Redox Biology 14 (2018) 645–655

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Oxidative stress-driven pulmonary inflammation and fibrosis in a mouse model of human ataxia-telangiectasia

Ruth Duecker[a,*], Patrick Baer[b], Olaf Eickmeier[a], Maja Strecker[a], Jennifer Kurz[a], Alexander Schaible[c], Dirk Henrich[c], Stefan Zielen[a], Ralf Schubert[a]

[a] Division for Allergy, Pneumology and Cystic Fibrosis, Department for Children and Adolescence, Goethe-University, Frankfurt/Main, Germany
[b] Division of Nephrology, Department of Internal Medicine III, Goethe-University, Frankfurt/Main, Germany
[c] Department of Trauma, Hand & Reconstructive Surgery, Goethe-University, Frankfurt/Main, Germany

ARTICLE INFO

Keywords:
Pulmonary inflammation
Lung fibrosis
Mice
Oxidative stress

ABSTRACT

Lung failure is responsible for significant morbidity and is a frequent cause of death in ataxia-telangiectasia (A-T). Disturbance in the redox balance of alveolar epithelial cells must be considered as a causal factor for respiratory disease in A-T. To investigate bronchoalveolar sensitivity to reactive oxygen species (ROS) and ROS-induced DNA damage, we used bleomycin (BLM) to induce experimental inflammation and fibrotic changes in the *Atm*-deficient mouse model.

BLM or saline was administered by oropharyngeal instillation into the lung of *Atm*-deficient mice and wild-type mice. Mice underwent pulmonary function testing at days 0, 9, and 28, and bronchoalveolar lavage (BAL) was analysed for cell distribution and cytokines. Lung tissue was analysed by histochemistry.

BLM administration resulted in a tremendous increase in lung inflammation and fibrotic changes in the lung tissue of *Atm*-deficient mice and was accompanied by irreversible deterioration of lung function. ATM (ataxia telangiectasia mutated) deficiency resulted in reduced cell viability, a delay in the resolution of γH2AX expression and a significant increase in intracellular ROS in pulmonary epithelial cells after BLM treatment. This was confirmed in the human epithelial cell line A549 treated with the ATM-kinase inhibitor KU55933.

Our results demonstrate high bronchoalveolar sensitivity to ROS and ROS-induced DNA damage in the *Atm*-deficient mouse model and support the hypothesis that ATM plays a pivotal role in the control of oxidative stress-driven lung inflammation and fibrosis.

1. Introduction

Ataxia-telangiectasia (A-T) is a rare autosomal recessive disorder caused by mutations in the ataxia-telangiectasia mutated (ATM) gene that results in the defective repair of DNA double-strand breaks. The clinical phenotype is characterised by progressive neurodegeneration, immunodeficiency, elevated risk of malignancies and a high rate of respiratory failure [1,2]. Mortality is generally due to respiratory diseases, which are a frequent cause of death in patients with A-T [3]. Primary symptoms include recurrent respiratory tract infections and bronchiectasis, aspiration and respiratory muscle abnormalities, as well as interstitial lung disease and pulmonary fibrosis [4–6].

It has been proposed that ongoing low-grade inflammation and oxidative stress might be an underlying mechanism leading to the clinical pathogenesis of A-T [7–9]. McGrath-Morrow et al. demonstrated that elevated serum IL-8 and IL-6 levels in A-T patients were found to be associated with lower lung function parameters [7,8]. Elevated IL-6 and IL-8 have also been described for other lung diseases, such as cystic fibrosis and chronic obstructive pulmonary disease (COPD), which both have serum and sputum cytokine profiles associated with their pathologic conditions [10–12]. In addition to inflammation, there is a growing body of evidence that reactive oxygen species (ROS) are involved in pulmonary disease in A-T. Barlow et al. showed that a loss of the ATM protein causes oxidative damage in target organs in *Atm*-deficient mice [13]. In addition, increased ROS and reduced anti-oxidative capacity were demonstrated in serum from patients with A-T [14,15]. A recent study from our group revealed a significantly higher amount of oxidative DNA damage based on analysis of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in the bronchial lavage fluid (BALF) of *Atm*-deficient mice compared to wild-type animals [9]. The use of antioxidants has been shown to reduce oxidative stress, increase lifespan and correct neurobehavioural deficits in these mice [16,17].

