Inhibition of pre-B cell colony-enhancing factor attenuates inflammation induced by hyperoxia in EA.hy926 cells

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Received November 6, 2015; Accepted June 9, 2017

DOI: 10.3892/ijmm.2017.3045

Abstract. The aim of this study was to investigate the role of pre-B cell colony-enhancing factor (PBEF) in the pathogenesis of bronchopulmonary dysplasia (BPD) using an established cell model of BPD. For this purpose, EA.hy926 cell cultures were divided into 4 groups as follows: the air group as the blank control, the hyperoxia group, the hyperoxia plus PBEF siRNA group and the hyperoxia plus scramble siRNA group. Cell viability and the generation of reactive oxygen species (ROS) were determined using respective kits. Moreover, the protein and mRNA expression levels of PBEF, interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) were also detected by corresponding methods. Compared with the hyperoxia group, the ROS levels in the hyperoxia plus PBEF siRNA group were significantly reduced (P<0.01). The silencing of PBEF increased cell viability compared with the hyperoxia group. The protein and mRNA expression levels of PBEF, IL-8 and TNF-α were all decreased in the hyperoxia plus PBEF siRNA group compared with the hyperoxia group (P<0.01). Our study thus demonstrates that the inhibition of PBEF attenuates oxidative stress and inflammation induced by hyperoxia in EA.hy926 cells, suggesting that PBEF may be a potential diagnostic and therapeutic target, which may be used for the development of novel treatment strategies for BPD.

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease, with a high morbidity and mortality in pre-term infants. It is the cause of prolonged hospitalization with serious social and economic consequences. Although the pathogenic and genetic basis of BPD remains incompletely understood, studies have suggested that inflammation-mediated damage and oxidative stress play an important role in the development of BPD (1-4). A number of cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-8, IL-1β, IL-6, intercellular adhesion molecule-1 (ICAM-1) and nuclear factor-xB (NF-xB) have been proposed to be involved in the development of lung inflammation, and to be closely related to the pathogenesis of BPD (5).

Pre-B-cell colony-enhancing factor (PBEF) was first identified by Samal et al in 1994 as a putative cytokine (6), which was involved in the maturation of B-cell precursors in the presence of IL-7, and also known as NAMPT. PBEF is synthesized and secreted by many types of cells, such as activated lymphocytes, epithelial cells and neutrophils (7). It was previously demonstrated that PBEF (visfatin) exerts pro-inflammatory effects by upregulating the production of the pro-inflammatory cytokines, IL-1β, TNF-α, IL-6, in human monocytes (8). Ye et al reported the first findings of PBEF expressed in lung tissues and to be overexpressed in acute lung injury (ALI) (9). Furthermore, PBEF is considered to be associated with the regulation of inflammation and endothelial barrier dysfunction in ALI (10). Kim et al demonstrated that PBEF stimulated reactive oxygen species (ROS) generation in endothelial cells, and antioxidants blocked PBEF-induced NF-xB activation and cell adhesion molecule (CAM) expression (11). PBEF expression is upregulated and plays an important role during the development of a variety of inflammation-related diseases, including ALI, sepsis, rheumatoid arthritis and atherosclerosis (7-9,12,13). Based on these results, we hypothesized that PBEF may be a potential modulator in the pathogenesis of BPD.

The EA.hy926 cell line is an permanent human endothelial cell line, which was derived from the fusion of human umbilical vein endothelial cell (HUVEC) line and A549 cell line (14). The EA.hy926 cell line has highly differentiated functions that are characteristic of lung adenocarcinoma cells and the vascular endothelium (15). Xu et al used these alveolar endothelial cells to construct the alveoli in vitro model (16). The EA.hy926 cells have been widely applied in the study of inflammation, oxidative stress and protein expression (17-20).

