Regulation of inflammatory response of macrophages and induction of regulatory T cells by using retinoic acid-loaded nanostructured lipid carrier

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ABSTRACT
Immunomodulatory function of all-trans retinoic acid (ATRA) has been gathering much attention for the therapy of autoimmune diseases. ATRA is a chemically unstable molecule which requires proper formulation for targeted delivery. Here we examined nanostructured lipid carrier (NLC) for the formulation of ATRA. NLC is a representative nanoparticle formulation especially suited for oral delivery. We established the preparation procedures of ATRA-containing NLC (NLC-RA) which minimizes the degradation of ATRA during the preparation process. NLC-RA thus obtained was taken up by macrophages and induced anti-inflammatory response via suppressing NF-κB signaling as well as via enhancing the production of anti-inflammatory cytokines. Moreover, NLC-RA enhanced differentiation of naïve T cells to regulatory T cells in the co-culture system with dendritic cells. These results suggest that NLC-RA is a promising alternative therapy for the autoimmune diseases especially intestinal bowel disease.

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Introduction

All-trans retinoic acid (ATRA), which is the active metabolite of vitamin A, is an agonist of nuclear receptors, retinoic acid receptors (RARs) [1–6]. ATRA induces macrophages and dendritic cells to express anti-inflammatory cytokines via RARs, which work on these cells to shift their phenotype to be anti-inflammatory and tolerogenic, respectively [1–6]. These anti-inflammatory macrophages and tolerogenic dendritic cells induce the naïve T cells to regulatory T (Treg) cells, which suppress excessive inflammatory responses [7] and induce immunotolerance toward antigens [1–6].

Because of these immunomodulatory functions of ATRA, therapeutic effects of ATRA have been examined for autoimmune diseases such as inflammatory bowel disease (IBD) [4–6,8], rheumatoid arthritis [9], asthma [10,11], and systemic lupus erythematosus [12,13]. However, chemical instability [14], poor water solubility [15], and short half-life in blood [16] of ATRA complicates its dosage formulation. To overcome these limitations, we tried to incorporate ATRA in nanostructured lipid carriers (NLC) that will improve stability and the therapeutic efficacy of ATRA. NLC is formulated from combination of a solid lipid with a liquid lipid to raise drug entrapment efficiency [17]. NLC has been originally developed for oral administration because of its stable nature in gastric fluid [18]. Thus, NLC will be suitable carrier to deliver ATRA to inflammatory lesions in gastrointestinal tract for IBD therapy.

Here we established preparation procedure of NLC containing ATRA (NLC-RA) to minimize the degradation of ATRA during preparation. As schematically shown in Figure 1, we evaluated the ability of NLC-RA to induce anti-inflammatory response in macrophages as well as differentiation of naïve T cells to Treg in vitro.

Materials and Methods

Materials

L-α-Phosphatidylcholine (PC) from soybean, Pluronic F-127 and α-tocopherol were purchased from Sigma-Aldrich. All-trans retinoic acid (ATRA) was purchased from TCI. L-(-)-Ascorbic acid, phosphotungstic acid and glycerol monostearate (GMS) were purchased from Wako. L-3-Phosphatidyl-L-serine sodium (PS) from bovine was purchased from Olbracht cedar research laboratories. 3,3’-dioctadecyloxycarbocyanine perchlorate (DiO) was purchased from Takara-Clontech.

