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Original article

Attenuation effects of heparin–superoxide dismutase conjugate on bleomycin-induced lung fibrosis *in vivo* and radiation-induced inflammatory cytokine expression *in vitro*

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Abstract

In this study, the effects of heparin–superoxide dismutase conjugate (heparin–SOD) on bleomycin-induced pulmonary fibrosis *in vivo* and on inflammatory cytokine expression *in vitro* were evaluated. To investigate the effects of heparin–SOD on pulmonary fibrosis, heparin–SOD was administered to bleomycin (BLM)-treated mice by intraperitoneal injection once a day and the hydroxyproline content in lung was determined per 7 days. The degree of fibrosis was assessed quantitatively using histopathologic features. The results showed that heparin–SOD inhibited BLM-induced lung fibrotic lesions as reflected by the decrease of lung hydroxyproline content and lung fibrosis grade 28 days after BLM instillation. The *in vitro* effects on the cytokine level expressed by irradiated 3T3 fibroblasts showed that heparin–SOD significantly lowered the levels of transforming growth factor-β1 and interleukin-1β. These results strongly demonstrated that heparin–SOD might be useful in the prevention and treatment of pulmonary fibrosis.

Keywords: Cu,Zn-superoxide dismutase; Heparin–superoxide dismutase; Pulmonary fibrosis; Bleomycin; Hydroxyproline

1. Introduction

Pulmonary fibrosis, the end-point of a heterogeneous group of disorders designated 'lung diseases', is characterized by fibroblast proliferation and extracellular matrix remodeling [1]. Up to one-half of the cases of pulmonary fibrosis are idiopathic pulmonary fibrosis (IPF). Unfortunately, despite intensive investigation, the results of therapy for IPF have still remained unsatisfactory [2]. IPF is a chronic inflammatory interstitial lung disease, characterized by the accumulation of alveolar macrophages, neutrophils and lymphocytes in the distal airspaces, and by parenchymal cell injury and fibrosis of the alveolae [3]. The etiology and pathogenesis of the disease are still unknown, but several inflammatory mediators have been implicated in the pathogenesis of IPF [4], including cytokines, chemokines, growth factors, and reactive oxygen species (ROS) [3–5]. An increased oxidant burden in the lungs in IPF is thought to arise from accumulation of inflammatory cells, such as activated alveolar macrophages and neutrophils, which shows an exaggerated release of reactive oxygen intermediates (ROI) [5,6]. Radiotherapy is an essential mode of treatment for nearly half of all cancer patients, but formation of overdose free radicals such as superoxide anion radical ($O_2^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$) is an unavoidable consequence in radiotherapy. These free radicals are very unstable and react rapidly with other groups or substances in

Abbreviations: SOD, Cu,Zn-superoxide dismutase; LMWH, low molecular weight heparin; BLM, bleomycin; ROS, reactive oxygen species; IPF, idiopathic pulmonary fibrosis.

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the body, leading to cell or tissue injury. This may be illustrated considering one of the many mechanisms by which oxidative stress can cause damage by stimulating the free radical chain reaction. Free radicals activate transforming growth factor-β1 (TGF-β1), one of the most important growth factors in the pathogenesis of fibrotic lung diseases, which promotes epithelial cell apoptosis [7,8].

There are no *in vitro* models that accurately reflect the development of IPF. However, many assay systems are available to help us to understand cellular responses under specific conditions. Many different animal models have been developed to investigate pulmonary fibrosis. The best characterized and most commonly used agent in inducing fibrosis is bleomycin (BLM), an antibiotic with anti-tumor activity derived from oxidant-induced DNA damage [7]. BLM is usually administered in saline with a single dose intratracheally, intranasally, intraperitoneally or intravenously. The single administration bleomycin model has been favored principally because it is relatively easy to perform, reproducible and presents with many of the histological, biochemical and molecular features of IPF. In addition, recent gene array studies demonstrated similarities in gene profiles in lung tissue from IPF patients and BLM-treated mice and rats [9,10]. Intratracheal instillation of BLM into the lungs of rodents causes alveolar cell damage, inflammatory response, fibroblast proliferation and subsequent collagen content deposition. Lesions observed in the early stages of lung damage induced by BLM resemble human fibrotic lung disease, both histologically and physiologically [11,12]. The availability of these animal models of interstitial pulmonary fibrosis also provides the opportunity to investigate novel pharmacological approaches to prevent this disease [13,14].

