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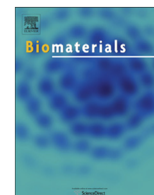

Bilirubin nanoparticles ameliorate allergic lung inflammation in a mouse model of asthma

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ABSTRACT

Although asthma, a chronic inflammatory airway disease, is relatively well-managed by inhaled corticosteroids, the side effects associated with the long-term use of these agents precipitate the need for alternative therapeutic options based on differing modes of action. Bilirubin, a potent endogenous antioxidant, and anti-inflammatory molecule have been shown to ameliorate asthmatic symptoms; however, its clinical translation has been limited owing to its water insolubility and associated potential toxicity. Here we report the first application of bilirubin-based nanoparticles (BRNPs) as a nanomedicine for the treatment of allergic lung inflammatory disease. BRNPs were prepared directly from self-assembly of PEGylated bilirubin in aqueous solution and had a hydrodynamic diameter of ~100 nm. Because allergen-specific type 2 T-helper (Th2) cells play a key role in the pathogenesis and progression of allergic asthma, the effects of BRNPs on Th2 immune responses were investigated both *in vivo* and *in vitro*. BRNPs after intravenous injection (i.v.) showed much higher serum concentration and a longer circulation time of bilirubin than the intraperitoneal injection (i.p.) of BRNPs or unconjugated bilirubin (UCB). The anti-asthmatic effects of BRNPs were assessed in a mouse model of allergen-induced asthma. Compared with UCB, treatment with BRNPs suppressed the symptoms of experimental allergic asthma and dramatically ameliorated Th2-related allergic lung inflammation. Consistent with these results, BRNPs caused a reduction of Th2 cell populations and the expression of related cytokines by antibody-stimulated $CD4^+$ T cells *in vitro*. Therefore, our results establish BRNPs as an important immunomodulatory agent that may be useful as a therapeutic for allergic lung inflammatory disease and other immune-mediated disorders.

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1. Introduction

Asthma is a chronic inflammatory airway disease associated with reversible airflow obstruction, persistent airway hyperresponsiveness (AHR), and airway remodeling that impacts more than 300 million individuals globally [1,2]. To date, the best option for asthma treatment is inhaled corticosteroids, which can keep asthma symptoms under control on a day-to-day basis. However, long-term use of such corticosteroids can cause various side effects, and thus there is a need for alternative therapeutic options based on a mode of action different from those of existing drugs. Recently, Ohrui et al. reported that asthma symptoms are transiently relieved during jaundice, suggesting a beneficial role of increased bilirubin levels in serum [3]. In addition, it has previously been shown that 'unconjugated' bilirubin (UCB) ameliorates vascular cell adhesion molecule-1 (VCAM-1)-mediated airway inflammation [4].

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Furthermore, bilirubin, the final metabolite of the heme catabolic pathway, has been shown to be a potent antioxidant and anti-inflammatory molecule [5]. In fact, its anti-inflammatory efficacy has been demonstrated in a variety of inflammatory disease models, ranging from chronic to acute inflammation [6–8]. Despite this promising efficacy, clinical applications of UCB have been limited owing to its water insolubility and associated potential toxicity to neuronal cells and erythrocytes [9]. Very recently, we developed bilirubin-based nanoparticles (BRNPs) with a diameter of ~100 nm, composed of entirely PEGylated bilirubin, that is freely water dispersible [10]. Whereas BRNPs retain the key anti-inflammatory effects of the parent UCB, as demonstrated in a mouse colitis model, they do not cause jaundice (deposition of UCB aggregates in various tissues). Encouraged by the intrinsic immune modulatory effects of UCB and previous findings of its beneficial actions in asthma patients, we hypothesized that BRNPs might be effective in treating asthma. Here we report the first application of BRNPs as a nanomedicine for the treatment of an allergic lung inflammatory disease. In the present study, the anti-asthmatic effects of BRNPs were assessed in a mouse model of allergen-induced asthma. In particular, given the key role of allergen-specific type-2 T-helper (Th2) cells in the pathogenesis and progression of allergic asthma, the effects of BRNPs on Th2 immune responses were investigated both *in vivo* and *in vitro*. Thus, the therapeutic efficacy of both intravenously (i.v.) and i. p. injected BRNPs was compared with that of UCB (i.p.), used as a positive control, in most experimental settings [11].

