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Specific accumulation of orally administered redox nanotherapeutics in the inflamed colon reducing inflammation with dose–response efficacy

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ABSTRACT

Although current medications for ulcerative colitis (UC) are effective to some extent, there are still some limitation of their use due to the non-specific distribution, drug metabolism in the gastrointestinal tract, and severe adverse effects. In our previous studies, we developed oral redox nanoparticles (RNP^O) that specifically accumulated and scavenged overproduced reactive oxygen species (ROS) in an inflamed colon. However, the mechanism leading to specific accumulation of RNP^O in an inflamed colon is still unclear. In this study, we investigated the cellular uptake of RNP^O into ROS-treated epithelial colonic cells *in vitro*, and compared to the untreated cells, found a significantly increased uptake in ROS-treated cells. *In vivo*, we discovered that orally administered RNP^O were not internalized into the cells of a normal colon. A significant amount of disintegrated RNP^O was detected in the cells of an inflamed colon of dextran sodium sulfate (DSS)-induced colitis mice, resulting in scavenging of ROS and suppression of inflammation with low adverse effects. Furthermore, we confirmed a significant reduction of disease activity and a robust dose response efficacy following RNP^O treatment in acute DSS-induced colitis mice, outperforming the positive control 5-aminosalicylic acid. Oral administration of RNP^O is a promising approach to develop a new therapy for UC disease.

1. Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), affects millions of patients worldwide [1–4]. It has been reported that the intestinal mucosa of patients with IBD is characterized by overproduction of reactive oxygen species (ROS) and an imbalance of important antioxidants, leading to oxidative damage. Self-sustaining cycles of oxidant production may amplify inflammation and mucosal injury [5–8]. For many years, corticosteroids and 5-aminosalicylic acid (5-ASA) have been used for patients with UC [9,10]; however, these medications are not always effective due to their drawbacks including low solubility, non-specific distribution, and adverse effects after systemic absorption.

In a previous study, we described a novel ROS scavenging nanotherapy for the treatment of UC *via* oral administration [11]. We designed a redox polymer, methoxy-poly(ethylene glycol)-*b*-poly[p-4-(2,2,6,6-tetramethylpiperidine-1-oxyl)oxymethylstyrene] (MeO-PEG-*b*-PMOT), which is an amphiphilic block copolymer with stable nitroxide radicals in the hydrophobic segment as a side chain *via* an ether linkage; it forms core–shell-type micelles (RNP^O) with a diameter of 40 nm by self-assembling in aqueous environments regardless of pH (Fig. 1A). We have confirmed that orally administered RNP^O substantially accumulates in the colon area, but is not absorbed into the bloodstream and does not affect the intestinal bacterial population [11,12]. However, the mechanism underlying the specific accumulation of RNP^O in an inflamed colon is still unclear. In addition, a dose-dependent improvement in disease activity by RNP^O has not been pharmacologically proven in preclinical models.

This study aims to investigate the specific accumulation of RNP^O in an inflamed colon and the RNP^O dose–response relationship to disease activity in a dextran sodium sulfate (DSS)-induced colitis mice model. We found that RNP^O specifically internalized in intestinal cells pretreated with ROS *in vitro*, compared to untreated cells. Furthermore, it was confirmed *in vivo* that orally administered RNP^O specifically internalized in inflamed colon tissues; they slightly localized inside the epithelial cells, while no internalization of RNP^O was observed in normal healthy cells. In this investigation, a significant dose-dependent therapeutic effect on DSS-induced colitis in mice was observed after oral treatment with RNP^O.

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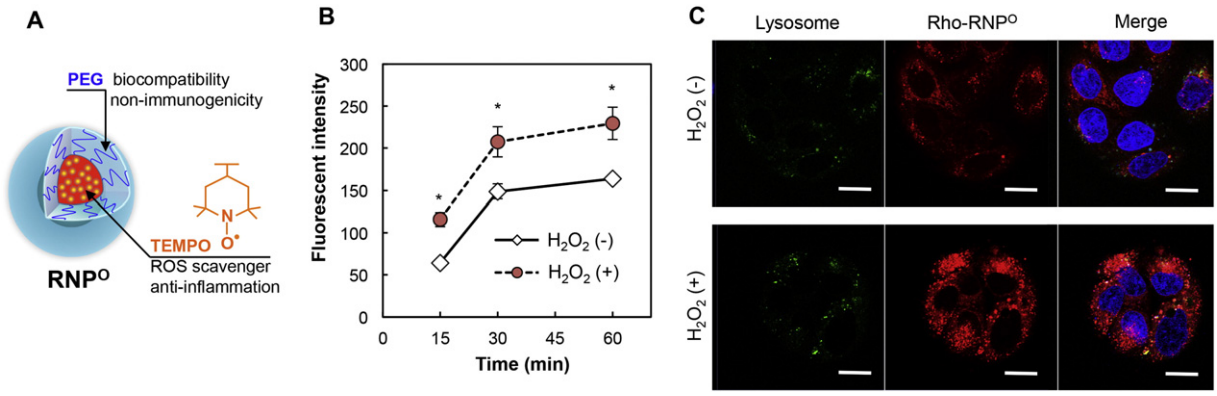

Fig. 1. (A) Schematic illustration of redox nanoparticle (RNP^O). (B) The fluorescent intensities of Rhodamine-labeled RNP^O (Rho-RNP^O) in untreated Caco-2 cells and H_2O_2-treated Caco-2 cells. H_2O_2 (200 μM) was incubated with cells for 2 h prior to adding Rho-RNP^O. (C) The fluorescence images of untreated Caco-2 cells and H_2O_2-treated Caco-2 cells after 30-min incubation with Rho-RNP^O. Rho-RNP^O (red), lysosomes (green, stained by Lysotracker Green DND-26), and nuclei (blue, stained by Hoechst 33342) were imaged. Scale bars, 20 μm. The data are expressed as mean ± SEM from three independent experiments, $^*p < 0.05$.

