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HMGB1 promotes HLF-1 proliferation and ECM production through activating HIF1-α-regulated aerobic glycolysis

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ABSTRACT

Aerobic glycolysis is a crucial event in fibroblast differentiation, and extracellular matrix (ECM) production in the progression of pulmonary fibrosis (PF). Abnormal high mobility group protein B1 (HMGB1) activation is involved in the pathogenesis of PF. However, whether aerobic glycolysis contributes to HMGB1-induced fibroblast proliferation and ECM production in PF has not yet been determined. In this study, we investigated the effects of HMGB1 on human embryonic lung fibroblast (HLF-1) proliferation, ECM production, and aerobic glycolysis. The lactate dehydrogenase inhibitor oxamic acid (OA), and PFKFB3 inhibitor 3PO were used to block certain crucial steps of aerobic glycolysis. As a result, we observed an increase of HMGB1 in bronchoalveolar lavage fluid (BALF) in bleomycin (BLM)-treated rats as compared to non-treated rats (control group). A concentration-dependent increase of HLF-1 proliferation and expression of α-SMA and α-collagen I were observed in the HMGB1 group, as well as increases of LDHA activation, glucose uptake levels, glycolytic rate, lactate level, and ATP production. OA and 3PO, or suppression of HIF1-α, blocked the effects of HMGB1. In summary, HMGB1 promotes fibroblast proliferation and ECM production though upregulating expression of HIF1-α to induce an increase of aerobic glycolysis.

1. Introduction

Pulmonary fibrosis (PF) occurs as an idiopathic disorder, or as a result of inflammatory states or injuries [1–3]. It is a, progressive, multifactorial, and lethal lung disease of undefined etiology that is characterized by inflammation and alveolar epithelial cell injury, excessive proliferation of fibroblasts, and significant deposition of interstitial extracellular matrix (ECM) [3,4].

High-mobility group box 1 (HMGB1) is a highly conserved DNA shepherding protein that is abundant in the cell nucleus [5]. Growing evidence has suggested that HMGB1 is an important factor in the respiratory diseases, including PF [6–9]. In lung tissues from patients with PF, HMGB1 is predominantly expressed in alveolar macrophages, epithelial cells, and infiltrating inflammatory cells [9,10]. HMGB1 has been shown to mediate mediates epithelial-to-mesenchymal transition in mouse type II alveolar epithelial cells (AECs) and human proximal tubular epithelial cells [11,12]. On the other hand, HMGB1 is also a key cytokine that regulates HLF-1 cell proliferation [13]. Accumulated data support the conclusion that HMGB1 exerts pro-fibrotic effects.

Aerobic glycolysis is a very important method of energy generation. Recently, Kottmann and colleagues reported that lactic acid was increased in the lungs of individuals with idiopathic PF (IPF) as compared with disease-free controls [14]. Furthermore, myofibroblasts (fibroblast exposed to transforming growth factor β [TGF-β]) exhibit high levels of aerobic glycolysis, and lactic acid may activate TGF-β, a pro-fibrotic cytokine [15]. Importantly, partial blockade of glycolysis with 3PO suppressed the pro-fibrotic effects of TGF-β *in vitro*, and attenuated the development of lung fibrosis in both the TGF-β- and bleomycin (BLM)-induced murine models [15]. These findings demonstrate that aerobic glycolysis plays an important role in the progression of PF. In addition, some studies have highlighted the importance of hypoxia inducible factor-1 alpha (HIF1-α) in the regulation of aerobic glycolysis [16,17]. Other studies have also reported the expression and activation of HIF1-α were upregulated by HMGB1 [18,19]. Moreover, Roth W reported that a metabolic shift from tricarboxylic acid cycle to glycolysis for the maintenance of energy production was induced by HMGB1 in cancer cells [20]. These indicated that HMGB1 may enhance aerobic glycolysis by HIF1-α to promote PF.

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The purpose of this study was to investigate the role and mechanism of HIF1-α-induced aerobic glycolysis in HMGB1-mediated fibroblast proliferation and ECM production during the development of PF. We determined the HMGB1 concentration in the bronchoalveolar lavage fluid (BALF) in a rat model of BLM-induced PF. In addition, human embryonic lung fibroblasts (HLF-1) were used to determine if the uptake of exogenous HMGB1 triggered cells proliferation and ECM production, and whether HMGB1 was involved in the regulation of aerobic glycolysis. Suppression of HIF1-α expression was performed to determine its role in HMGB1-induced HLF-1 proliferation and ECM production.