As first, Eickmeier et al. induced lung inflammation in *Atm*-deficient

* Correspondence to: Department for Children and Adolescence Goethe University, Theodor-Stern-Kai 7, 60590 Frankfurt/Main, Germany.
E-mail address: RuthPia.Duecker@kgu.de (R. Duecker).

https://doi.org/10.1016/j.redox.2017.11.006
Received 1 August 2017; Received in revised form 13 October 2017; Accepted 7 November 2017
Available online 10 November 2017

mice by using an acute lung injury model [18]. The data indicated that restricted lung function and a high sensitivity to hydrochloric acid induced inflammation in these mice. However, this short-term model did not analyse the role of ROS in pulmonary inflammation, and fibrotic changes in the lung tissue were not investigated. To take a closer look at the role of ROS-induced inflammation and fibrotic changes in respiratory disease in A-T, we used the bleomycin (BLM) model, which is widely used in rodents to induce pulmonary fibrosis [19]. BLM is a chemotherapeutic antibiotic produced by the bacterium *Streptomyces verticillus* that plays an important role in the treatment of different cancer types [20,21]. Pulmonary side effects of BLM treatment are the induction of single- and double-strand DNA breaks by the chelation of metal ions and the generation of superoxide and hyperoxide free radicals [22].

Revealing the origin of A-T lung disease and its progression may help in the discovery and development of new therapeutic interventions. Therefore, the aim of the present study was to investigate bronchoalveolar sensitivity to ROS and ROS-induced DNA damage using the pulmonary bleomycin model in the *Atm*-deficient mouse.

2. Materials and methods

2.1. Animals

Atm-deficient mice (Atmtm1Awb; 8–10 weeks old), in a 129SvEv background, were used as the animal model. The animal studies were performed according to the protocols approved by the German Animal Subjects Committee (Gen. Nr. FK/1001). Mice were housed in plastic cages on a 12-h light/12-h dark cycle with access to food and water ad libitum until harvest. Weight was taken every single day (0 − 28) throughout the treatment. All surgery was performed under ketamine/xylazine anaesthesia (20% ketamine, CuraMED GmbH, Karlsruhe, Germany; 8% xylazine, Bayer Vital GmbH, Leverkusen, Germany), and every effort was made to minimise suffering.

2.2. Bleomycin model

Bleomycin sulphate (Sigma-Aldrich, Germany) was dissolved in sterile 0.9% saline and administered as a single dose of 0.75 mg/kg body weight (a dose chosen based on our data from a preliminary dosage experiment, data not shown) by oropharyngeal instillation into the lung of lightly anaesthetised (2.0% Isoflurane, Abbott GmbH, Germany) *Atm*-deficient mice and wild-type mice on day 0. Control animals received a body weight adjusted dose of saline alone. Oropharyngeal aspiration (OA) was performed as described by De Vooght et al. [23]. Briefly, mice were held vertically, the tongue was pulled out with forceps, and the fluid was placed onto the distal part of the oropharynx while the nose was gently closed. Bronchoalveolar lavage, pulmonary function tests and perfusion were performed and lung tissue was harvested at day 0, 9 and 28 post-dosing.

2.3. Cell Culture

A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1% v/v penicillin/streptomycin (Gibco by Life Technologies, Germany) and 10% v/v foetal bovine serum (FBS) (Sigma-Aldrich, Germany) [24]. Proliferative cultures were incubated at 37 °C in a humidified, 5% CO_2 incubator, and subculture was carried out by washing the cell monolayers twice with calcium and magnesium-free phosphate-buffered saline (PBS) followed by the addition of 0.25% trypsin/EDTA solution (Life Technologies, Germany) and incubation at 37 °C until the cells detached. Trypsin was inactivated by the addition of growth medium before seeding into fresh T25 flasks at densities of 2–4 $\times 10^4$ cells/cm ^{2}. The medium was changed every 2–4 days. The passages used for the following experiments were 6–12.