In the present study, we aimed to explore the role of PBEF in BPD and its mechanisms of action by establishing an in vitro cell model of BPD. siRNA targeting PBEF (PBEF siRNA) was used to knockdown PBEF in the EA.hy926 cells in order to observe the effects of PBEF on inflammation and apoptosis caused by hyperoxia.
Cell proliferation and viability detection by CCK8. The EA.hy926 cells were plated at a density of 1x10^4 cells/well in a 96-well plate. The cells in the air group were cultured for 24, 48 and 72 h in a 37°C humidified incubator. The EA.hy926 cells were divided into 4 groups as follows: the air group, the hyperoxia group, the hyperoxia plus PBEF siRNA group and the hyperoxia plus scramble siRNA group. The cells in the air group were cultured for 24, 48 and 72 h in a 5% CO_2, 95% air, 37°C incubator. The cells in the hyperoxia group were cultured for 24, 48 and 72 h in the 37°C triple gas mixture. The cells in the hyperoxia plus PBEF siRNA group were transfected with PBEF siRNA, and cultured for 24, 48 and 72 h in the 37°C triple gas mixture. The cells in the hyperoxia plus scramble siRNA group were transfected with scramble siRNA, and cultured for 24, 48 and 72 h in the 37°C triple gas mixture.

Assessment of ROS generation. The ROS activities were measured using a specific assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The cells were washed twice with phosphate-buffered saline (PBS), and 1 ml H_2O_2 (100 μM) was added to the cells in the air group as the positive control, and 1 ml 2,7'-dichloro-fluorescein-diacetate (DCFH-DA; 10 μM) was added to the cells in the air group, the hyperoxia group and the hyperoxia plus PBEF siRNA group. The cells were then incubated at 37°C for 30 min. Following the removal of the mixture from the Petri dish, the cells were washed twice with PBS. The fluorescence was measured using an MD5 microplate reader. The fluorescence was measured using an MD5 microplate reader.

Quantitative PCR (qPCR). Total RNA was isolated from the cells using TRIzol reagent (Takara Bio) following the manufacturer's instructions. Single-strand cDNA was synthesized using 2 μg of total RNA by the reverse transcription (RT) reaction using the PrimeScript RT reagents kit (Takara Bio). Real-time (quantitative) PCR was conducted using an Applied Biosystems 7500 real-time PCR system according to the instructions provided with the SYBR Premix Ex Taq kit (Takara Bio). The thermal cycling parameters consisted of 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Relative expression values were normalized using an internal β-actin control. The fold-change of relative gene expression levels was calculated using the 2^(-ΔΔCq) method, and the primer sequences are listed in Table I.

Western blot analysis. Total protein was extracted from the cells with lysis buffer using the Total Protein Extraction Kit (Takara Bio) according to the manufacturer's instructions. Single-strand cDNA was synthesized using 2 μg of total RNA by the reverse transcription (RT) reaction using the PrimeScript RT reagents kit (Takara Bio). Real-time (quantitative) PCR was conducted using an Applied Biosystems 7500 real-time PCR system according to the instructions provided with the SYBR Premix Ex Taq kit (Takara Bio). The thermal cycling parameters consisted of 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Relative expression values were normalized using an internal β-actin control. The fold-change of relative gene expression levels was calculated using the 2^(-ΔΔCq) method, and the primer sequences are listed in Table I.
reagent kit (KeyGen, Nanjing, China). The protein concentration was measured by BCA protein assay (Keygen). Protein extract samples (30 µg/lane) were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore, Bedford, MA USA). The membranes were subsequently blocked with 5% non-fat milk solution, and incubated with monoclonal anti-β-actin (Cat. no. AF0003; Byotime Biotechnology, Jiangsu, China), anti-PBEF (Cat. no. sc-67020; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-TNF-α (Cat. no. BS6000) and anti-IL-8 (Cat. no. BS7145) (both from BioWorld Technology, Inc., St. Louis Park, MN, USA) antibodies, diluted at 1:1,000. Anti-rabbit (H+L) HRP diluted at 1:2,000 was used as the secondary antibody with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) for detection. The intensities of the protein bands were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. The results are presented as the means ± SD. Statistical analysis was analyzed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). Stimulated samples were compared with the controls by an unpaired Student’s t-test. One-way analysis of variance (ANOVA) was used for multiple-group comparisons. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability. The cells in the air group were cultured for 24, 48 and 72 h in a 5% CO₂, 95% air, 37°C incubator, while the cells in the hyperoxia group and the hyperoxia plus PBEF siRNA group were cultured for 24, 48 and 72 h in the 37°C triple gas mixture. The results of CCK8 assay revealed that hyperoxia significantly decreased cell viability by 16% after 24 h, 17% after 48 h and 21% after 72 h compared with the air group, while cell viability in the hyperoxia plus PBEF siRNA group was significantly higher than that of the hyperoxia group (P<0.05; Fig. 1). These results indicate that PBEF silencing suppresses cell damage induced by hyperoxia.

