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Carbon monoxide-bound hemoglobin-vesicles for the treatment of bleomycin-induced pulmonary fibrosis

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ABSTRACT

Carbon monoxide (CO) has potent anti-inflammatory and anti-oxidant effects. We report herein on the preparation of a nanotechnology-based CO donor, CO-bound hemoglobin-vesicles (CO-HbV). We hypothesized that CO-HbV could have a therapeutic effect on idiopathic pulmonary fibrosis (IPF), an incurable lung fibrosis, that is thought to involve inflammation and the production of reactive oxygen species (ROS). Pulmonary fibril formation and respiratory function were quantitatively evaluated by measuring hydroxyproline levels and forced vital capacity, respectively, using a bleomycin-induced pulmonary fibrosis mice model. CO-HbV suppressed the progression of pulmonary fibril formation and improved respiratory function compared to saline and HbV. The suppressive effect of CO-HbV on pulmonary fibrosis can be attributed to a decrease in ROS generation by inflammatory cells, NADPH oxidase 4 and the production of inflammatory cells, cytokines and transforming growth factor-β in the lung. This is the first demonstration of the inhibitory effect of CO-HbV on the progression of pulmonary fibrosis *via* the anti-oxidative and anti-inflammatory effects of CO in the bleomycin-induced pulmonary fibrosis mice model. CO-HbV has the potential for use in the treatment of, not only IPF, but also a variety of other ROS and inflammation-related disorders.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive type of fibrous interstitial pneumonia with an unknown cure, except for lung transplantation. Patients with IPF have an estimated median survival of 2–5 years [1–3]. Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is currently the only orally administered drug approved for clinical use in the treatment of IPF in both the EU and Japan. Recently, the CAPACITY (Clinical Studies Assessing Pirfenidone in idiopathic pulmonary fibrosis: Research of Efficacy and Safety Outcomes) program showed that pirfenidone has a favorable benefit–risk profile, and, as a result, represents an appropriate treatment option for patients with IPF [4]. On the other hand, this multinational, double-blind, placebo-controlled study (CAPACITY006) also showed that pirfenidone treatment does not completely improve the clinically meaningful effects on forced vital capacity (FVC) and survival benefit [4]. In addition, it is well-known there are some significant side effects associated with the use of

Abbreviation: CO, carbon monoxide; HbV, hemoglobin-vesicles; CO-HbV, CO-bound hemoglobin-vesicles; IPF, idiopathic pulmonary fibrosis; FVC, forced vital capacity; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; CO-RM, CO-releasing molecules; HbCO, carboxyhemoglobin; RBC, red blood cell; PEG, polyethylene glycol; BLM, bleomycin; 8-OH-dG, 8-hydroxy-2′-deoxygenase; NO_2-Tyr, nitrotyrosine; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; Nox4, nicotinamide adenine dinucleotide phosphate oxidase 4; Poldip2, polymerase delta interacting protein 2; EMT, epithelial–mesenchymal transition.

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pirfenidone, which include photosensitivity (more than 50% of patients) [5]. Therefore, the development of drugs designed to suppress the progression of this disease or to improve respiratory function is of great importance.

Since new pathogenic pathways and mediators of IPF are discovered, the progression of IPF appears to result from a complex combination a number of factors, including inflammation, reactive oxygen species (ROS) and transforming growth factor (TGF)-β. Although recent studies have suggested that the repeated administration of drugs with either anti-oxidative or anti-inflammatory properties would be expected to be useful in the treatment of IPF [6,7], the clinical use of these agents for IPF have not been approved worldwide. This is likely because these agents targeted only one of the many pathogenesis pathways, and, because mechanism responsible for the development of IPF is complex, little alleviation occurs. Therefore, a shift in the effective treatment strategy for IPF from agents that block a single functional action to an agent that can address multiple functions is clearly needed.

Carbon monoxide (CO) possesses anti-inflammatory, anti-oxidant and anti-proliferative effects, and has attracted interest as a possible clinically viable medicinal agent [8,9]. Similar to medical gasses that are routinely used in clinical situations, such as nitric oxide and oxygen, clinical applications of CO take the form of inhaled gaseous therapy and the use of CO-releasing molecules (CO-RM) [10,11]. In fact, several studies have demonstrated the efficacy of inhaled CO and CO-RM in preclinical animal models such as disorders related to inflammation and redox [12–14]. In addition, it was reported that inhaled CO and CO-RM also exerts protective effects in the case of several types of lung diseases, including pulmonary hypertension, asthma and ischemia reperfusion [15–17]. Taking these findings into consideration, CO holds enormous potential for use in the treatment of pulmonary disorders, including IPF. However, CO-RM rapidly liberates CO, with a half-life of 1–21 min, which is extremely short in terms of producing a significant therapeutic impact [18]. To achieve a sustainable therapeutic effect of CO, the continuous or repeated administration of CO-RM would be required. In addition, although high serum carboxyhemoglobin (HbCO) levels can cause several toxicity [19], it is difficult to control the serum HbCO levels as the result of inhaled CO and avoid CO intoxication. Therefore, it should be noted that an alternative pathway for the therapeutic delivery of CO to the lungs is essential in the successful clinical application of CO.

