 cells (Fig. 3B). It will be important to develop applications *in vivo*.

Antohe *et al.* reported that systemically administered folate-fluorescent conjugates accumulated in protrusions of atherosclerotic plaques populated by macrophages, suggesting that activated macrophages can be efficiently targeted *in vivo* by using a folate conjugate.¹⁴⁾ Paulos *et al.* reported that folate-linked imaging agents could be selectively delivered to arthritic joints, allowing the visualization of RA.⁷⁾ These reports suggested that a folate-linked therapeutic agent could

be selectively delivered to activated macrophages *in vivo*, allowing the treatment of RA. We also confirmed that the folate moiety promoted the association of FITC-f-BSA with RAW264.7 cells treated with LPS (Figs. 1C, D). Therefore, the FR expression and folic acid can be utilized to target activated macrophages in humans and mice.

In conclusion, we have demonstrated that NP-F effectively delivered a NFκB decoy into the cytoplasm, which inhibited the translocation of NFκB into the nucleus of LPS-activated macrophages, compared with commercial transfection reagent, DMRIE-C. To our knowledge, this is the first report that folate-linked vector is effective for DNA delivery into activated macrophage-targeting. This information is of value for the design of carriers for NFκB decoys that will enable therapy for rheumatoid arthritis to be carried out.

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