2. Methods

2.1. Animals

Male Wistar rats (age, 8 weeks; weight, 200–300 g) were provided by the Central Animal Care Facility of NanChang University. The animals were housed in a controlled environment (12 h light/12 h dark at 22–24 °C), with access to a standard diet and tap water ad libitum. All animals received humane care in compliance with the Chinese Animal Protection Act, which is in accordance with the National Research Council criteria.

2.2. Animal treatments

A PF rat model was used as previously reported [21]. Briefly, 20 male Wistar rats were randomly divided into 2 groups of 10 rats each: a control group and a BLM-treated group. Under pentobarbital anesthesia, rats in the control group rats received a single intratracheal instillation of 200 mL of sterile saline, while rats in the BLM group received the same instillation of 200 mL sterile saline except that it contained 5 mg/kg of BLM sulfate. Animals in both groups received a constant subcutaneous infusion of saline. After 28 days, bronchoalveolar lavage fluid (BALF) was collected from all rats in both groups, and immediately centrifuged at 500*g* at 4 °C for 15 min. An enzyme-linked immunosorbent assay (ELISA) kit (USCN Life Science, Wuhan, China) was used to measure the level of HMGB1 in the cell-free supernatant of the first BAL sample according to the kit manufacturer's instructions. HMGB1 content was expressed as ng/mL of BALF [22].

2.3. Histological examination

Following sacrifice, the right lungs of the rats were removed and fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. The paraffin-embedded tissue samples were sectioned into 5-μm slices, stained with hematoxylin & eosin (H&E), and examined under a light microscope (DP73; Olympus). The Ashcroft score was used to determine the degree of fibrosis in the lung specimens [23].

2.4. Cell culture

Human embryo lung fibroblasts (HLF-1) were purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL streptomycin, and 100 μg/mL penicillin at 37 °C in a humidified atmosphere of 5% CO_2. HMGB1 (Sigma Aldrich, St. Louis, MO) was added to the media at concentrations of 100, 200, and 200 ng/mL, respectively [5].

2.5. Small RNA interference

The siRNA duplexes of HIF1-α (siHIF1-α) and negative control (siNC) used in the study were purchased from Genechem Co., Ltd. (Shanghai China). Transfection of siRNA was carried out according to the Lipofectamine™ 2000 (Invitrogen, USA) instructions. After 48 h of transfection, the efficiency of the different HIF1-α siRNA sequences was determined by Western blot analysis.

2.6. Western blotting

Western blot studies were performed as described previously [24]. The primary antibodies were: smooth muscle actin (α-SMA), α-collagen I, and HIF1-α (Abcam, USA), and β-actin (Proteintech, USA). IRDye800-conjugated anti-rabbit IgG, and IRDye700-conjugated anti-mouse IgG (LiCor, USA) were used as secondary antibodies. Signal intensities were analyzed using the Odyssey infrared Image System (LiCor, USA). The densitometry results were first normalized with that of β-actin, and then compared with the control to obtain relative fold changes. The mean value for each blot band was averaged from 3 independent experiments.

2.7. Cell proliferation assay

Cell proliferation was examined using a methythiazol tetrazolium (MTT) assay. The absorbance was recorded at 490 nm using a Spectramax/M5 multi-detection reader (Molecular Devices, USA), and calculated as a ratio against untreated cells [5].

2.8. Metabolic studies

All metabolic studies, including glucose consumption, glycolytic rate, lactate production, and ATP production were performed with kits according to the manufacturer's instructions (Biovision). Briefly, a total of 1×10^6 cells per well were seeded in 6-well plates for 24 h, with or without pharmacological manipulations. The cells were washed, harvested, and homogenized in assay buffer, and the medium was collected to assess glucose consumption. Samples were mixed with respective reaction buffers, and product concentration was determined by fluorescence at excitation/emission = 535/590 nm in a microplate reader. All results were normalized to cell number for quantification [25].

2.9. Lactate dehydrogenase a (LDHA) activation assay

LDHA activity was determined as described previously [26]. Briefly, cells were cultured in 3 cm dishes, and homogenized in PBS on ice. The homogenates were added to buffer containing 0.2 M Tris-HCl (pH 7.3), 6.6 mM NADH, and 30 mM sodium pyruvate and mixed. The mixture was incubated for 5 min to achieve temperature equilibration, and rate of decreased absorbance at 340 nm was determined in a microplate reader.

2.10. Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM) ($n \geq 3$), and analyzed using one-way ANOVA followed by Newman-Keuls post-hoc comparisons. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Increase of HMGB1 levels in BLM-induced PF

Rats in the BLM-treated group exhibited higher Ashcroft scores compared with the control animals. The BLM-treated animals exhibited characteristic histological changes of PF in lung tissues, including thickening of the alveolar walls, areas of inflammatory