Bax-inhibiting peptide attenuates bleomycin-induced lung injury in mice
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ABSTRACT
Bax is a pro-apoptotic member of the Bcl-2 family of proteins, and plays a central role in mitochondria-dependent apoptosis. Several lines of evidence have implied that Bax is involved in both epithelial apoptosis and fibroblast proliferation in idiopathic pulmonary fibrosis; however, the mechanisms remain unknown. Bax-inhibiting peptide V5 (BIP-V5) exhibits membrane permeability and inhibits the activation of Bax.

The purpose of this study was to investigate whether the control of Bax activity by BIP-V5 reduces the degree of bleomycin-induced lung injury. C57BL/6J mice were administered bleomycin and BIP-V5 intratracheally on day 0. Bronchoalveolar lavage fluid and lung tissue were obtained on day 7. Human pulmonary alveolar epithelial cell lines (A549 cells) and mouse pulmonary alveolar epithelial cells (LA-4 cells) were stimulated with bleomycin to induce apoptosis.

Administration of BIP-V5 improved the survival rate and degree of bleomycin-induced lung injury by suppressing Bax activation in mice. BIP-V5 treatment decreased bleomycin-induced apoptosis of alveolar epithelial cell lines (A549 cells and LA-4 cells) by suppressing Bax activation. These results indicate that administration of BIP-V5 may constitute a novel therapeutic strategy against lung injury.

KEY WORDS: Bax, Lung injury, Apoptosis

INTRODUCTION
Idiopathic pulmonary fibrosis (IPF) is a fatal disease defined as a specific form of chronic fibrosing interstitial pneumonia and has the histological appearance of usual interstitial pneumonia. The median survival of patients with IPF has been reported as 3–4 years from the onset of respiratory symptoms (American Thoracic Society and European Respiratory Society, 2002). Although the prognosis of this disease is relatively poor, the etiology of IPF remains unknown, and the efficacy of current therapeutic interventions is unsatisfactory (Behr, 2012; Richeldi et al., 2011). Therefore, a novel strategy for IPF treatment needs to be established.

Alveolar epithelial cells are known as the primary site of lung damage in pulmonary fibrosis (Kuwano et al., 2004). It is thought that recurrent and persistent epithelial damage and its insufficient repair disrupt normal epithelial–fibroblast interactions and evoke fibroblast proliferation and pulmonary fibrosis (Gross and Hunninghake, 2001). Apoptosis is observed in the lung epithelial cells of patients with IPF (Barbas-Filho et al., 2001; Kuwano et al., 1996) and may be involved in alveolar epithelium injury (Hagimoto et al., 1997; Maeyama et al., 2001). Therefore, alveolar epithelial cell apoptosis is a potential target in IPF.

The BCL-2 protein family plays a pivotal role in apoptosis, which is a cell suicide system that is essential for development, tissue homeostasis, and immunity (Czabotar et al., 2014). Bcl-2 family members act as anti- or pro-apoptotic regulators, producing hetero- or homodimers. Bcl-2-associated X protein (Bax) is the main pro-apoptotic member of the Bcl-2 family, and is involved in mitochondria-dependent apoptosis. In healthy cells, Bax is located in the cytosol and, after the initiation of apoptotic signaling, Bax changes its conformation, produces homodimers and translocates to the mitochondrial membrane. This translocation results in the release of cytochrome c, followed by the activation of the mitochondrial apoptosis pathway. Various factors, including heat and hydrogen peroxide, stimulate Bax activation. In addition, Bax can be activated by binding to other pro-apoptotic Bcl family proteins, such as BH3-only proteins (Bid, Bim, Noxa, PUMA, etc.). The expression of Bax is upregulated by p53, and Bax has been reported to be involved in p53-mediated apoptosis (Platka et al., 2005).

The expression of Bax is increased in the pulmonary alveolar epithelial cells of patients with IPF (Platka et al., 2005). Attenuation of lung fibrosis has been shown in Bax knockout mice (Kang et al., 2007); therefore, Bax may constitute a potential therapeutic target of alveolar epithelial injury in IPF.