Recent developments in nanotechnology-based carriers, namely, Hemoglobin-vesicles (HbV), would offer great potential for effective CO delivery, and could lead to strategies in the development of new CO donors. To date, several preclinical trials have evaluated the histology, biochemical analysis and pharmacokinetic properties after the single or repeated administration of a putative dose of HbV in rodent, pig and monkey [20–24]. The results show that HbV possesses good biological compatibility (low complement activation) and is promptly metabolized (no accumulation in the body) even after a massive single or repeated infusion. Furthermore, the size of HbV is controlled at ca. 250 nm, because it can prevent capillary plugging, renal excretion and vascular wall permeability. Fortunately, CO easily and stably binds to hemoglobin (Hb) in the form of HbV as well as red blood cell (RBC), because the cellular structure of HbV most closely mimics the characteristics of a natural RBC, in which a highly concentrated Hb is encapsulated within a liposome with polyethylene glycol (PEG). In addition, in a previous study, we reported that CO was exhaled within 6 h after administering CO-bound HbV (CO-HbV) to hemorrhagic-shocked rats [25]. These findings led us to the hypothesis that HbV has the potential for use a carrier of CO to the lungs. Given the known therapeutic effects of CO-HbV on IPF, we decided to first evaluate whether CO-HbV could protect against IPF using an IPF animal model of bleomycin (BLM)-induced pulmonary fibrosis. In subsequent experiments, we investigated the reason why CO-HbV functions to suppress the progression of IPF.

2. Materials and methods

2.1. Preparation of HbV and CO-HbV solution

HbVs and CO-HbV were prepared under sterile conditions, as previously reported [26]. In short, the Hb solution was purified from outdated donated RBC, which was provided by the Japanese Red Cross Society (Tokyo, Japan), and the oxyhemoglobin converted into HbCO by bubbling with CO gas. The lipid bilayer was a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan) at a molar ratio of 5/5/1, and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG (NOF Corp., Tokyo, Japan) (0.3 mol%). The CO-HbV particles were prepared by the extrusion method, and suspended in a physiological salt solution, filter-sterilized (Dismic, Toyo-Roshi, Tokyo, Japan; pore size, 450 nm). By illumination with visible light under an oxygen atmosphere, CO-HbV was converted to HbV. The HbV particles suspended in physiological salt solution bubbled with nitrogen for storage. The average diameters of the HbV and CO-HbV were maintained at approximately 250 nm *via* stepwise extrusion through cellulose acetate membrane filters with a final pore size of 0.2 μm (Fig. 1). The HbV and CO-HbV suspended in physiological salt solution were at [Hb] = 10 g/dL and [lipid] = 9.0 g/dL. The HbCO rate in CO-HbV was nearly 100%, while that in HbV was less than 5%.

2.2. Production of BLM-induced pulmonary fibrosis mice model

All animal experiments were conducted in accordance with the guidelines of Kumamoto University for the care and use of laboratory animals. To create BLM-induced pulmonary fibrosis model mice, Sea-ICR mice (6 weeks, male; Kyudo Co.,Ltd, Saga, Japan) were intratracheally treated with BLM (5 mg/kg; Nippon Kayaku, Tokyo, Japan) in PBS (1 ml/kg) under anesthesia with chloral hydrate (500 mg/kg) as previous report [27]. Saline, HbV, or CO-HbV was administered *via* the tail vein at 30 min before BLM treatment and 24 h after BLM treatment.

2.3. Plasma biochemical parameters

At 7 and 14 days after the HbV injection, BLM-induced pulmonary fibrosis model mice were anesthetized with ether and collected blood. Blood samples were immediately centrifuged (3000 g, 10 min) to produce plasma. The plasma samples were then ultracentrifuged to remove HbV (50,000 g, 30 min), because HbV interferes with some of the laboratory tests [28]. All plasma samples were stored at −80 °C until used. All plasma samples were analyzed by Clinical Chemistry Analyzer (JEOL, JCA-BM6050, Tokyo, Japan).

2.4. Histological and immunohistochemical analyses

The whole lungs were removed and fixed with 10% phosphate buffered formalin. The tissue was then dehydrated at room temperature through a graded ethanol series and embedded in paraffin. The prepared tissues were cut into 4-μm-thick sections for histological and immunohistochemical evaluation. Hematoxylin and Eosin (HE) stain and Masson's trichrome stain were performed as previously described [27]. The immunostaining for 8-hydroxy-2′-deoxygenase (8-OH-dG) and nitrotyrosine (NO_2-Tyr) were performed as described in a previous report with minor modifications [29]. In short, the primary antibody reaction was conducted below 4 °C overnight, and the secondary antibody reaction at room temperature for 90 min. In addition, the primary antibody containing NO_2-Tyr (Millipore, Tokyo, Japan, cat#: AB5411) and 8-OH-dG [15A3] (Santa Cruz, California, USA, cat#: sc-66036) was diluted 50 fold prior to use. The secondary antibodies for 8-OH-dG and NO_2-Tyr were Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Invitrogen, Eugene, USA, cat#: AB11008) and Alexa Fluor 546 goat anti-rabbit IgG (H + L) (Invitrogen, Eugene, USA, cat#: AB11010), respectively. In each case the secondary antibody was diluted 200 times before use. After the reaction, the slide was observed using Microscope (Keyence, BZ-8000, Osaka, Japan).

2.5. Determination of hydroxyproline level in lung tissues

On day 14 after BLM administration, the left lung was removed and hydroxyproline content was determined as described previously [30]. The absorbance was measured at 550 nm to determine the amount of hydroxyproline.

2.6. Measurement of lung mechanics and FVC

Measurement of lung mechanics and FVC were performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ), as described previously [31]. Mice were mechanically ventilated at a rate of 150 breaths/min, using a tidal volume of 8.7 ml/kg and a positive end-expiratory pressure of 2–3 cm H_2O. Total respiratory system elastance and tissue elastance were measured by the snap shot and forced oscillation techniques, respectively.

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