2. Materials and methods

2.1. Materials

Anti-IFN-γ (clone AN-18; eBioscience, San Diego, CA, USA), anti-IL-4 (clone 11B11; BD Biosciences, San Diego, CA, USA), anti-IL-5 (clone TRFK5; BD Biosciences), anti-IL-13 (clone eBio13A; eBioscience), and anti-IL-17A (clone eBio17CK15A5; eBioscience) were purchased for use as capture antibodies. Recombinant IFN-γ (BD Biosciences) and IL-4 (BD Biosciences), as well as anti-IL-5 (BD Biosciences), anti-IL-13 (Peprotech, Rocky Hill, NJ, USA) and IL-17A (Peprotech), were purchased for use as ELISA standards. Biotin-conjugated detection antibodies included anti-IFN-γ (clone R4-6A2; eBioscience), anti-IL-4 (clone BVD6-24G2; BD Biosciences), anti-IL-5 (clone TRFK4; BD Biosciences), anti-IL-13 (clone eBio1316H; eBioscience), and anti-IL-17A (clone eBio17B7; eBioscience) were purchased for use as detection antibodies. Allophycocyanin (APC)-Cy7-conjugated anti-CD8 (clone eBio53–6.7; eBioscience); phycoerythrin (PE)-Cy7-conjugated anti-CD4 (clone GK1.5; eBioscience), Alexa Fluor 700 (AF700)-conjugated anti-CD44 (clone IM7; eBioscience), Percp-eFluor710-conjugated anti-ST2 (clone RMST2-2; eBioscience), and eFluor506-labeled anti-Fixable Viability dye (eBioscience) were used for flow cytometry. PE-conjugated anti-IL-4 (clone 11B11; BD Biosciences) was used for ICCS.

2.2. Synthesis of BRNPs

PEG-BR was synthesized as described previously [10]. PEG-BR (2 μmol) was first dissolved in chloroform (200 μL), then a film layer was formed by evaporating the solvent, after which the film layer was hydrated with distilled water and sonicated for 10 min to yield uniform-sized BRNPs. The hydrodynamic size and zeta potential of the BRNPs were characterized using a Nanosizer ZS90 (Malvern Instruments, Ltd., Malvern, UK). The morphology of BRNPs was examined by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) using Tecnai TF30 ST (FEI Co., Hillsboro, OR, USA) and Magellan XHR 400L (FEI Co.) systems, respectively. The diluted solution of BRNPs was used for *in vitro* and *in vivo* studies.

2.3. Mice

Wild-type (WT) C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred in our specific pathogen-free animal facility. Six-to seven-week-old female mice were used for this study. Animal care and experimental procedures were performed with the approval of the Animal Care and Use Committee of Korea Advanced Institute of Science and Technology (KAIST, KA2013-32).

2.4. Pharmacokinetics study

BRNPs (4 mM; 50 mg/kg) or UCB (4 mM; 11.6 mg/kg) were injected via the i. v. or i. p. route into mice (6-week-old female WT mice; n = 5/group). At a predetermined time, blood was collected from the orbital plexus after mice had been sacrificed by exposure to isoflurane gas. Blood samples were mixed in BD Microtainer tubes (BD Biosciences) and centrifuged at 15,000× g for 5 min. The supernatants (serum) were collected and the serum concentration of the bilirubin component of the BRNPs or the UCB was measured using a Hitachi 7080 automated blood chemistry analyzer (Tokyo, Japan) at the Laboratory Animal Research Center of Chungbuk National University.

2.5. Induction of allergic asthma

A. oryzae protease allergen was purchased (Sigma-Aldrich, St. Louis, MO, USA) and reconstituted to 1 mg/mL using sterile PBS. OVA from Sigma-Aldrich was reconstituted to 0.5 mg/mL using sterile PBS. APO allergen was mixed at a 1:9 (v/v) ratio immediately before administration. Mice received five intranasal challenges with 50 μL of APO allergen every 4 days (days 0, 4, 8, 12, and 16). For the intranasal challenge, mice were lightly anesthetized by isoflurane inhalation (Abbott Laboratory, Abbott Park, IL, USA). For evaluation of the therapeutic effects of BRNPs, BRNPs were given i. p. or i. v. before the APO allergen challenge.

2.6. Measurement of AHR

AHR was measured with a flexiVent system (SCIREQ Inc., Montreal, Canada). Briefly, 16 h after the final intranasal challenge, mice were anesthetized with pentobarbital (Hanlim Pharma Co, Seoul, Korea) via i. p. injection of 0.15 mL/10 g body weight and intubated with a 20-gauge cannula. After intubation, mice were injected with 0.1 mL/10 g body weight of pancuronium (0.1 mg/mL; Sigma-Aldrich) and ventilated with the flexiVent system. Airway responsiveness was assessed by administering incremental doses of nebulizing methacholine (0, 1, 3, 9, 18, 27 mg/mL; Sigma-Aldrich) and measuring resistance every 30 s. After measuring AHR, BALF samples were obtained by washing the lungs with PBS (1 mL, 4 °C) delivered via a tracheal tube.

2.7. Measurement of secreted glycoprotein

Secreted glycoprotein levels in BALF were measured by modified ELISA using jacalin, a glycoprotein-binding lectin. Briefly, a glycoprotein mucin standard derived from the porcine stomach (Sigma-Aldrich) and BALF samples were serially 2-fold diluted in PBS, beginning at a 1:100 dilution. Forty microliters of each sample were transferred to a flat-bottom ELISA plate (Greiner, Kremsmunster, Austria) and incubated at 4 °C overnight. After washing, the plates

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