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Research paper

Assay system development to measure the concentration of sargramostim with high specificity in patients with autoimmune pulmonary alveolar proteinosis after single-dose inhalation

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ABSTRACT

During a clinical trial of a *Saccharomyces cerviciae*-derived recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF), sargramostim, in patients with autoimmune pulmonary alveolar proteinosis (aPAP), we conducted a pharmacokinetic study of single-dose sargramostim inhalation. Several problems were encountered whereby sargramostim formed an immune-complex with GM-CSF autoantibodies (GMAbs) immediately after entering the body; thus, we could not measure the concentration of sargramostim using a commercial high sensitivity enzyme-linked immunosorbent assay (ELISA). Moreover, the ELISA could not discriminate inhaled sargramostim from intrinsic GM-CSF. To solve these problems, we developed a novel ELISA system with a capture antibody that is specific for sargramostim and a detection antibody capable of binding with GM-CSF. This system quantified the serum sargramostim concentration, but not *E. coli*-, CHO-, or HEK293T-derived human recombinant GM-CSF. Using this system, serum pharmacokinetics were estimated in five patients after inhalation of 250 μg sargramostim, with a mean Cmax of 9.7 ± 2.85 pg/ml at a Tmax of 2 ± 1.22 h.

1. Introduction

Autoimmune pulmonary alveolar proteinosis (aPAP) is a rare lung disease characterized by excessive surfactant accumulation within alveoli and terminal bronchioli. In most cases, onset occurs in middle age, and 5% of patients develop severe respiratory failure (Inoue et al., 2008). We previously reported that the consistent presence of granulocyte-macrophage colony stimulating factor (GM-CSF) autoantibodies (GMAbs) in the blood and lungs of patients is the causative agent of the disease (Kitamura et al., 1999). As GM-CSF promotes the maturation of myeloid precursor cells and maintains the function of alveolar macrophages (Metcalf, 2008; Esnault and Malter, 2002), GMAbs neutralize the bioactivity of GM-CSF in the lungs, impairing alveolar macrophage-mediated pulmonary surfactant clearance, and thus causes aPAP.

Intrinsic GM-CSF is a glycoprotein that consists of 127 amino acid residues, with four cysteines involved in two disulfide bonds that form a compact globular structure comprised of four α-helices joined by loops (Hashimoto et al., 2014). Recombinant human GM-CSF (rhGM-CSF) preparations produced from *E. coli* and *S. cerevisiae* are now available for the treatment of myeloid reconstitution after autologous or allogeneic bone marrow transplantation and neutropenia induced by chemotherapy during the treatment of acute myeloid leukemia. *E. coli*-derived rhGM-CSF, molgramostim, is non-glycosylated, consists of 127 amino acid residues, has a molecular weight of 14.5 kDa, and is methylated at the N-terminal (Forno et al., 2004). *S. cerevisiae*-derived rhGM-CSF, sargramostim, is a glycoprotein with the same 127 amino acid sequence as molgramostim, but contains leucine at position 23 instead of arginine (Dorr, 1993).

Based on the etiology, several clinical trials of GM-CSF inhalation have been conducted with variable response rates ranging from 40 to 62%. From 2004 to 2008, 35 patients have completed 24 weeks of GM-CSF inhalation, of whom 23 cases responded with a mean reduction in the alveolar–arterial gradient ($AaDO_2$) of 12.3 mm Hg (Tazawa et al., 2010; Arai et al., 2004; Price et al., 2006; Yamamoto et al., 2008; Robinson et al., 2009; Rodriguez Portal et al., 2009). Currently, an investigator-initiated clinical trial of sargramostim inhalation therapy is being carried out for the purpose of pharmaceutical approval. Originally, sargramostim was developed for intravenous use; thus, no pharmacokinetic data of its inhalation has been available until now. Prior to the investigator-initiated clinical trial of sargramostim inhalation in

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patients, the Pharmaceutical and Medical Devices Agency (PMDA) advised the present research team to conduct a pharmacokinetic study after single-dose inhalation in both healthy subjects and patients, and to monitor the serum sargramostim concentrations in patients in a phase II study. However, sargramostim forms an immune-complex with GMAbs immediately after entering the lungs and blood of patients, such that a commercially available, high-sensitivity enzyme-linked immunosorbent assay could not be used to measure the concentration. Moreover, the commercial ELISA cannot discriminate between inhaled sargramostim and intrinsic GM-CSF.

In this study, we developed a sargramostim-specific monoclonal antibody to use in a novel ELISA system that measures the serum sargramostim concentration, even when it is in the form of an immune complex. Using this system, the pharmacokinetics of sargramostim was successfully evaluated in both healthy subjects and patients.

2. Materials and methods

2.1. Subjects

This study was approved by the Institutional Review Board at Niigata University Medical Dental Hospital (IH28001). The clinical trial of the pharmacokinetics of inhaled sargramostim was filed with the PMDA (NTU28-0852). Five patients with aPAP aged between 20 and 80 years were enrolled in this study. Patients with severe disease, namely those with disease severity scores of 3–5 according to the AJRCCM criteria were excluded from the study. Nine healthy males aged between 20 and 45 years were also enrolled in this study. Subjects with a body mass index (BMI) $>$ 25 and $<$ 18 were excluded from the study. All subjects gave written informed consent to participate in this study. Serum samples were stored at −80 °C until use.

