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Short communication

An endostatin-derived peptide orally exerts anti-fibrotic activity in a murine pulmonary fibrosis model

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ABSTRACT

Objective: Pulmonary fibrosis causes high morbidity and mortality in affected individuals. Recently, we showed that parenteral or intratracheal administration of a peptide derived from endostatin, called E4, prevents and ameliorates fibrosis using different models of dermal and pulmonary disease. No marketed orally delivered peptide drugs are currently available for progressive pulmonary fibrosis; however oral delivery of drugs is the preferred route for treating most chronic diseases. Thus, we investigated whether oral administration of E4 peptide exerted anti-fibrotic activity in a murine pulmonary fibrosis model.

Methods: Bleomycin (1.2 mU/g body weight) was intratracheally administrated to male 6–8-week-old C57BL/6J mice. E4 peptide (20, 10, 5, and 1 μg/mouse) or scrambled control peptide (20 μg/mouse) was orally administered on the same day as bleomycin. In some experiments, E4 peptide (10 and 5 μg/mouse) was orally administered three times on days 0, 3, and 6 post-bleomycin treatment. Lungs were harvested on day 21 for histological analysis and hydroxyproline assay.

Results: Histological analysis and hydroxyproline assay revealed that bleomycin successfully induced pulmonary fibrosis, and that 20 μg of oral E4 peptide ameliorated the fibrosis. The lower doses of E4 peptide (10, 5, and 1 μg) were insufficient to exert anti-fibrotic activity when given as a single dose. Multiple doses of E4 peptide efficiently exerted anti-fibrotic activity even at lower doses.

Conclusion: E4 peptide shows oral bioavailability and exerts anti-fibrotic activity in a bleomycin-induced pulmonary fibrosis model. We suggest that E4 peptide is a novel oral drug for fibroproliferative disorders.

1. Introduction

Fibrosis is a pathological process characterized by fibroblast activation and proliferation and increased deposition of extracellular matrix (ECM) proteins such as fibronectin and collagen in an organ or tissue. Pulmonary fibrosis results in end-stage organ failure and loss of function, consequently causing high morbidity and mortality in individuals with a progressive pulmonary fibrotic disease such as idiopathic pulmonary fibrosis (IPF) or a systemic fibrotic disease such as systemic sclerosis (SSc) [1,2]. Unfortunately, there are currently no effective therapies that reverse organ fibrosis. Organ transplantation remains a viable option for a small number of patients. Recently, two drugs were approved by the FDA for the treatment of IPF [3,4], and although these drugs reduce the rate of progression of the disease, neither drug reverses lung fibrosis. Thus, the need to identify an effective therapy for lung fibrosis is undiminished.

Endostatin is a 20 kDa C-terminal fragment of collagen XVIII, which was originally identified as an inhibitor of endothelial proliferation, angiogenesis, and tumor growth [5]. We recently showed that a peptide derived from the C-terminus of endostatin, called E4, prevented and ameliorated fibrosis *in vitro*, *in vivo*, and *ex vivo* [6]. We also demonstrated that E4 peptide prevented and reversed TGF-β- and bleomycin-induced dermal and pulmonary fibrosis, and that the E4 peptide is effective at reducing dermal and pulmonary fibrosis if given subcutaneously, intraperitoneally, or intratracheally, respectively [6].

Oral delivery of drugs is the preferred route for treating most chronic diseases [7]. Oral administration offers advantages in terms of ease of administration, lower manufacturing costs, and increased patient compliance [8]. The use of peptides and proteins as therapeutic agents is rapidly expanding in various fields such as neurology, oncology, endocrinology, and hematology. To date, two orally available small compounds, Nintedanib, an intracellular inhibitor that targets multiple tyrosine kinases, and Pirfenidone, which has anti-fibrotic, anti-inflammatory, and anti-oxidant properties, have been approved for use in patients with IPF [9,10]. However, no marketed orally delivered peptide or protein drugs are available for progressive pulmonary fibrotic diseases.

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Therefore, in the present study, we investigated whether orally administrated E4 peptide exerts anti-fibrotic activity in a murine pulmonary fibrosis model, and thus whether oral administration of E4 peptide would be a viable therapeutic strategy.

2. Materials and methods

2.1. Bleomycin induced pulmonary fibrosis model

Pulmonary fibrosis was induced in mice as previously described with some modifications [6]. Briefly, bleomycin (1.2 mU/g body weight) in a total volume of 50 μl PBS was intratracheally administrated to male 6–8-week-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA). E4 peptide was synthesized in the Peptide Synthesis Core Facility, University of Pittsburgh Genomics & Proteomics Core Laboratories, as a biotinylated peptide to protect the N-terminus from degradation with a C-terminal amidation to protect the carboxy terminus as previously described [6]. Biotinylated-E4 (20 μg/mouse) or biotinylated-scrambled peptide (20 μg/mouse) in 100 μl H_2O was orally administered on the same day as bleomycin *via* gavage. In some experiments, to identify the minimal dose of E4 peptide that can exert anti-fibrotic activity, biotinylated-E4 (20, 10, 5, and 1 μg/mouse) was orally administered on the same day as bleomycin *via* gavage. In some experiments, biotinylated-E4 (10 and 5 μg/mouse) was orally administrated three times on days 0, 3, and 6 post-bleomycin treatment. Lungs were harvested on day 21 for histological analysis and hydroxyproline assay. Bronchoalveolar lavage (BAL) fluid was collected from a group of mice on day 5 and cell counts were evaluated using a hemocytometer. All experiments were done under a protocol approved by the IACUC of the Medical University of South Carolina.