Preparation of NLC-RA

NLCs containing ATRA were prepared by an oil in water emulsion method. A total of 1.0 mL of a chloroform-toluene (v/v = 1/1; density 1.1 g/cm³) mixed solution containing GMS (30 mg/mL), PC (4 mg/mL), PS (1 mg/mL), α-tocopherol (2 mg/mL), and ATRA (500 µg/mL) was prepared. Then this organic solution was added to an aqueous solution (24 mL) containing Pluronic F127 (1.25 mg/mL) and ascorbic acid (0.42 mg/mL). The two-phase solution was mixed by homogenizer (T25 digital Ultra turax, IKA, Germany) at 22,000 rpm for 15 min and sonicated with a probe sonicator.
(Ultrasonic Disrupter UD 201 (TOMY) equipped with a TP 040 standard probe tip) for 5 min (10% power, 20 kHz, 50 W). Then the organic solvent was evaporated from the solution by stirring (300 rpm) overnight. Fluorescently labeled NLCs were similarly prepared by mixing with DiO (10 μg/mL) in the organic phase.

**NLC characterization**

**Size and ζ-potentials measurements of NLC**
The size, polydispersity index, and ζ-potential of the NLCs were measured with a dynamic light scattering spectrophotometer (Zeta sizer Nano series, Malvern Instruments, UK) at 25°C. To measure the size and polydispersity index, the NLC dispersions were diluted with water to a concentration of 0.25 mg/mL of GMS. For measurement of the ζ-potential, the NLC dispersions were diluted with 10 mM HEPES buffer (pH 7.4) to a concentration of 0.25 mg/mL of GMS.

**Transmission electron microscopy**
A NLC dispersion was dropped on a 150 mesh-copper grid, then the NLC was stained with 2% phosphotungstic acid. The grid was air dried at room temperature, then observed by using a transmission electron microscopy (TEM) (JEOL JEM-2010, USA) operated at 120 keV.

**Quantification of intact ATRA in NLC**
The prepared NLC dispersion (1 mL) was ultracentrifugation at 220,000 g for 45 min. After collecting the supernatant, the NLC pellet was dissolved in methanol/chloroform at a ratio of 1:1. The amount of intact ATRA in NLC and supernatant (aqueous phase) was quantified by Jasco V-670 spectrophotometer (Japan) at maximum wavelength (350 nm). Entrapment efficiency was defined as follows:

\[
\text{Entrapment efficiency (%) = } \frac{\text{ATRA in particle} + \text{ATRA in aqueous phase}}{\text{ATRA in feed}} \times 100\%
\]

**In vitro study**

**Cell culture**
RAW 264.7 macrophages transfected with secreted alkaline phosphatase (SEAP) as a reporter gene under the transcriptional control of an NF-κB response element (Novusbio) were maintained in complete Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) and 4 mM L-glutamine supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 1 mM sodium pyruvate, and 500 μg/mL G418. The cells were cultured in a humidified atmosphere containing 5% CO₂ and 95% air at 37 °C.
Cellular uptake of NLC
RAW 264.7 macrophages transfected with SEAP (1 \times 10^4 cells/well) were seeded in a 96-well glass surface plate with complete DMEM. After 24 h of incubation, the cells were washed twice with DPBS. Complete DMEM containing 0.1 mg/mL NLC-DiO was added to the well. After 24 h of incubation at 37°C, the cells were observed for the uptake of NLC-DiO by LSM700 confocal laser scanning fluorescence microscopy (Carl Zeiss).

Toxicity of NLC
RAW 264.7 macrophages transfected with SEAP (4 \times 10^4 cells/well) were seeded in a 48-well plate with complete DMEM. After 24 h of incubation, the medium was replaced with complete DMEM containing NLC or NLC-RA (1.68-16.8 \mu M of ATRA; 0.05-0.5 mg/mL of NLC) and incubated for 24 h at 37°C. Cell viability was assessed using the LDH Assay Kit-WST (DOJINDO Laboratories).

Suppression NF-\kappa B-mediated inflammatory response
RAW 264.7 macrophages transfected with SEAP (4 \times 10^4 cells/well) were cultured in a 48-well plate with complete DMEM. After incubation overnight, the medium was replaced with complete DMEM containing NLC-RA (3.36 \mu M of ATRA; 0.1 mg/mL of NLC). After 6 h, LPS (final conc. 20 ng/mL) was added and the plate was incubated for 18 h. After incubation, the supernatants were mixed with an equal volume of alkaline phosphatase substrate (1 mg/mL p-nitrophenylphosphate) and incubated at room temperature for 1.5 h and then 3 N NaOH was added to stop the reaction. The optical density of the solution was measured at 405 nm with a microplate reader (Wallac ARVO.SX 1420 Multilabel counter).

