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Treatment of chemotherapy-induced neutropenia in a rat model by using multiple daily doses of oral administration of G-CSF-containing nanoparticles

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ABSTRACT

Chemotherapy-induced neutropenia often increases the likelihood of life-threatening infections. In this study, a nanoparticle (NP) system composed of chitosan and poly(γ-glutamic acid) conjugated with diethylene triamine pentaacetic acid (γPGA-DTPA) was prepared for oral delivery of granulocyte colony-stimulating factor (G-CSF), a hematopoietic growth factor. The therapeutic potential of this NP system for daily administration of G-CSF to treat neutropenia associated with chemotherapy was evaluated in a rat model. *In vitro* results indicate that the procedures of NP loading and release preserved the structural integrity and bioactivity of the G-CSF molecules adequately. Those results further demonstrated the enzymatic inhibition activity of γPGA-DTPA towards G-CSF against intestinal proteases. Additionally, the *in vivo* biodistribution study clearly identified accumulations of G-CSF in the heart, liver, bone marrow, and urinary bladder, an indication of systemic absorption of G-CSF; its relative bioavailability was approximately 13.6%. Moreover, significant glucose uptake was observed in bone marrow during G-CSF treatment, suggesting increased bone marrow metabolism and neutrophil production. Consequently, neutrophil count in the blood increased in a sustained manner; this fact may help a patient's immune system recover from the side effects of chemotherapy.

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1. Introduction

Cytotoxic chemotherapy suppresses the production of neutrophils, increasing the likelihood of life-threatening infections [1]. As a protein produced by recombinant DNA technology, granulocyte colony-stimulating factor (G-CSF) has been increasingly used to treat chemotherapy-induced neutropenia [2]. As a hematopoietic growth factor, G-CSF predominantly promotes the differentiation and proliferation of neutrophilic granulocyte progenitor cells by interacting with their cell surface G-CSF receptor (G-CSFR) [3]. To maintain its therapeutic effects, G-CSF is normally administered *via* daily injections repeated over several days during chemotherapy [4]. However, frequent injection is inconvenient and painful for patients, possibly causing infections at the injection site [5].

As an attractive alternative to parenteral injection, oral route of drug administration both avoids the pain and side effects associated with daily injections, as well as offers a self-administration capability with a high degree of patient compliance [5]. However, orally administering protein drugs often creates a limited bioavailability, owing to their presystemic degradation and inadequate permeation through the intestinal epithelium [6]. Consequently, an adequate delivery vehicle is necessary to protect the therapeutic proteins from inactivation in the gastrointestinal (GI) tract and improve their oral bioavailability.

Our recent work presented a pH-responsive nanoparticle (NP) vehicle composed of chitosan (CS) and poly(γ-glutamic acid) conjugated with diethylene triamine pentaacetic acid (γPGA-DTPA) for

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the oral delivery of insulin [7]. The CS can adhere to the intestinal surface to impart transient opening of the tight junctions (TJs) between epithelial cells, thereby increasing its paracellular permeability [8]. As a well-known complexing agent, DTPA can chelate divalent metal ions, subsequently inhibiting intestinal proteases and disrupting epithelial TJs [9,10]. Our experimental results demonstrated the effectiveness of CS/γPGA-DTPA NPs as permeation enhancers and their ability to significantly enhance the intestinal absorption of insulin, ultimately lowering the blood glucose in diabetic rats.

By extending the results of above research, this work evaluates an approach in which the CS/γPGA-DTPA NP system is used for daily administration of G-CSF *via* an oral route during one cycle of chemotherapy in a rat model. The effectiveness of γPGA-DTPA in protecting G-CSF from intestinal enzymatic degradation is evaluated. Additionally, the structural integrity of G-CSF molecules released from test NPs is investigated by determining their molecular weight. Meanwhile, their bioactivity is assessed by a NFS-60 cell proliferation assay, in which free-form G-CSF is used as a control. Previous research has used NFS-60 cells, a murine myeloblastic cell line with the G-CSFR on their cell surface, as an *in vitro* model to evaluate the G-CSF bioactivity [11,12]. The biodistribution of G-CSF, as orally delivered *via* CS/γPGA-DTPA NPs, is examined by single-photon emission computed tomography (SPECT)/computed tomography (CT). Its subsequent stimulation of glucose uptake in bone marrow is observed using positron emission tomography (PET)/CT. Moreover, their pharmacokinetic (PK) profiles of plasma G-CSF level and pharmacodynamic (PD) effects on producing a sustained increase in circulating neutrophilic granulocytes are analyzed as well.