A recent study suggested that antioxidant therapy for idiopathic pulmonary fibrosis might be useful [15,16,1]. It has been reported that administering superoxide dismutase (SOD) to the lung prior irradiation by inhalation could protect the lung against functional damage and that the mRNA transcription of inflammatory cytokines demonstrated parallel reductions [17–20]. Because of the shortcomings of proteins used as medicines, such as short half-life, low tissue affinity, antigenicity and instability, increasing attention has been given to chemical modification of proteins[21]. In our laboratory, Cu,Zn-superoxide dismutase (Cu,Zn-SOD) had been chemically modified with low molecular weight heparin (LMWH), and our previous study had proved that LMWH–SOD conjugate, which has a lower immunogenicity and higher anti-inflammatory activity, also gained higher stabilities than Cu,Zn-SOD towards acid, alkali, heat and trypsin treatment [22]. Our previous study had shown that the administration of the heparin–SOD actually leads to increased levels of SOD activity in the blood or other tissues *in vivo*, particularly in the lungs. Heparin–SOD was also prepared using the same strategy. The principal aim of this paper is to evaluate the potential role of heparin–SOD in oxidative lung injury induced by BLM in mice *in vivo* and the effect on inflammatory cytokine expression *in vitro*. This was assessed using biochemical and histological methods.

2. Materials and methods

2.1. Chemicals

Bleomycin A5 hydrochloride (Tianjin Hebei Pharmaceutical Factory, China); L-hydroxyproline standard, trypsin (Sigma); paradimethylaminobenzaldehyde (PDAB) (Shanghai Reagent Factory, China); RPMI-1640 culture medium, fetal bovine serum (Gibco BRL Life Technologies); TGF-β1 enzyme-linked immunoassay kit (Bender Co.); interleukin (IL)-1β enzyme-linked immunoassay kit (Biosource Co.). Other chemicals and reagents were of analytical grade.

2.2. Animals

Pathogen-free male Kunming mice, weighing 23–27 g, offered by Experimental Animal Center of Shandong University, were used in the experiments. The animals were housed in animal facilities accredited by the Shandong Council on Animal Care and treated in accordance with approved protocols. Animals were maintained in a specific pathogen-free environment that was temperature-controlled (23 ± 2 °C) and humidity-controlled (60 ± 10%), under a 12:12 h light/dark cycle. The animals used in this study were handled and treated in accordance with the strict guiding principles of the National Institution of Health for Experimental Care and Use of Animals. The experimental design and procedures were approved by the Institutional Ethical Committee for Animal Care and Use of Shandong University, People's Republic of China.

2.3. Model building of mouse pulmonary fibrosis

The method of one time intratracheal injection with BLM A5 hydrochloride to duplicate mouse lungs was employed to make lung injury model. For injection, BLM was dissolved in sterile saline. Some were injected intratracheally (0.15 U/mouse in 50 μl saline) after exposure of the trachea under ketamine anesthesia as described previously [23–27].

2.4. Grouping

150 male Kunming mice were divided randomly into six groups: 1, sham operated group; 2, Cu,Zn-SOD medication group; 3, heparin–SOD medication group; 4, heparin and Cu,Zn-SOD mixture (heparin + SOD) medication group; 5, control group; 6, heparin medication group. The administration dosages concerning Cu,Zn-SOD to the above groups were according to Cu,Zn-SOD enzymatic activity (20,000 U/kg), and heparin dosage of the heparin + SOD medication group was according to heparin proportion of heparin–SOD medication group. The day after bleomycin treatment, the above corresponding samples were administered to each group by intraperitoneal injection once daily with a volume of 0.5 ml; the control group was injected with isotonic Na chloride of the same volume. Each group included 25 mice. Five mice of each group were euthanized