The murine alveolar epithelial cells and the human alveolar epithelial cell line A549 were stimulated with 100 mU bleomycin sulphate (Calbiochem, Germany) for 24 h. Additionally, A549 cells were incubated with the ATM-kinase inhibitor, 10 μM KU55933 (Selleckchem, Germany) 1 h prior to BLM stimulation.

2.4. Primary murine alveolar epithelial cells

The isolation of primary, whole lung cells was performed as described by Corti et al. [25]. Briefly, mice were sacrificed by CO_2 asphyxiation, the thorax was removed, and the diaphragm was carefully punctured and removed to expose the heart. The right ventricle was perforated with a 26 G cannula and perfused with a 20 mL syringe filled with cold phosphate-buffered saline (PBS) until free of blood. To expose the trachea, the salivary glands were removed, and a 22 G cannula was inserted into the trachea and fixed with thread around the trachea and the catheter. A total of 1.5 mL of dispase (Corning, USA; pre-warmed to 37 °C) was carefully instilled into the lung so that all the lobes were fully expanded. After that, 0.5 mL of 1% low-melt agarose (Bio&Sell, Germany) was also instilled into the lung. Backflow was avoided by leaving the syringe on the catheter. After cutting the trachea, the lung, heart and thymus were removed, and the lung was incubated in 3 mL of dispase at room temperature for 45 mins on a bench rocker. Lung tissue was minced using the gentleMACS Dissociator (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol with 3 mL of gentle MACS Buffer (5 mL DMEM, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 20 mM HEPES, 120 U DNase). For further cell proliferation assays (XTT assay), 2.5 $\times$ 10^4 cells were used, and 2 $\times$ 10^5 cells were used for FACS analysis.

2.5. Flow cytometry

The expression of specific cell surface markers on primary pulmonary epithelial cells was determined using a BD FACSVerse cytometer (Beckman Coulter, Inc., CA, USA). Cell suspensions were transferred to FACS tubes and washed twice with phosphate-buffered saline (PBS; Life Technologies, Darmstadt, Germany). Then, 0.2 $\times$ 10^6 cells in 100 μL of PBS were blocked with CD16/32 for 10 min, and the surface was stained for 20 min in the dark with the following antibodies: SPC-FITC, CD45-PE, CD326-PECy7, CD31-APC, Sca-1-FITC, CD11c-PECy7 (BD Bioscience, San Jose, USA). After a washing step, 300 μL of PBS was added, and the samples were measured. FACSuite Software was used to analyse the data.

2.6. XTT assay (Supplement)

Viability was measured in A549 cells and primary murine AEC2 cells via the XTT assay. Cells were then seeded in 96-well plates at a specified density (A549 25,000 cells/well, AEC2 5 $\times$ 10^4 cells/well) and were incubated with different concentrations (1 mU, 10 mU and 100 mU) of bleomycin sulphate (Calbiochem, Germany). Ethanol (70%) served as the positive control. The XTT solution (Applichem, Germany) was added, and the cells were incubated further for 4 h at 37 °C and colorimetrically quantified (absorbance 450 nm, reference 630 nm).

2.7. Reactive oxygen species (ROS)

A549 cells or whole-lung cells, isolated from 6 to 8-week-old *Atm*-deficient and wild-type mice (2 $\times$ 10^5), were untreated or treated with 100 μM bleomycin for 24 h, collected and suspended in 1 mL PBS containing 5 μM DCF-DA (2′, 7′ –dichlorofluorescein diacetate, Molecular Probes, Inc., OR, USA) for 15 min. Additionally, murine cells were surface-stained with αCD326-PE-Cy7 (BD Bioscience, San Jose, USA), αSPC-APC (Bioss, USA) and αSca-1-PE (Biolegend, San Diego, USA) and analysed by flow cytometry on a BD FACSVerse cytometer (Beckman Coulter, Inc., CA, USA) using the BD FACSuite Software. The