2. Materials and methods

2.1. Materials

The 85%-deacetylated CS (MW 60 kDa) was obtained from Koyo Chemical (Japan), while γPGA (MW 55 kDa) was purchased from Vedan (Taichung, Taiwan). The G-CSF was acquired from Mycenax Biotech (Miaoli, Taiwan). Other chemicals and reagents used were of analytical grade and obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Inhibition of G-CSF degradation by γPGA-DTPA

The γPGA-DTPA was synthesized based on a method described in our previous publication [7]. Its ability in protecting G-CSF from enzymatic degradation was studied using the proximal portion of an intestinal tract freshly isolated from rats (Wistar, about 250 g). The isolated intestinal segments were longitudinally dissected (1 cm^2), followed by mounting onto a modified Franz diffusion cell [13]. The intestinal tissues were then incubated with a Krebs–Ringer buffer solution containing free-form G-CSF (1.1 mg/mL, 0.5 mL) and γPGA-DTPA (11 mg/mL, 0.5 mL) at 37 °C. Next, the amount of intact G-CSF remaining was quantified by withdrawing a 50 μL of test solution at different time intervals and immediate analysis by high-performance liquid chromatography (HPLC; LC-NetII/ADC, JASCO, Tokyo, Japan). The G-CSF solution without γPGA-DTPA was used as a control.

2.3. Preparation and characterization of G-CSF-containing NPs

During preparation of G-CSF-containing NPs (G-CSF NPs), an aqueous G-CSF (1.1 mg/mL, 0.25 mL, pH 4.0) was premixed with an aqueous CS solution (1.2 mg/mL, 2.5 mL, pH 6.0) in the presence of sodium tripolyphosphate (TPP, 1.25 mg/mL, 0.25 mL) by magnetic stirring at 4 °C for 6 h. An aqueous γPGA-DTPA (10 mg/mL, 75 μL, pH 7.4) was subsequently added into the mixed solution and blended thoroughly at room temperature in order to form the CS/γPGA-DTPA NPs containing G-CSF; the obtained G-CSF NPs were collected by centrifugation (10,000 rpm at 4 °C for 50 min). The loading efficiency (LE) and content (LC) of G-CSF in the as-prepared NPs were determined by assaying the amount of free G-CSF in the supernatants using HPLC [14]. To reveal their intestinal stability, the particle size and zeta potential value of G-CSF NPs at distinct pH values, in which the pH environments in the GI tract were simulated, were assessed using a Zetasizer (3000HS, Malvern Instruments, Worcestershire, UK); meanwhile, changes in their morphology were examined by transmission electron microscopy (TEM, JEOL, Tokyo, Japan).

2.4. In vitro release study

In vitro release studies were performed by incubating G-CSF NPs, enclosed in dialysis bags (cellulose membrane, MWCO 50,000, Sigma), in phosphate buffered saline (PBS) with various pH values at 37 °C under mild agitation (100 rpm) in a water bath. At predetermined time intervals, samples were withdrawn from the incubation medium and the amount of G-CSF that was released from test NPs was quantified by HPLC. The amount of released G-CSF was expressed as a percentage of the total G-CSF associated with test NPs as calculated from their LE [14].