Bax-inhibiting peptide V5 (BIP-V5) is a cell-permeable pentapeptide, which was created to mimic Ku70, which is a subunit of Ku, a DNA-dependent protein kinase holoenzyme, which binds Bax through residues 578–583 (Yoshida et al., 2004). BIP-V5 has a Bax-binding domain and acts to prevent the activation of Bax in the cytosol. Similar to Ku70, BIP-V5 interacts with Bax and prevents its conformational change (Gomez et al., 2007). In the present study, we investigated the roles of Bax in lung injury in vivo and in vitro using BIP-V5.

RESULTS
BIP-V5 attenuates bleomycin-induced lung injury in mice
The survival of the mice treated with bleomycin (BLM) and BIP-V5 was significantly better than that of the mice in the BLM and negative control (NC) group (14-day cumulative survival rate, 75% versus 25%, respectively) (Fig. 1A). Extreme inflammatory cell infiltration in the lung interstitium, thickening of the alveolar septa, collapse of alveolar spaces, and proliferation of fibroblasts were observed on day 7 after BLM and NC instillation (Fig. 1B). BIP-V5
treatment alleviated all of these changes (Fig. 1B), and also decreased the pathological grade of inflammation (Fig. 1C). On day 7, the protein concentration and total number of cells in the bronchoalveolar lavage fluid (BALF) of mice treated with BLM and BIP-V5 were significantly lower than those in the BALF of the mice treated with BLM and NC (Fig. 2A,B). The transforming growth factor (TGF)-β1 levels in the BALF of mice treated with BLM were significantly increased. BIP-V5 treatment significantly decreased BLM-induced TGF-β1 levels in the BALF (Fig. 2C).

**BIP-V5 suppresses apoptosis in bleomycin-induced lung injury**

The number of apoptotic cells detected using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining is considered to reflect the degree of lung injury (Kuwano et al., 1999). Some bronchial and alveolar epithelial cells and cells in inflammatory lesions revealed evidence of apoptosis as evaluated by TUNEL staining on day 7 after BLM instillation. The number of TUNEL-positive cells in BIP-V5-treated mice was significantly lower than that in NC-treated mice (Fig. 3A,B). The total number of cells was quite different between NC and BIP with BLM. However, the cells that increased were mainly inflammatory cells, and the TUNEL-positive cells were mainly epithelial cells. Thus, we counted the surfactant protein-C (SP-C)-positive cells, which indicate type II alveolar epithelial cells (Fig. 3C,D), and TUNEL-positive cell counts were normalized using SP-C-positive cell counts in the lung fields. Even after normalization using the SP-C-positive cell count, TUNEL-positive cell counts were lower when treated with BIP-V5 and BLM than when treated with NC and BLM (Fig. 3E).

**BIP-V5 suppresses epithelial cell apoptosis in vitro**

Activated Bax, which was detected by the anti-Bax 6A7 antibody, specifically recognizes conformational Bax protein changes (Yamaguchi and Wang, 2001). Activated Bax was significantly expressed in the lung tissues 7 days after BLM instillation, and was attenuated by BIP-V5 treatment (Fig. 4A). To further elucidate the relationship between Bax and apoptosis of alveolar epithelial cells, we used a human alveolar epithelial cell line (A549 cells). Activated Bax was significantly expressed in the cytoplasm of A549 cells 24 h after BLM instillation, and was attenuated by BIP-V5 treatment (Fig. 4B), same as in vivo. A549 cells underwent apoptotic changes 24 h after exposure to BLM, and BIP-V5 pretreatment significantly decreased these apoptotic changes (Fig. 5A). We also showed the inhibiting effect of BIP-V5 on BLM-induced apoptosis in a mouse lung epithelial cell line, LA-4 (Fig. 5B,C).

Furthermore, we assessed p53 phosphorylation at ser15, ser20 and ser46, which are required for the induction of apoptotic proteins. P53 is a protein present upstream of Bax and is also necessary in mitochondrial apoptosis (Plataki et al., 2005). Exposure to BLM upregulated the phosphorylation of p53 at ser15, ser20 and ser46, and BIP-V5 treatment significantly decreased the phosphorylation of p53 at ser46 (Fig. 6A,B).