Gene expression of cytokines
RAW 264.7 macrophages transfected with SEAP (4 \times 10^5 cells/well) were seeded in a 6-well plate with complete DMEM. After incubation overnight, the medium was replaced with complete DMEM containing NLC-RA (3.36 \mu M of ATRA; 0.1 mg/mL of NLC). After 6 h, LPS (final conc. 10 ng/mL) was added and the cells were
incubated for 18 h. The total RNA from the cells was prepared using Isoplus reagent (Takara). The samples were reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen) and the synthesized cDNA was used as a template in qPCR experiments performed with a LightCycler 1.5 (Roche Diagnostics, Germany) and analyzed with LightCycler Manager software 3.5 (Roche Diagnostics, Germany). The relative expression level was calculated by the $\Delta\Delta$Ct method using Gapdh as a reference gene. All primers were purchased from Takara-Clontech Laboratories (Japan). The sequences of the primer sets are listed in Supplementary Table S1.

**Production of cytokines**

RAW 264.7 macrophages transfected with SEAP (1 $\times$ 10^4 cells/well) were seeded in a 48-well plate with complete DMEM. After 24 h of incubation, the medium was replaced with complete DMEM containing NLC or NLC-RA (8.4 $\mu$M of ATRA; 0.25 mg/mL of NLC). After 24 h, LPS (final conc. 10 ng/mL) was added and the cells were incubated for 24 h. The cell culture supernatant was collected then cytokines were quantified by ELISA kit following the manufacturer’s protocol (R&D systems).

**Induction of regulatory T cell differentiation**

Dendritic cells (DC) and naïve T cells were isolated from C57BL mice spleen cells by magnetic separation (Miltenyi Biotec). DC and naïve T cells were co-cultured in 96-well U-bottom plates (BD Falcon). DC and naïve T cell were seeded at 5 $\times$ 10^3 cells/well and 1 $\times$ 10^5 cells/well respectively. Cultures were maintained in complete RPMI containing anti-CD3 antibody (20 $\mu$g/mL), TGF-β (0.4 ng/mL), and IL-2 (40 ng/mL). NLC-RA or free ATRA (final conc. 1-100 nM of ATRA; 0.02-2 $\mu$g/ml of NLC) was added to complete medium then cells were harvested after 3 days incubation for flow cytometric analysis. Co-cultured DC and T cell were stained by using antibodies; BV510-CD45 (BioLegend) and eF450-MHC II (eBiosciences) for DC, FITC-TCRβ (BD Biosciences), PE-CD4 (eBiosciences) and eF660-Foxp3 (eBiosciences) for T cell, respectively. FVS-780 (BioLegend) was used to stain dead cells. The cells were analysed by a LSR II Flow Cytometer (BD Biosciences). The process to identify the regulatory T cell fraction from the co-cultured cells is shown in Figure S3.

**Results and Discussion**

**Preparation and characterization of NLC**

The conventional preparation procedures of NLC use high temperature to melt solid lipid component to prepare pre-emulsion, then the size of the pre-emulsion is reduced by ultrasonication or high-pressure homogenizer [19,20]. Because ATRA is sensitive to high temperatures [14], the conventional preparation procedures of NLC are not suited. To avoid high temperatures, we prepared NLC containing ATRA (NLC-RA) by using organic solvent to dissolve lipids and ATRA to prepare the pre-emulsion at ambient temperature. The formulation of NLC consists of a core matrix of glycerol monostearate and $\alpha$-tocopherol as a solid and a liquid lipid, respectively. $\alpha$-Tocopherol is antioxidant, which is expected to suppress inflammation by neutralizing reactive oxygen species (ROS) [21,22]. The anti-oxidative function of
α-tocopherol may contribute to protect ATRA from radical species which generate during preparation of NLC. As stabilizers of NLC, we used combination of a neutral polymer (Pluronic F127) and phospholipids. All the components of NLC we selected are approved materials for clinical application.