Assessment of ROS levels. As shown in Fig. 2, the average ROS levels were 77.156±8.465, 121.077±15.795 and 100.857±9.567 in the air, hyperoxia and hyperoxia plus PBEF siRNA groups. The ROS levels in the hyperoxia group and the hyperoxia plus PBEF siRNA group were significantly higher than those in the air group at 24, 48 and 72 h (P<0.01), while the ROS levels in the hyperoxia plus PBEF siRNA group was significantly lower than those in the hyperoxia group (P<0.01). The ROS levels in the hyperoxia group at 48 and 72 h were significantly higher compared to those at 24 h (P<0.01). The results demonstrate that the silencing of PBEF reduces the ROS levels induced by hyperoxia in the EA.hy926 cells.

Changes in the mRNA expression of PBEF, IL-8 and TNF-α detected by qPCR. As shown in Fig. 3, qPCR analysis revealed that the PBEF, IL-8, TNF-α mRNA expression levels in the hyperoxia group were significantly higher than those in the air group at each time point (P<0.01); however, these mRNA expression levels were significantly decreased in the hyperoxia plus PBEF siRNA group compared with the hyperoxia group at each time point (P<0.01). In the hyperoxia plus PBEF siRNA group, it was shown that PBEF siRNA knocked down PBEF mRNA expression in the EA.hy926 cells (>65% decrease, P<0.01; Fig. 3). Scramble siRNA alone had no effect on PBEF expression. The TNF-α and PBEF gene expression levels in the hyperoxia group and the hyperoxia plus PBEF siRNA group at 48 and 72 h were significantly higher than those at any time point. These results provided evidence that the mRNA expression of PBEF, IL-8 and TNF-α is increased in the hyperoxia group, and the silencing of PBEF reduces the ROS levels induced by hyperoxia.
expression of PBEF was significantly increased in the hyperoxia group, and the silencing of PBEF inhibited IL-8 and TNF-α mRNA expression which was induced by hyperoxia.

Detection of PBEF, IL-8 and TNF-α protein expression in EA.hy926 cells by western blot analysis. The results from western blot analysis revealed that the protein expression of PBEF, IL-8 and TNF-α in the hyperoxia group was increased compared with the air group (P<0.01), while the protein expression of PBEF, IL-8, TNF-α in the hyperoxia plus PBEF siRNA group was significantly lower than that in the hyperoxia group (P<0.01; Fig. 4). In the hyperoxia plus PBEF siRNA group, PBEF siRNA knocked down PBEF protein expression in the EA.hy926 cells (>65% decrease, P<0.01; Fig. 4A and B). Scramble siRNA alone had no effect on PBEF expression compared with the hyperoxia group. The TNF-α and PBEF protein expression levels in the hyperoxia group and the hyperoxia plus PBEF siRNA group at 48 and 72 h were significantly higher than the levels at 24 h (P<0.01). No significant differences were observed between the hyperoxia group and the hyperoxia plus scramble siRNA group at any time point (Fig. 4). These results demonstrated that hyperoxia increased the expression of inflammatory cytokines in the EA.hy926 cells and the knockdown of PBEF expression attenuated the promoting effects of hyperoxia on cytokine expression, indicating that PBEF is critically involved in the regulation of the inflammatory response to hyperoxia in EA.hy926 cells.