Apoptosis-inducing conditions have been reported to increase the production of TGF-β1 (Solovyand and Keski-Oja, 2006). Therefore, alveolar epithelial cells were employed to verify whether the concentration of TGF-β1 was elevated under BLM stimulation. The concentration of TGF-β1 was elevated in the culture supernatants of A549 cells treated with BLM. The TGF-β1 concentration was significantly decreased following premedication with BIP-V5 (Fig. 5D). Furthermore, the A549 cells underwent apoptotic changes 24 h after stimulation with TGF-β1, and BIP-V5 pretreatment significantly decreased TGF-β1-induced apoptotic changes (Fig. 5E).

**DISCUSSION**

A few studies have described the effect of local administration of BIP-V5 in vivo (Han et al., 2011; Wang et al., 2010). Because BIP-V5 is membrane permeable, dissolution in saline and subsequent intratracheal administration of BIP-V5 may have a direct effect on lung epithelial cells. We demonstrated that BIP-V5 has a protective effect on bleomycin-induced lung injury in mice. BIP-V5 suppressed Bax activity in bleomycin-treated mice lung tissues and bleomycin-treated alveolar epithelial cell lines. In addition, we confirmed that BIP-V5 suppressed expression of total Bax in bleomycin-treated mice lung tissues (Fig. S1); however, the reason was unclear.

**In vitro** experiments revealed that BIP-V5 suppressed BLM-induced apoptosis of A549 cells by inhibiting not only Bax activity but also p53 phosphorylation of ser46. P53 requires the phosphorylation of ser46, as well as ser15 and ser20, to induce pro-apoptotic proteins such as Bax. P53 not only acts as a transcriptional factor that regulates downstream target genes of Bax but also interacts directly with Bax (Selvakumaran et al., 1994; Westphal et al., 2014). This interaction between Bax and p53 may be related to BIP-V5 suppression of p53 phosphorylation of ser46 in vitro.

Various kinds of cytokines and growth factors participate in the pathological mechanism of IPF. TGF-β1 is one of the most...
important molecules involved in lung fibrosis (Higashiyama et al., 2007) and is produced from various kinds of cells, such as macrophages, lymphocytes, epithelial cells and mesenchymal cells (Khalil et al., 1993; Lo Re et al., 2011). In particular, epithelial cells produce TGF-β1 during apoptosis (Guo et al., 2012). TGF-β1 stimulation induces apoptosis in epithelial cells, and epithelial injury persists as long as TGF-β1 is produced. BIP-V5 inhibits TGF-β1 levels in BALF in vivo, TGF-β1 production, and TGF-β1-induced apoptosis in alveolar epithelial cells under BLM stimulation in vitro. Our results reveal that BIP-V5 has the potential to cease the vicious cycle of epithelial injury caused by TGF-β1.

In conclusion, the control of epithelial cell damage appears to be the key to therapeutic strategies against lung injury. Bax is suggested as an important intracellular molecule involved in both alveolar epithelial cell injury, and the administration of BIP-V5 may be a potent therapy against lung injury.

MATERIALS AND METHODS

Animal treatment

All experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kyushu University, and were approved by the Ethical Committee of Kyushu University Faculty of Medicine. Nine-week-old female C57BL/6J mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and used in all experiments, except for a survival experiment in which 7-week-old females were used. After weighing, the mice were anesthetized with an intraperitoneal injection of ketamine sodium (Daiichi Sankyo Co. Ltd., Tokyo, Japan) and xylazine sodium (Wako Pure Chemical Industries Ltd., Tokyo, Japan), and were intratracheally administered 50 μl of bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan) in sterile saline. We used 2 U of bleomycin/kg body weight in all experiments. BIP-V5 (4 μmol/body weight; Merck Chemicals International, Kenilworth, NJ, USA) was intratracheally injected on day 0 with the bleomycin instillation. Control mice were injected with NC peptide (4 μmol/body weight; Merck Chemicals International) instead of BIP-V5. After the treatments, the mice were returned to their cages and allowed food and water ad libitum. The mice were anesthetized and sacrificed on day 7 after bleomycin instillation.

Histopathology of lung tissues

Histopathology was performed as previously described (Hamada et al., 2008). The pathological grades were determined according to the following criteria: 0, no lung abnormalities; 1, presence of inflammation and fibrosis involving <25% of the lung parenchyma; 2, lesions involving 25–50% of the lung; and 3, lesions involving >50% of the lung.
DNA damage and apoptosis in lung tissues

TUNEL staining was performed using a DeadEnd Colorimetric Apoptosis Detection System (Promega Corp., Madison, WI, USA) as previously described (Hamada et al., 2005).