2.2. Histological analysis

Lung tissues were fixed with 10% formalin and embedded in paraffin. Six micrometer sections of paraffin-embedded mouse lung tissues were stained with hematoxylin and eosin (H&E). Images were taken on a Motic BA410 Compound Microscope (Motic, British Columbia, Canada) using identical settings.

2.3. Hydroxyproline assay

To analyze the amount of collagen in mouse lung tissues, hydroxyproline content was measured as previously described [11].

2.4. Statistical analysis

All continuous variables were expressed as the mean ± standard deviation. All statistical analyses were done using IBM SPSS statistics 22 (IBM Corporation, Armonk, NY, USA). Comparison among 3 or more groups was performed using ANOVA followed by Mann–Whitney *U* test.

3. Results

3.1. Oral administration of E4 ameliorated bleomycin-induced pulmonary fibrosis in vivo

We reported that intratracheal and intraperitoneal administration of E4 peptide ameliorates bleomycin-induced pulmonary fibrosis *in vivo* [6]. We determined if oral administration of E4 peptide exerted anti-fibrotic activity in a similar bleomycin-induced pulmonary fibrosis model. As shown in Fig. 1A, bleomycin administration induced lung fibrosis *in vivo* and a single oral dose of 20 μg E4 peptide ameliorated fibrosis as assessed 21 days post-treatment. On the other hand, treatment with a scrambled peptide did not ameliorate lung fibrosis. Hydroxyproline assay also revealed that the amount of collagen in lungs from mice treated with bleomycin was significantly higher (61.7 ± 9.2 μg/mouse) than in lungs from mice treated with PBS (32.5 ± 7.1 μg/mouse), and that treatment with E4 peptide significantly reduced the amount of collagen in mouse lungs (48.0 ± 12.0 μg/mouse), as compared to bleomycin (61.7 ± 9.2 μg/mouse) or bleomycin and scrambled peptide (60.7 ± 15.0 μg/mouse) (Fig. 1B).

The bleomycin mouse model of lung fibrosis is characterized by an early inflammatory phase. To determine if E4 ameliorates the inflammation in the earlier phase after bleomycin treatment, we collected BAL fluid from bleomycin treated mice with and without E4 on day 5 post-treatment. Total cell counts as well as the numbers of individual cell types in BAL fluids were compared in mice treated with bleomycin and those with bleomycin + E4. The total number of immune cells, as well as the total number of neutrophils were comparable in the two experimental groups (Fig. 1C). Similarly, the numbers of monocytes and lymphocytes were comparable in the two groups (data not shown).

Thus, these data suggest that oral administration of E4 peptide exerts anti-fibrotic activity in a bleomycin-induced pulmonary fibrosis model. They also reveal that E4 treatment exerts its anti-fibrotic activity without any effect on the inflammatory phase, suggesting that the peptide's anti-fibrotic activity is not due to an anti-inflammatory effect.

3.2. Identification of the minimally effective dose of orally-administered E4 in bleomycin-induced pulmonary fibrosis in vivo

To identify the minimal effective dose of E4, four different doses of the peptide were administered to mice treated with bleomycin. As shown in Fig. 2A, hydroxyproline assay revealed that the amount of collagen in lungs from mice treated with bleomycin was significantly higher (65.7 ± 10.1 μg/mouse) than in lungs from mice treated with PBS (34.7 ± 10.7 μg/mouse). Further, treatment with a single dose of 20 μg of E4 peptide significantly reduced the amount of collagen in mouse lungs (48.0 ± 12.0 μg/mouse) ($P = 0.003$). However, single doses consisting of 10, 5, and 1 μg of E4 peptide had no beneficial effect for bleomycin-induced fibrosis. Therefore, the lowest effective single dose of E4 peptide that orally exerts anti-fibrotic activity is 20 μg in the bleomycin-induced pulmonary fibrosis model.

We then examined whether repeated administration of lower doses of E4 peptide, that were ineffective as a single dose, could ameliorate bleomycin-induced pulmonary fibrosis. Thus, 10 and 5 μg of E4 peptide were orally administrated three times on days 0, 3, and 6 post-bleomycin treatment. As shown in Fig. 2B, hydroxyproline assay revealed that treatment with three doses of 10 μg of E4 peptide significantly reduced the amount of collagen in mouse lungs (43.1 ± 13.8 μg/mouse) compared to bleomycin treated mice (68.6 ± 12.7 μg/mouse) ($P = 0.008$). However, 5 μg of E4 peptide had no anti-fibrotic effect. This result suggests that repeated administration of a lower dose of E4 peptide is effective at ameliorating lung fibrosis.

4. Discussion

Our results demonstrate that oral administration of E4 peptide exerts anti-fibrotic effects in a bleomycin-induced pulmonary fibrosis model, and that E4 treatment exerts its anti-fibrotic activity independently of the inflammatory phase. The minimal effective oral dose of E4 given as a single administration is 20 μg, while a lower dose of 10 μg was effective when administered three times in the bleomycin model of pulmonary fibrosis.

The use of peptides/proteins is accepted in medical practice. In spite of that, to date, few orally delivered peptide and protein drugs are available. Examples of these include desmopressin, cyclosporin, vancomycin, linaclotide, and pancreatin [8]. Most recently, several oral peptide or protein drugs, including insulin, calcitonin, and interferon-α, are being evaluated for their oral bioavailability and efficacy [12]. The reported success of oral insulin is particularly interesting as it is comparable in size to our peptide [13,14]. In addition, other collagen-derived peptides have shown efficacy when administered orally. Collagen peptides have