Discussion

Our findings demonstrate that the levels of the pro-inflammatory cytokines, PBEF, TNF-α and IL-8, as well as the ROS level were increased in the EA.hy926 cells following exposure to hyperoxia, and the knockdown of PBEF using siRNA in
the EA.hy926 cells significantly inhibited the expression of the cytokines mentioned above, as well as the ROS levels. Furthermore, hyperoxia induced the EA.hy926 cell apoptosis, and the silencing of PBEF suppressed the cell growth inhibition induced by hyperoxia. These results provide new insight into the role of PBEF in the inflammatory pathways and functional abnormalities associated with BPD.

There is considerable experimental and clinical evidence that pro-inflammatory cytokines play a crucial role in the pathogenesis of BPD, including IL-6, IL-8, IL-10, TNF-α.
and ICAM-1 (22,23). TNF-α and IL-8 are among important early mediators of BPD. TNF-α has been reported to be involved in many inflammatory diseases, such as acute respiratory distress syndrome, BPD and chronic obstructive pulmonary disease (24,25). It has been shown that TNF-α is present in increased amounts in the bronchoalveolar lavage fluid (BALF) of rats with BPD (23). The role of TNF-α in pulmonary physiology has been investigated by Robriquet et al., including the activation of oxidative stress and the induction of cellular inflammatory reactions (26).

Among the pro-inflammatory cytokines, IL-8 is regarded as another important mediator in the pathogenesis of BPD (22). In fact, IL-8 was first purified and molecularly cloned by Matsushima et al. in 1988 as a neutrophil chemotactic factor from lipopolysaccharide-stimulated human mononuclear cell supernatants (27). IL-8 has been reported to induce actin fiber formation and intercellular gap formation in endothelial cells (28). Duncan et al. demonstrated that the TNF-α mediated IL-8 production attenuated neutrophil apoptosis and thus potentially prolonged neutrophil migration into the lungs and damage to lung tissues (29). It has also been demonstrated that PBEF may play a vital role in the augmentation of pulmonary epithelial cell IL-8 expression induced by TNF-α (10).

The data of the present study indicate that hyperoxia significantly induces PBEF, IL-8 and TNF-α expression at both the mRNA and protein level in EA.hy926 cells (Figs. 3 and 4), suggesting that PBEF, IL-8 and TNF-α are involved in the inflammatory process during the pathogenesis of BPD. The knockdown of PBEF expression by PBEF siRNA markedly blunted TNF-α and IL-8 expression in the EA.hy926 cells. These results support the concept that PBEF may be an inflammatory signal transducer of TNF-α and IL-8 or other inflammatory cytokines. These conclusions can be demonstrated by evidence in non-lung tissue studies. Jia et al. reported that the silencing of PBEF prevented the suppression of neutrophil apoptosis caused by TNF-α, IL-8 and other mediators (7). Furthermore, it has been suggested that the treatment of amnion-like epithelial cells with recombinant human PBEF significantly increased IL-8 and IL-6 expression (30). Taken together, these data indicate that PBEF may play a crucial role as an inflammatory cytokine during the pathogenesis of BPD.

