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Research paper

Inflamed site-specific drug delivery system based on the interaction of human serum albumin nanoparticles with myeloperoxidase in a murine model of experimental colitis

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ABSTRACT

To develop a new strategy for inflamed site-specific drug delivery in the colon for the treatment of ulcerative colitis (UC), we leveraged on the interaction between myeloperoxidase (MPO) and human serum albumin (HSA) and prepared nanoparticles (HSA NPs) conjugated with 5-aminosalicylic acid (5-ASA). The 5-ASA-HSA NPs (nine molecules of 5-ASA per HSA molecule) were uniform particles with an average particle size of 190 nm, a zeta potential of - − 11.8 mV, and a polydispersity index of 0.35. This was considered a suitable particle characteristic to pass through the mucus layer and accumulate into the mucosa. The specific interaction between the 5-ASA-HSA NPs and MPO was observed using quartz crystal microbalance analysis *in vitro*. In addition, the 5-ASA-HSA NPs group containing one thousandth of the dose of the 5-ASA (75 μg/kg) showed significantly lower disease activity index values and colon weight/length ratios in UC model mice as similar to large amount of neat 5-ASA group (75 mg/kg), indicating that the therapeutic effect of the 5-ASA-HSA NP formulation was confirmed *in vivo*. Microscopic images of tissue sections of colon extracted from UC model mice demonstrated that HSA NPs and MPO were both localized in the colon, and this specific interaction between HSA NPs and MPO would be involved the in the therapeutic effect *in vivo*. Furthermore, in the 5-ASA and 5-ASA-HSA NPs groups, some inflammatory damage was observed in the colon, but the degree of damage was mild compared with the control and HSA NPs groups, suggesting mucosal repair and replacement with fibrous granulation tissue had occurred. Therefore, these data demonstrated that an HSA NP formulation has the potential to specifically deliver 5-ASA to an inflamed site where MPO is highly expressed.

1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are two major forms of inflammatory bowel disease (IBD), and are chronic intestinal immune-mediated disorders of unknown etiology [1]. In sites of intestinal inflammation, granulocytes and macrophages produce high levels of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, which are directly involved in the pathogenesis of IBD [2,3]. Although several therapeutic strategies are used for the treatment of IBD, none have been found to be totally effective. Conventional therapies for IBD focus on suppression and control of inflammation using 5-aminosalicylic acid (5-ASA), corticosteroids, and immune-modulating drugs, such as azathioprine and mercaptopurine [4]. Recently, novel cytokine antagonist therapies targeting TNF-α and IL-6 have been found to be effective in certain IBD patients [5]. Such targeted inhibition of inflammatory processes may provide better therapeutic options for patients with IBD. To date, various formulations with specific delivery to the large intestine have been developed and sustained-release formulations using undissolved and pH-dependent dissolved polymers containing 5-ASA are in clinical use [6,7]. However, some recent reports have demonstrated that physical conditions in the large intestine in UC and CD patients widely vary

Abbreviations: AOPPs, advanced oxidation protein products; 5-ASA, 5-aminosalicylic acid; CD, Crohn's disease; CDDS, colon drug delivery system; DAI, disease activity index; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; DSS, dextran sulfate sodium; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HSA, human serum albumin; IBD, inflammatory bowel diseases; IL-1β, interleukin-1β; MPO, myeloperoxidase; NHS, *N*-hydroxysuccinimide; QUM, quartz crystal microbalance; TNF-α, tumor necrosis factor; UC, ulcerative colitis

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among patients, including differences in enzyme activity [8], the intestinal transit times of drugs, and the pH [9,10]. These variations suggest that therapeutic effects might not be obtained, and serious side effects caused by released 5-ASA in the non-inflamed area might occur, in some patients. Furthermore, as the daily therapeutic dose of 5-ASA needs to be approximately 3600 mg, it is quite difficult to change formulations containing 5-ASA. Therefore, development of a new colon drug delivery system (CDDS) that can specifically deliver a small amount of 5-ASA to the inflamed site is desirable.

At the inflamed site of the large intestine in IBD patients, high levels of myeloperoxidase (MPO) are released from activated neutrophils. MPO is known to mediate the reaction between hydrogen peroxide and chloride ions to produce hypochlorous acid as an antioxidant against pathogens in the body [11,12]. However, in conditions caused by chronic inflammation in IBD, instead of an antioxidative effect, MPO causes damage at the site of inflammation. The levels of advanced oxidation protein products (AOPPs), used as a biomarker of chronic inflammation, which are generated by oxidation of interior proteins by hypochlorous acid derived from MPO, were reported to be elevated in IBD patients compared with healthy subjects. AOPPs upregulated expression of monocyte chemoattractant protein-1 and transforming growth factor-β1 in the large intestine [13], demonstrating that chronic accumulation of AOPPs promotes progress of IBD. These findings indicate that MPO is an aggravating factor involved in the pathogenesis and progress of IBD. Therefore, a CDDS that can specifically deliver a drug to an inflamed area where MPO is highly localized would be useful in terms of increased therapeutic effects and decreased side effects of drugs, such as 5-ASA.