2.2. Materials

For ELISA development, sargramostim was purchased from the Sanofi-Genzyme Corporation (Boston, MA). For the inhalation study of the patients and healthy subjects, vials containing 250 μg of sargramostim were provided by the Sanofi-Genzyme Corporation. A biotinylated anti-human GM-CSF antibody was purchased from Abcam (Cambridge, UK). The *E. coli*-derived rhGM-CSF was purchased from Amoytop Biotech (Fujian, China), and the HEK293T-derived rhGM-CSF was purchased from HumanZyme Inc. (Chicago, IL). Chinese Hamster Ovary (CHO) cell derived rhGM-CSF was kindly provided from Japan Chemical Research, Co. Ltd. Four peptides encoding the major GM-CSF α-helices were purchased from GeneScript (New Jersey, NJ). The alkaline phosphatase labeling kit-NH2 was purchased from Dojindo (Kumamoto, JPN). Biotin labeling kit-NH2 was purchased from Dojindo. A high sensitivity GM-CSF enzyme linked immunosorbent assay kit (Human GM-CSF Quantikine HS ELISA Kit) was purchased from R&D system, Inc.(Minneapolis, MN).

2.3. Establishment of sargramostim-specific monoclonal antibodies

Before immunization to mouse, 100 μg of sargramostim was denatured by heating at 95 °C for 5 min in 1 mM dithiothreitol/phosphate buffered saline (PBS). Monoclonal antibodies specific for sargramostim were established according to a method called “the mouse iliac lymph node method” in ITM Co. Ltd. (JPN). Briefly, mice were anesthetized and injected intramuscularly at the right and left tail base with an emulsion (0.1 ml in total) containing the heat denatured sargramostim and Freund's complete adjuvant. After 15 days, iliac lymph node cells and murine myeloma cells (SP2/0-Ag14) were fused in the presence of polyethylene glycol (PEG), and hybridomas reactive to sargramostim were selected using the limiting dilution method. Four clones, 33-8F, 33-10D, 3-8F, and 40-1H, were selected to react with sargramostim. Of these, clone 40-1H reacted with only sargramostim and not with *E. coli*-derived GM-CSF. Subsequently, BALB/C mice were inoculated intraperitoneally with a 40-1H or 33-8F hybridoma. After 8 weeks, the ascites were collected and applied to a protein A column to purify the monoclonal antibodies. The isotypes of 40-1H, 33-8F, 3-8F, and 33-10D were IgG2a, IgG1, IgG3, and IgG2b, respectively. Alkaline phosphatase labeling of the 33-8F monoclonal antibody was performed using an alkaline phosphatase labeling kit (Dojindo), according to the manufacturer's instructions.

2.4. Binding ability of the four monoclonal antibodies with various recombinant GM-CSFs

1 μg/ml of the monoclonal antibodies 40-1H, 33-8F, 33-10D, and 3-8F in 50 μl of phosphate buffered saline (PBS) was added into each well of a micro-ELISA plate (Maxisorp™ flat-bottom, clear, 96-well plates, Nunc, Roskilde, Denmark) previously coated with rabbit anti-mouse IgG (10 ng/ml) and incubated at room temperature for 60 min. After washing three times with PBS containing 0.1% Tween® 20 (PBST), 1 ng/ml of biotinylated sargramostim, biotinylated *E.coli*-derived, or biotinylated CHO-derived rhGM-CSF in 50 μl of PBST was added and incubated at room temperature for 60 min. The plate was washed again three times with PBST before adding horseradish peroxidase (HRP) conjugated streptavidin (2.5 μg/ml, 50 μl) and incubating at room temperature for 30 min. To visualize the reaction, the plate was again washed three times with PBST, 50 μl of OPD solution (Thermo Fisher Scientific, MA) was added, followed by 100 μl of 1 M H_3PO_4 to stop the color development. Measurement of the optical density was at 490 nm.

2.5. Epitope mapping of monoclonal antibody 40-1H

Two μg/ml of the four major peptides (peptide 1: 3-32AA, peptide 2: 39-77AA, peptide 3: 82-105AA, and peptide 4: 110-126AA) were coated onto micro ELISA plates at 4 °C overnight. After washing three times, each well was blocked with Stabilicoat (Stabilicoat® Immunoassay Stabilizer, Surmodics, Inc., MN) and incubated for 2 h at room temperature. One hundred ng of the monoclonal antibodies 40-1H, 33-8F, 33-10D, or 3-8F in 50 μl of PBS was added to each well and incubated for 40 min at room temperature. After washing three times with PBST, 0.2 μg/ml of peroxidase labeled anti-mouse IgG (Sigma-Aldrich) was added and incubated for 30 min at room temperature. Finally, the plate was washed four times before the color was developed with TMB solution (TMB Substrate Solution, Thermo Fisher Scientific, Waltham, MA), stopped with 0.5 M H_2SO_4, and the optical density was measured at 450 nm.

2.6. Selection of the second antibody for the sargramostim-specific ELISA

Monoclonal antibodies (100 ng/ml) were incubated with sargramostim (50 μg/ml) coated on a 96-well plate for 1 h, then each well was treated with 4 M Urea, washed with PBST, and incubated with a 10,000-fold diluted peroxidase-labeled rabbit anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) in PBS for 1 h. After washing with PBST three times, the color was developed using TMB solution (Thermo Fisher Scientific), and the absorbance was measured at 450 nm. This method provided the relative avidity index, which was calculated using the following formula: (absorbance reading after urea wash)/(absorbance reading without urea wash) × 100.

2.7. Extraction and purification of sargramostim from sera using a polyclonal anti-GM-CSF antibody (Fig. 1a-g)

A streptavidin coated plate (Fig. 1a) (Immobilizer streptavidin F96 clear; Thermo Fisher Scientific) was hydrophilized by washing with PBST three times. One hundred μl of biotinylated GM-CSF polyclonal antibody (1 μg/ml) was then transferred to the plate and incubated at 25 °C for 1 h (Fig. 1b), followed by washing with PBST three times.