2. Material and methods

2.1. Preparation of RNP^O

RNP^O was prepared using a self-assembling MeO-PEG-*b*-PMOT block copolymer, as previously reported [11,13].

2.2. Cellular uptake of RNP^O in vitro

The experiment was carried out using Rhodamine-labeled RNP^O (Rho-RNP^O) to analyze the cellular uptake of these nanoparticles using a fluorescent confocal microscope. The human intestinal Caco-2 cells (Riken RCB0988; Saitama, Japan) were seeded in 12-well plates at a density of 5×10^4 cells per well. After 2 d of culturing, the Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich) was replaced with fresh media, and the Rho-RNP^O solution (100 μg/mL) was added. At predetermined time intervals, the cells were washed 3 times with fresh DMEM media. Photographs of cellular uptake were taken and analyzed using a fluorescent confocal microscope system (Zeiss LSM 700, Carl Zeiss Microscopy GmbH, Jena, Germany) under oil immersion at 63× magnification.

2.3. Animal

All experiments were carried out using 7 to 8-week-old male ICR mice (32–35 g) purchased from Charles River Japan, Inc. (Yokohama, Japan). The mice were maintained in the experimental animal facilities at the University of Tsukuba under controlled temperature (23 ± 1 °C), humidity (50 ± 5%) and lighting (12 h light–dark cycles). The animals were provided free access to food and water. All of the experiments were performed according to the guidelines for the care and use of Laboratory animal resource center of the University of Tsukuba (Animal experimental plan #12-199).

2.4. Accumulation of orally administered RNP^O in the colon

Localization of RNP^O in the colon was determined using fluorescent Rho-RNP^O. Mice were sacrificed at predetermined times after oral administration of 1 mL of low-molecular-weight (LMW) fluorescent Rhodamine or Rho-RNP^O (5 mg/mL). The feces in the colon were gently removed with phosphate buffered saline (50 mM, pH 7.4), and 7-μm-thick colon sections were prepared. Localization of LMW Rhodamine or Rho-RNP^O was observed using a fluorescent microscope (Leica, Japan).

2.5. Specific cellular internalization of RNP^O in vivo

The cellular isolation procedure was based on a previous report with modifications [14]. At 4, 5, and 6 h after oral administration of RNP^O (300 mg/kg), colon tissues were collected from normal mice and mice with DSS-induced colitis. Then, the colons were opened longitudinally and washed with phosphate buffer saline (PBS) to remove residues, followed by mechanical fragmentation. Colonic tissues were treated with collagenase (10 mg/mL; Wako Pure Chemical Industries, Osaka, Japan) for 30 min at 37 °C with slow agitation, followed by centrifugation at 10,000 rpm at 4 °C for 5 min. Cell pellets were gently resuspended in acetic acid (0.1 M, pH 3). Samples were centrifuged to separate extracellular RNP^O and intracellular internalized RNP^O. Supernatants and cell pellets were oxidized with potassium ferricyanide (10 mM; Kanto Chemical Co., Inc, Tokyo, Japan) for electron spin resonance (ESR; ES-TE25X, Jeol, Tokyo, Japan) measurements.

2.6. Induction of colitis by DSS and drug administration

Colitis mice were induced by supplementing 3% (wt/vol) DSS (5,000 Da; Wako Pure Chemicals) in the drinking water for 7 d. During 7-d DSS administration, RNP^O was administered daily *via* oral gavage at low (50 mg/kg), medium (100 mg/kg), and high (300 mg/kg) doses, denoted RNP^O(50), RNP^O(100), and RNP^O(300), respectively. The positive control 5-ASA was suspended in 0.5% (wt/vol) carboxymethyl cellulose and administered *via* oral gavage at a dose of 35 mg/kg, which is a dose equivalent of 200 mg/kg RNP^O (35 mg/kg of nitroxide radicals) as used in a previous study [11].

2.7. Colon length and disease activity index (DAI)

During 7-d treatment period, body weight was measured daily. Following 7 d of treatment, mice were sacrificed under sodium pentobarbital anesthesia (40 mg/kg), and the entire colon (from cecum to rectum) was quickly collected. Colon length was measured using a vernier caliper. DAI is the summation of the stool consistency index (0–3), fecal bleeding index (0–3), and weight loss index (0–4), as previously reported [15].

2.8. Histological assessment

After gently washing with physiological saline, 1 cm of the distal colon was fixed in 4% (vol/vol) buffered formalin for 1 d and 70% (vol/vol) alcohol for 2 d prior to paraffin embedding. Then, 7-μm-thick sections of the distal colon were prepared and stained with hematoxylin and eosin. Histology of the colon was evaluated using a microscope.