There is increasing evidence to indicate that oxidative stress is one of the most important events in the development of BPD (31,32). It has been reported that oxidative stress plays an important role in endothelial dysfunction and vascular injury (33). Oxidative stress is caused by an imbalance between ROS generation and the antioxidant defense system (34). The balance is likely to be broken when the overexpression or inadequate clearance of ROS becomes uncontrollable, resulting in severe damage to DNA, protein and lipids (34-36). ROS influence a number of cellular responses by affecting several intracellular signaling cascades, which contribute to altered inflammation and vascular remodeling (37). The premature infant is particularly sensitive to ROS-induced damage due to deficient antioxidant stores at birth, as well as impaired upregulation in oxidant stress response. Therefore, the premature infant is at high risk of the development of ROS-induced diseases, such as BPD, necrotizing enterocolitis, periventricular leukomalacia and retinopathy of prematurity (34). In this study, the ROS levels in the hyperoxia group were significantly higher than those in the air group (P<0.01), while the inhibition of PBEF expression by PBEF siRNA significantly blunted hyperoxia-induced ROS production compared with the hyperoxia group (Fig. 2). These results indicated that PBEF affects intracellular oxidative stress.

Kim et al. demonstrated that PBEF stimulated ROS generation in endothelial cells, and antioxidants blocked PBEF-induced NF-κB activation and CAM expression (11). In addition, the interacting partners of PBEF in oxidative stress have been identified, including NADH dehydrogenase subunit 1 (ND1), ferritin and interferon-induced transmembrane 3 (IFITM3) (38). Taken together, the above-mentioned data suggest that the interaction between PBEF and oxidative stress may be a potential mechanism in the pathogenesis of BPD.

It is well known that apoptosis is critical for normal lung development and function (39,40) and plays a key role in remodeling of lung tissue by clearing excess epithelial and mesenchymal cells following injury (41,42). However, abnormal apoptotic activity may contribute to the pathophysiology of a number of lung diseases, including BPD. Kazzaz et al. reported that apoptosis was clearly induced in the lungs of mice exposed to hyperoxia (43). Normally, lung alveolar epithelium forms a tight barrier to restrict the movement of proteins and liquid from the interstitium into the alveolar spaces. If the alveolar epithelium is damaged, both the permeability of endothelial cells and epithelial cells changes, and this may lead to major alveolar flooding with high-molecular-weight proteins, with prolonged changes in gas exchange (44,45). In this study, the viability of the cells in the hyperoxia group was significantly decreased compared with that of the cells in the air group, and the induction of apoptosis by hyperoxia was observed in the cultured cells. Exposure to high oxygen concentrations leads to oxidative stress and activates the inflammatory response, which may cause cell death. However, the knockdown of PBEF increased cell viability in the hyperoxia plus PBEF siRNA group. The exact mechanisms behind this effect remain unknown; however, the silencing of PBEF attenuated oxidative stress and the inflammatory response, and this may suppress the cell damage caused by hyperoxia. Recently, a study demonstrated that PBEF was involved in the process of microvascular endothelial cell apoptosis through the Fas/FasL-mediated extrinsic apoptotic pathway (46). Ming et al. demonstrated that PBEF promoted the apoptosis of pulmonary microvascular endothelial cells and regulated the expression of inflammatory factors and the expression of aquaporin 1 (AQP1) through the MAPK pathways (47). These results indicate that the inhibition of PBEF signaling may be a potential strategy with which to attenuate endothelial cell apoptosis.

In conclusion, this study demonstrated that hyperoxia significantly induced PBEF, IL-8 and TNF-α expression at both the mRNA and protein level in EA.hy926 cells. The knockdown of PBEF expression by PBEF siRNA significantly blunted TNF-α and IL-8 mRNA and protein production, and attenuated the hyperoxia-induced increase in ROS levels in EA.hy926 cells. Since inflammation and oxidative stress are the hallmarks of the pathogenesis of BPD, these results suggest that PBEF plays a key role as a pro-inflammatory cytokine in the dysregulation of alveolar epithelial cell barriers in the development of BPD. These results lend further support to
the potential of PBEF to serve as a diagnostic and therapeutic target in future studies of BPD.

Acknowledgements

The present study was supported by the Guangdong Province Science and Technology Plan Project (00465510217884051 to W.M.H.)

References


