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Senescence-associated secretory phenotype in a mouse model of bleomycin-induced lung injury

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ABSTRACT

Bleomycin produces DNA damage, apoptosis and senescence, all of which play crucial roles in the development of pulmonary fibrosis. Recently, close attention has been paid to a DNA damage-induced phenotypic change (senescence-associated secretory phenotype; SASP) as a trigger for the secretion of various mediators which modify the processes of tissue injury, inflammation, repair and fibrosis. We characterized the SASP in a murine model of bleomycin-induced lung injury. Mice were intratracheally administered bleomycin or control saline, and the lungs were obtained on days 7, 14 and 21. The occurrence of DNA damage and the SASP in the lungs was examined by immunostaining. γH2AX immunostaining of the bleomycin-treated lungs revealed double-strand breaks (DSBs), largely within E-cadherin-positive, β4-integirn-positive alveolar epithelial cells. The DSBs were associated with phosphorylation of ATM/ATR, a central signal transducer mediating the DNA damage response, and upregulation of the cyclin-dependent kinase inhibitor $p21^{CIP1}$. The DSBs persisted for at least 21 days after the bleomycin exposure, although it began to wane after 7 days. A subpopulation of the γH2AX-positive, DNA-damaged cells exhibited the SASP, characterized by overexpression of IL-6, TNFα, MMP-2 and MMP-9, in association with the phosphorylation of IKKα/β and p38 MAPK. Persistent DNA damage and the SASP are induced in the process of bleomycin-induced lung injury and repair, suggesting that these events play an important role in the regulation of inflammation and tissue remodeling in bleomycin-induced pneumopathy.

1. Introduction

The murine model of bleomycin-induced lung fibrosis is widely used as the preferred model system to study pulmonary fibrosis, a progressive and untreatable disease that, according to estimates, affects approximately 80,000 individuals in the United States (King et al., 2011; Raghu et al., 2006). Bleomycin is a member of the glycopeptide antibiotic family that exerts potent antitumor activity (Chen and Stubbe, 2005). The cytotoxic effects of bleomycin are thought to be related to the ability of the drug to mediate single-stranded DNA breaks (SSBs), and also the more lethal double-stranded DNA breaks (DSBs), in the presence of specific cofactors, such as a transition metal [Fe(II) or Cu(I)], oxygen, and an electron reductant. Cells sense DNA damage and trigger various signal transduction cascades (DNA damage response) that ultimately lead to different fates, including DNA repair, cell-cycle arrest, cell senescence, mitotic cell death, and apoptosis (Chen and Stubbe, 2005).

When DSBs are induced, the histone H2A variant H2AX becomes rapidly phosphorylated at serine 139 by ataxia telangiectasia mutated kinase/ataxia telangiectasia and Rad3-related kinase (ATM/ATR) and DNA-dependent protein kinase (DNA-PK) (Mah et al., 2010). This modified form of H2AX, called γH2AX, is easily identified by staining with antibodies and serves as a reliable and sensitive indicator of DSBs (Mah et al., 2010). Recently, DSBs have attracted much attention as a trigger for the secretion of various mediators (senescence-associated secretory phenotype; SASP), which modify inflammation, tissue repair, fibrosis and carcinogenesis (Acosta et al., 2008; Freund et al., 2010; Kuilman et al., 2008, 2010; Rodier et al., 2009; Rodier and Campisi, 2011). It has been reported that many phlogogenic substances (IL-1α, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor [GM-CSF], growth-regulated oncogene [GRO]α, monocyte chemotactic protein [MCP]-2, -3, matrix metalloproteinase [MMP-1, -2, -3, -12, -13, and -14]) are produced by cells that become senescence as a result of the occurrence of DSBs of the DNA and the subsequent DNA

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damage response, which activates nuclear factor-κB (NFκB) and p38-mitogen-activated protein kinase (p38 MAPK) (Acosta et al., 2008; Freund et al., 2010; Kuilman et al., 2008, 2010; Rodier et al., 2009; Rodier and Campisi, 2011).

Bleomycin produces extensive DNA damage and apoptosis, both of which are thought to be crucial processes underlying the development of pulmonary fibrosis (Hagimoto et al., 1997; Harrison et al., 1989; Kuwano et al., 1999, 2000, 2001; Mishra et al., 2000). Cell senescence is reported to occur, in addition to apoptosis, when the DNA damage is less severe (Aoshiba et al., 2003; Shivshankar et al., 2012). Recent studies, including our own have shown that alveolar epithelial cells undergo senescence in the lungs of a murine model of bleomycin-induced pneumopathy (Aoshiba et al., 2003; Shivshankar et al., 2012). Furthermore, an in vitro study has shown that A549 alveolar type II-like cells that became senescent after bleomycin exposure exhibited the SASP, characterized by overexpression of IL-6 (Aoshiba et al., 2012). However, the SASP in bleomycin-induced lung injury and fibrosis had not yet been characterized.