Immunohistochemistry of lung tissue

Immunohistochemistry was performed using a Histofine SAB-PO Kit (Nichirei, Tokyo, Japan). Anti-SP-C (FL-197) antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was used as the first antibody. SP-C-positive cells were counted in all fields (40–55 fields) of the lung using a light microscope with 200× magnification.

Bronchoalveolar lavage

The bronchoalveolar lavage method and analyses were performed as previously described (Hamada et al., 2008). Total protein concentrations in...
BALF were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA).

**Measurement of TGF-β1 levels in BALF**

BALF were centrifuged, and the supernatant was stored at −80°C until use. TGF-β1 levels in BALF were measured with a cytokine-specific enzyme-linked immunosorbent assay (ELISA) kit purchased from R&D Systems (Minneapolis, MN, USA).

**Cell culture and treatment**

The A549 human lung epithelial cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) using 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), penicillin and streptomycin in an incubator with 5% CO2 at 37°C. A549 cells were stimulated using bleomycin to induce apoptosis. The cells were pretreated with BIP-V5 before apoptotic stimulation. The cells were harvested 24 h after the addition of bleomycin and subsequently prepared for flow cytometry, immunoprecipitation and western blot.

The LA-4 murine lung epithelial cell line was cultured in F-12K (Kaighn’s Modification of Ham’s F-12 Medium) medium (Thermo Fisher Scientific, UK) using 10% FBS, penicillin, and streptomycin in an incubator with 5% CO2 at 37°C. LA-4 cells were stimulated using bleomycin to induce apoptosis (Stoner et al., 1975).

The cells were pretreated with BIP-V5 before apoptotic stimulation. The cells were harvested 12-24 h after the addition of bleomycin and subsequently prepared for flow cytometry.

**Flow cytometry**

Apoptosis of A549 cells and LA-4 cells was assessed with Annexin V and propidium iodide (Roche Diagnostics, Basel, Switzerland). A549 cells were analyzed on an EPICS XL flow cytometer (Coulter, Luton, UK), and LA-4 cells were analyzed on a FACS Verse instrument (BD Bioscience).

**Western blot analysis**

Cellular protein extract from mouse lung tissue and cell line were prepared by homogenization in radioimmunoprecipitation assay buffer. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to hydrophobic polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA), and the membranes were incubated using anti-Bax (N-20) antibody (Santa Cruz Biotechnology), PI, propidium iodide.
Fig. 6. Effect of BIP-V5 on p53 signaling. (A) Western blot analysis of the phosphorylation of p53 in A549 cells stimulated with BLM. (B) Quantification of Western blotting. Each column represents the mean±s.e.m. (n=3). *P<0.05.

Immunoprecipitation followed by SDS-PAGE and immunoblotting
Homogenized A549 cells and mice lung tissues were incubated with Bax 6A7 antibody (Santa Cruz Biotechnology), anti-GAPDH antibody (Cell Signaling Technology), anti-p53 ser15 antibody (Cell Signaling Technology), anti-p53 ser20 antibody (Cell Signaling Technology) and anti-p53 ser46 antibody (Cell Signaling Technology) in blocking buffer. After rinsing, the membranes were incubated with biotinylated anti-rabbit or mouse IgG. The blots were detected using the enhanced chemiluminescence method (Bio-Rad Laboratories). The signals were measured using ImageJ public domain software (https://imagej.nih.gov/ij/) and standardized to GAPDH.

Statistical analyses
Survival curves (Kaplan–Meier plots) were compared using the log-rank test. Pathological grades were compared using the Mann–Whitney U test. Comparisons of the numbers or contents of other results were carried out using the one-way ANOVA test followed by Tukey’s multiple comparison test. P values less than 0.05 were considered to indicate statistical significance. All statistical analyses were performed using JMP Pro 10.0 (SAS Institute Japan, Tokyo, Japan).

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary information
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Fig. S1 Effect of Bax-inhibiting peptide V5 on the amount of total Bax expression in vivo.

(A) Western blot for total amount of Bax in lung tissues was performed on day 7. Data are collected from three separate experiments and are expressed as mean ± S.E.M. *p < 0.05, **p < 0.01. BLM, bleomycin; NC, negative control; BIP, Bax-inhibiting peptide V5.