2.5. In vitro G-CSF transport study

The ability of CS/γPGA-DTPA NPs to enhance the transport of G-CSF *via* opening the epithelial TJs was investigated *in intro* in Caco-2 cell (human colon adenocarcinoma) monolayers [14]. The grown cell monolayers were incubated with test NPs containing G-CSF at distinct pH environments (pH 6.6, 7.0, or 7.4 in the donor compartment and pH 7.4 in the receiver compartment) to replicate the physiological conditions in different segments of the small intestine [15]. Samples (50 μL) were collected from the receiver compartment at distinct time intervals, and their G-CSF concentrations were measured by using ELISA (Human G-CSF Instant ELISA; eBioscience, San Diego, CA, USA).

2.6. Structural integrity and bioactivity of the G-CSF released from test NPs

The structural integrity of G-CSF molecules that had been released from the test NPs in a neutral pH environment due to their pH-sensitivity (the released G-CSF) was exposed by measuring their molecular weight, using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Micromass, Manchester, UK). The MALDI-TOF MS allows for characterization of the protein structures at various levels, including their covalent structure, conformation, dynamics and interaction with physiological partners [16]. Free-form G-CSF molecules were used as a control group.

This study also attempted to determine their ability to bind to the cell surface G-CSFR, by co-culturing test NPs containing the fluorescein isothiocyanate-labeled G-CSF (FITC-G-CSF) with NFS-60 cells at pH 7.4. Twenty four hours later, cells were washed three times with PBS, fixed in 4% paraformaldehyde, incubated with a Cy3-conjugated antibody (rabbit anti-G-CSFR; Abcam, Cambridge, MA, USA) overnight at 4 °C, and then examined *via* a fluorescence microscope (Axio Observer Z1, Carl Zeiss, Göttingen, Germany).

Once binding to G-CSFR, bioactivity of the released G-CSF in stimulating the proliferation of NSF-60 cells was also evaluated [17]. Briefly, NFS-60 cells were suspended in the RPMI 1640 medium containing 10% fetal bovine serum (FBS) and then aliquoted to 96-well tissue culture plates (50 μL/well, 2×10^5 cells/mL). To deactivate its containing proteolytic enzymes, the RPMI 1640 medium was heated for 1 h at 59 °C right before use followed by cooling to 37 °C for cell culture application [18]. Free-form G-CSF and G-CSF NPs (0.01, 0.1, or 1.0 ng/mL G-CSF; 50 μL/well) were then individually added to each well of the culture plate and incubated at 37 °C in an incubator for 48 h. Next, cell proliferation assay was performed by using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) [19]. The absorbance of each test well, which is directly proportional to the number of living cells in culture, was read at 490 nm using a multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). The NFS-60 cells without any treatment and those treated with empty NPs were used as the controls.

2.7. Animal studies

Animal studies were performed according to the "Guide for the Care and Use of Laboratory Animals", as prepared by the Institute of Laboratory Animal Resources, National Research Council and published by the National Academy Press in 1996, and approved by the Institutional Animal Care and Use Committee of National Tsing Hua University (protocol number 10046).

2.7.1. Biodistribution study

Dynamic biodistributions of the G-CSF orally delivered *via* CS/γPGA-DTPA NPs in rats were assessed by using a high-resolution dual-modality NanoSPECT/CT system (Bioscan, Washington DC, USA), allowing their non-invasive visualization and characterization of biological processes in a temporal manner [20]. In this study, G-CSF was radiolabeled with Iodine-123 (^{123}I) by the iodogen precoated tubes (Pierce Iodination Tubes, Thermo Fisher Scientific, Rockford, IL, USA) as per the manufacturer's instructions. Radiolabeled NPs were subsequently prepared using the as-prepared ^{123}I-G-CSF.

Following oral administration of test NPs containing the radiolabeled ^{123}I-G-CSF in rats (Wistar, ~250 g), dynamic SPECT images were acquired. Details of radiotracer distribution were then examined by collecting additional CT images for anatomical reference. Rats treated with free-form ^{123}I-G-CSF *via* oral ingestion or subcutaneous (SC) injection were used as the controls ($n = 3$ for each group). Animal imaging was also performed under the controlled temperature (37 °C) and anesthesia (1.5% isoflurane in 100% oxygen). Details of the protocol used in the image scanning can be found in our earlier study [21].

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