We tried to establish preparation conditions to obtain NLC with small size (~100 nm) with high entrapment efficiency of ATRA. Nanoparticle with size of about 100 nm is suitable to target inflammatory regions in gastrointestinal tract [20]. Table 1 summarizes characteristics of NLC-RA prepared by various conditions. Homogenization alone or ultrasonication alone did not produce NLC-RA with small particle size (run 1-4). Moreover, the ultrasonication alone reduced the entrapment efficacy of ATRA (run 3, 4). In contrast, combination of the homogenization and the ultrasonication (run 5) produced NLC-RA with a mean size of about 130 nm, narrow size distribution, and high entrapment efficacy of ATRA. Morphology of NLC-RA prepared by the condition of run 5 was found to be spherical shape with sizes of approximately 50-200 nm (Figure 2), which is matched with the result of dynamic light scattering. We concluded that the condition of run 5 is optimum for the preparation of NLC-RA. By using this condition, empty NLC (NLC) and fluorophore containing NLC (NLC-DiO) were prepared (Table 2). Size, polydispersity index and ζ-potential of each NLC are almost constant irrespective of the content. The

| Run | Condition | Homogenization | Ultrasonication | Size (nm) | Polydispersity index | ATRA in NLC (µg) | ATRA in aq. phase (µg) | Entrapment efficacy (%) |
|-----|-----------|----------------|-----------------|----------|----------------------|-----------------|------------------------|------------------------|
| 1   |            | 15 min         | –               | 427 ± 20 | 0.66 ± 0.04         | 365.6 ± 24.6   | 128.0 ± 0.5           | 98.7 ± 4.8             |
| 2a  |            | –              | 10%, 5 min      | –        | –                   | –               | –                      | –                      |
| 3   |            | –              | 10%, 15 min     | 429 ± 22 | 0.64 ± 0.02         | 213.0 ± 10.8   | 56.8 ± 1.0            | 53.9 ± 2.1             |
| 4   |            | –              | 20%, 10 min     | 179 ± 9  | 0.44 ± 0.02         | 204.8 ± 6.1    | 77.4 ± 0.7            | 56.4 ± 1.3             |
| 5   |            | 15 min         | 10%, 5 min      | 130 ± 9  | 0.29 ± 0.02         | 312.3 ± 5.5    | 49.1 ± 4.7            | 72.3 ± 1.4             |

Stable NLC dispersion was not obtained (NLC was aggregated after evaporation of organic solvent).

Figure 2. TEM image of NLC-RA. Scale bar: 100 nm.
concentration of each component in NLC-RA is summarized in Table S2. The process to determine the concentration of each component is summarized in Figure S1.

**Cellular uptake and cytotoxicity of NLC**

We evaluated cellular uptake of NLC-DiO by RAW 264.7 macrophages. The uptake of NLC-DiO was assessed by confocal laser scanning microscopy at 24 h after incubation. As shown in Figure 3, the strong green fluorescence of DiO was observed in the macrophages. We examined the cytotoxicity of NLC and NLC-RA against macrophages after 24 h incubation. Despite the high cellular uptake of NLC, we observed no cytotoxicity of either NLC or NLC-RA below the concentration of 0.5 mg/mL (Figure 4), showing cytocompatible nature of NLC.
We investigated the effect of NLC-RA on inhibition of NF-κB signaling triggered by LPS stimulation in macrophages. NF-κB is one of the key transcription factors in activation of macrophages during inflammation process [23]. Here we utilized RAW 264.7 macrophages stably transfected with secreted alkaline phosphatase (SEAP) reporter gene under the transcriptional control of an NF-κB response element. As shown in Figure 5, both NLC and NLC-RA showed suppression of NF-κB signaling.