Using a murine model of bleomycin-induced pneumopathy, we demonstrated that a proportion of alveolar epithelial cells that undergo DNA damage and show DSBs exhibit the SASP, characterized by increased secretion of IL-6, TNFα, MMP-2 and MMP-9, suggesting that cells with DNA damage contribute to persistent inflammation and lung remodeling in bleomycin-induced lung injury and fibrosis.

2. Materials and methods

2.1. Animal treatment

The animal experiments were performed according to the Helsinki Declaration and approved by the Animal Care and Use Committee of Tokyo Women's Medical University. An animal model for bleomycin-induced pulmonary fibrosis was generated, as described previously (Aoshiba et al., 2000, 2003). Six-week-old male C57/BL6J mice were anesthetised with an intraperitoneal injection of sodium pentobarbital (Abbott Lab., North Chicago, IL, USA), followed by intratracheal administration of 50 μL of a saline solution containing bleomycin hydrochloride (2.5 mg kg^{-1} body weight; Nippon Kayaku, Co., Tokyo, Japan). On days 7, 14 and 21 after the bleomycin injection, the mice were sacrificed by intraperitoneal injection of an overdose of pentobarbital. As control, mice were intratracheally administered 50 μL of saline and sacrificed on day 7. The lungs of the mice were inflation-fixed in situ for 5 min with 10% neutral buffered formalin (NBF) at a pressure of 25 cm H_2O and immersion-fixed in NBF for 24 h. Formalin-fixed tissue was embedded in paraffin and sectioned (3 μm). Mid-sagittal sections of the left lungs were stained with hematoxylin and eosin or processed for immunohistochemistry and immunofluorescence.

2.2. Immunohistochemistry and immunofluorescence

The primary antibodies used in this study are listed in Table 1. For immunohistochemistry, the primary antibodies were detected with a secondary antibody conjugated with a horseradish-peroxidase (HRP)-labeled polymer (Histofine® Simple Stain, Nichirei Biosciences, Tokyo, Japan). To expose the immunoreactive epitopes of the antigens, the sections were autoclaved in a citrate buffer (pH 6.0) for 20 min before application of the primary antibodies. Endogenous peroxidase activity was quenched by exposure to 3% hydrogen peroxide for 20 min. The immunoreactants were detected with a diaminobenzidine substrate. For double staining, the prior antibody complexes were erased by immersing the slides in glycine–HCl buffer (pH 2.2) for 1 h, and the sections were then immunostained for the secondary antigen. Immunoreactants against the secondary antigen were detected by using the HistoGreen® substrate detection kit for peroxidase (AbCys, Paris, France). For immunofluorescent staining, the primary antibodies were reacted with secondary anti-IgG antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich Japan, Tokyo, Japan). Images were acquired using an Olympus BX60 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a digital camera, and processed with a computerized color image analysis software (WinRoof Version 3.5; Mitani Corporation, Fukui, Japan) and Adobe Photoshop software (San Jose, CA, USA).

The lung tissue sections were assessed in three steps. In the first step, lung tissue sections were immunostained for γH2AX, phosphorylated ATM/ATR, or $p21^{CIP1}$. For each slide, 5 microscopic fields at a magnification of 100× were randomly selected and the number of positively stained cells within each field was counted. Data are expressed as the number of positively stained cells per unit area (mm^2). In the second step, the lung tissue sections were double-immunostained for γH2AX and cell-lineage/activated signal transduction/SASP markers. For each slide, more than 200 cells showing positive staining for γH2AX at a magnification of 200× were randomly selected and each of the positive cells was examined for positive or negative staining for each marker. The percentage of the total number of γH2AX-positive cells that were positive for each marker was calculated by dividing the number of cells that were double-positive for γH2AX and the respective cell markers by the total number of γH2AX-positive cells. In the third step of immunohistochemical assessment, lung tissue sections double-stained for γH2AX and E-cadherin were observed at a magnification of 200×, and more than 5000 cells that were positive for E-cadherin in the lung parenchyma were examined for the expression of γH2AX. We calculated the percentage of E-cadherin-positive cells that were positive for γH2AX by dividing the number of cells double-positive for γH2AX and E-cadherin by the total number of E-cadherin-positive cells.

2.3. Statistical analysis

Data are mean ± SEM. Differences were evaluated for significance by a one-way analysis of variance (ANOVA), followed by post hoc analysis with the Tukey–Kramer test for multiple comparisons. $P < 0.05$ was considered to denote significance.

3. Results

3.1. Histologic evidence of bleomycin-induced lung injury and fibrosis

The lungs of the mice intratracheally instilled with saline and sacrificed on day 7 were histologically normal (Fig. 1). In the bleomycin group, by day 7 after the intractracheal bleomycin instillation, thickening of the alveolar septa as a result of edema and infiltration by lymphocytes and some neutrophils, and collapse of the alveolar air spaces were observed. On day 14, thickening of the alveolar septa by fibroblast proliferation and collagen deposition was observed. By day 21, thickening of the alveolar septa by fibrosis became more intense, and was associated with extensive remodeling of the alveolar structure. These changes, including infiltration by inflammatory cells and the development of pulmonary fibrosis following bleomycin administration, are similar to previously reported histologic findings (Aoshiba et al., 2000; Aso et al., 1976).