Example of candidates for CDDS by means of MPO interaction may be included in MPO monoclonal and polyclonal antibodies. However, as the MPO antibody has been reported to be involved in the pathogenesis of MPO-anti-neutrophil cytoplasmic antibody-associated allergic granulomatous vasculitis and crescentic glomerulonephritis [14], the use of MPO antibodies as treatments for inflammatory disorders has a high risk of inducing the pathogenesis of other diseases. As an alternative to MPO antibodies, Tiruppathi et al. have reported that human serum albumin (HSA) interacts with MPO and MPO was able to be delivered from the blood stream into the extracellular region [15]. HSA is the most abundant plasma protein and contributes to the maintenance of osmotic pressure, plasma pH, and the Donnan effect in the capillaries [16,17]. HSA is also an attractive macromolecule for drug delivery because it is biodegradable, nontoxic, and nonimmmunogenic [17]. Although HSA has been widely used as a drug delivery carrier, there are no reports of the use of HSA as an inflamed site-specific CDDS carrier.

HSA is, however, degraded by proteolytic enzymes in the gastrointestinal tract, as well as denatured by low pH conditions in stomach [18], hence it is difficult to orally administer a drug that will reach the colon with an intact form of HSA. Langer et al. have reported that the sensitivity of HSA to proteolysis by variety of different enzymes was suppressed by nanoparticulates of HSA prepared by a desolvation technique in which the active site for enzymatic degradation of HSA was internalized in the nanoparticles [19]. Thus, nanoparticulates of HSA may provide a useful technique for intact delivery at inflamed sites in the colon. In addition, nano-order sized particles have been reported to be highly diffused and taken-up at the inflamed site of the colon [20–22], resulting in a high accumulation of particles, compared with micro-order sized particles, because the average pore size of the mucus layer is less than 200 nm [23]. Therefore, use of HSA nanoparticles would be useful for colon specific drug delivery because these nanoparticles would be less susceptible to enzymatic proteolysis and highly accumulated at the site of inflammation, resulting in a decrease in the required dose of the drug.

In the present study, we demonstrated for the first time, that HSA nanoparticles conjugated with 5-ASA showed anti-inflammatory effects 1,000 times greater than neat 5-ASA. Confocal microscopy observations showed that HSA and MPO were co-localized at the inflamed site of the colon *in vivo*, suggesting that HSA nanoparticles have potential as an CDDS carrier to inflamed sites for the treatment of inflammatory diseases.

2. Materials and methods

2.1. Materials and animals

HSA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 5-ASA, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) and *N*-hydroxysuccinimide (NHS) were purchased from Tokyo Chemical Co., Ltd. (Tokyo, Japan). Glutaraldehyde solution was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dextran sulfate sodium (DSS, 36000–50000 molecular weight, colitis grade) was purchased from MP Biochemicals, Llc. (Santa Ana, CA, USA). Recombinant mouse MPO was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and Slow Fade® diamond antifade mountant were purchased from Thermo Fisher Scientific, Inc. (Yokohama, Japan). Mayer's hematoxylin solution and 1% eosin Y solution were purchased from Wako Pure Chemical Industries, Ltd. Polyclonal rabbit anti-MPO antibody was purchased from Abcam (Cambridge, UK). Goat anti-rabbit IgG (H + L) highly cross-absorbed secondary antibody, Hilyte Fluor 555-labeled, was purchased from AnaSpec Inc. (Fremont, CA, USA). Lightning-link Rapid Dylight 488 kit was purchased from Novus Biologicals (Littleton, CO, USA). All other chemicals were of the highest grade commercially available, and all solutions were prepared in deionized and distilled water.

Male BALB/c mice (24–26 g, 8 weeks) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Preparation of HSA nanoparticles (NPs) and 5-ASA conjugated HSA NPs (5-ASA-HSA NPs)

HSA NPs were prepared by a modified desolvation technique as described previously [24,25]. Fifty milligrams of HSA was diluted in 0.5 ml of 10 mM NaCl solution (pH 9.0) under stirring at 500 rpm to prepare the HSA solution. To prepare the HSA NPs, ethanol, as a desolvating agent, was added to an HSA solution at a rate of 0.5 ml/min using a syringe pump (YSP-101, YMC Co., Ltd., Kyoto, Japan) for 30 s, stirring at 500 rpm at room temperature, and the suspension was stirred for 5 min. This intermittent addition was repeated until the solution just became turbid. The total amount of ethanol added was 1.0 ml. The resultant dispersions were passed through a high-pressure homogenizer (M110-E/H, Microfluidizer®, Microfluidics Co., Newton, MA, USA) at 100 MPa with five pass cycles. Then, 8% glutaraldehyde (29.4 μl) was added to induce particle crosslinking. The crosslinking process was performed under stirring at 500 rpm for 24 h, and this process was terminated by removing the glutaraldehyde by extensive dialysis using a cellulose dialysis bag (Eidia Co. Ltd., Tokyo, Japan) against water. The resultant HSA NP solution was frozen at −40 °C for 3 h and the frozen sample was then lyophilized in a glass chamber for 24 h using a vacuum pump accompanied by a vapor condenser (−20 °C, 0.0225 Torr). Lyophilized HSA NPs were stored at −20 °C until use.

For the preparation of 5-ASA conjugated HSA NPs, 52.8 mg of 5-ASA and 104.9 mg of DMT-MM were diluted by a small amount of 10 mM NaCl solution (pH 9.0). This solution was added to the HSA NP solution which had been passed through a high-pressure homogenizer as mentioned above, and stirred at 500 rpm for 3 h at room temperature to conjugate 5-ASA to the HSA NPs. The reaction was terminated by extensive dialysis against water, and the resultant 5-ASA-HSA NP solution treated with 8% glutaraldehyde and lyophilized, as for the HSA NPs described above. The lyophilized 5-ASA-HSA NP samples were