Figure 5. Suppression of NF-κB signaling in LPS-stimulated RAW 264.7 macrophages by NLCs. Macrophages were treated with NLC and NLC-RA (0.1 mg/mL containing 3.36 μM ATRA). Data are the mean ± S.D (n = 3). *, p < 0.05; ***, p < 0.001

Figure 6. Modulation of gene expression of pro- and anti-inflammatory cytokines in RAW 264.7 macrophages by NLCs. Macrophages were treated with NLC and NLC-RA (0.1 mg/mL containing 3.36 μM ATRA) and gene expression was evaluated by qRT-PCR. Data are the mean ± S.D (n = 3). *, p < 0.05; **, p < 0.01.

**NLC-RA inhibits LPS-induced NF-κB signaling**

We investigated the effect of NLC-RA on inhibition of NF-κB signaling triggered by LPS stimulation in macrophages. NF-κB is one of the key transcription factors in activation of macrophages during inflammation process [23]. Here we utilized RAW 264.7 macrophages stably transfected with secreted alkaline phosphatase (SEAP) reporter gene under the transcriptional control of an NF-κB response element. As shown in Figure 5, both NLC and NLC-RA showed suppression of NF-κB signaling,
but NLC-RA has somewhat superior effect to NLC. The suppressive effect of empty NLC will be due to the anti-inflammatory effect of \(\alpha\)-tocopherol used as a component of NLC.

**NLC-RA alters cytokines gene expression in macrophages**

We assessed the effect of NLC-RA on the expression of pro- and anti-inflammatory cytokines from the LPS-stimulated RAW 264.7 macrophages. As shown in Figure 6, NLC-RA significantly suppressed the expression of mRNA of pro-inflammatory cytokines (IL-6, IL-1\(\beta\), TNF-\(\alpha\)), while strongly enhanced that of anti-inflammatory cytokines (IL-10, TGF-\(\beta\)). Similar results were obtained when the expression of the cytokines was detected as proteins; suppression of IL-6 and enhancement of IL-10 (Figure 7). IL-10 and TGF-\(\beta\) which were enhanced by NLC-RA are the most important anti-inflammatory cytokines in the resolution of inflammation and TGF-\(\beta\) is required to differentiate naïve T cells to Treg [24]. Thus, NLC-RA will have effect to induce Treg as well as to suppress the inflammation.
**Induction of Treg by NLC-RA**

We examined effect of NLC-RA in the differentiation of naïve T cells to Treg by using the co-culture system of naïve T cells and dendritic cells both of which were isolated from mice spleen. As shown in Figure 8A, NLC-RA could induce Treg and its induction efficacy of Treg was comparable to that of free ATRA. NLC-RA did not induce cytotoxicity to T cells and dendritic cells (Figure 8B). Thus, incorporation of ATRA into NLC did not compromise the induction efficacy of Treg, indicating that NLC matrix could be degraded in the dendritic cells to release ATRA.

**Conclusion**

We established preparation procedure of NLC containing ATRA (NLC-RA), which is compatible to chemically unstable ATRA. The size of NLC-RA was tuned to be ~100 nm, which is known to be suitable size for oral delivery to target inflammatory lesions of gastrointestinal tract. NLC-RA prepared by the procedure had cytocompatibility to macrophages even though its efficient cellular uptake. NLC-RA reduced the expression of pro-inflammatory cytokines while enhanced that of anti-inflammatory cytokines from the LPS-stimulated macrophages. NLC-RA efficiently promoted differentiation of naïve T cells to Treg in the co-culture system. These promising features of NLC-RA are suitable for therapy of inflammation diseases in gastrointestinal tract especially for IBD.

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