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Tacrolimus (FK506) has protective actions against murine bleomycin-induced acute lung injuries

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Abstract

The effects of tacrolimus on murine acute lung injury were tested, especially in comparison to dexamethasone. Acute lung injury was induced by intratracheal instillation of bleomycin. Oral tacrolimus significantly improved survival rates of bleomycin-exposed mice, while cyclosporin A or dexamethasone did not. After instillation of bleomycin (day 0), a migration of neutrophils into alveolar spaces peaked on day 3, with concomitant increases of chemokines. On day 6, marked morphological changes in the lungs were observed. All these changes were significantly inhibited by tacrolimus. Furthermore, DNA ladder and immunohistochemical analyses of lungs showed that apoptosis of lung cells appeared on day 6 and was abolished only by the treatment of tacrolimus. These results suggest that both anti-inflammatory and anti-apoptotic action of tacrolimus contribute to improvement of bleomycin-induced acute lung injury.

Keywords: Neutrophil; MCP-1 (Monocyte chemoattractant protein-1); Dexamethasone; Acute lung injury; Cyclosporin A; Tacrolimus

1. Introduction

Tacrolimus (FK506) is a macrolide isolated from *Streptomyces tsukubaensis* with molecular weight of 822.05 ($C_{44}H_{69}NO_{12}\cdot H_2O$). An immunosuppressive effect by tacrolimus involves inhibition of calcineurin, which leads to inhibition of translocation of nuclear factor of activated T cells into nucleus (Kino et al., 1987; Liu et al., 1991; Jain et al., 1993). This results in direct inhibition of the functions of T lymphocytes. Previously, we reported that tacrolimus, but not methylprednisolone, can improve survival rate, respiratory dysfunction, and edema formation in a canine acute lung injury model induced by lipopolysaccharide and phorbol myristate acetate, suggesting that it could prevent the development of acute lung injury (Koshika et al., 2001). It has been shown that massive infiltration of inflammatory cells (neutrophils rather than lymphocytes) into alveolar spaces may be involved in induction of the lipopolysaccharide and phorbol myristate acetate-evoked canine acute lung injury model (Hsu et al., 1986). In addition to these evidences, an increasing number of papers suggest that tacrolimus not only inhibits the function of T cells but also that of the cell types other than T cells (Yamashita et al., 1999; Squadrito et al., 2000).

Bleomycin-induced acute lung injury in mice has been used as a pulmonary fibrosis model. Histological features of the lungs are similar to those in human lung diseases, such as idiopathic pulmonary fibrosis (Adamson and Bowden, 1974). Nevertheless, development of severe acute lung injury occurs at 5–7 days post-bleomycin instillation in these murine models. Their pathological features include alveolitis and interstitial infiltration of inflammatory cells with epithelial cell injury. Various types of cells, including macrophages and neutrophils rather than lymphocytes, have

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been primarily implicated for potential roles in establishing bleomycin-induced acute lung injury and fibrosis (Kelly, 1990; Sharma et al., 1996; Smith et al., 1996; Gharaee and Phan, 1997; Okazaki et al., 2001). Also, it has recently been suggested that intratracheal instillation of bleomycin-evoked lung injury on the early stage in the rodent model has some histologic similarities to acute phase of acute respiratory distress syndrome or diffuse alveolar damage lung in human, rather than human pulmonary fibrosis (Gisella et al., 2001). These backgrounds lead us to regard bleomycin-treated mice as an appropriate model for investigating acute lung injury.

As there are few beneficial therapies against acute lung injury, development of drug to improve lung injury is needed. From this viewpoint, it is worthwhile to assess the beneficial effects of tacrolimus. Therefore, in this study, we evaluated the effects of tacrolimus on bleomycin-evoked murine acute lung injury and decrease in survival rate, in comparisons to dexamethasone, methylprednisolone and cyclosporin A, especially focusing on an acute phase of this model. Interestingly, tacrolimus, but not corticosteroids or cyclosporin A, showed a significant protective effect in this model. We present in vivo evidence of both anti-inflammatory and anti-apoptotic effects of tacrolimus on bleomycin-induced lung injuries and discuss possible mechanisms of these actions.

2. Materials and methods

2.1. Animals

All animal experimental procedures were performed according to guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical Co., Ltd. Six-week-old specific pathogen-free female C57BL/6J mice (weighing 17–20 g) were purchased from Charles River Japan, Inc. (Yokohama, Japan). Animals were kept in clean rooms and had free access to food and water ad libitum. Animals were acclimated for 1 week prior to start of experiments. Bleomycin sulfate (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 0.9% sterile saline at the concentration of 2 units/ml.

2.2. Bleomycin-induced acute lung injury model

Mice were anesthetized by intraperitoneal (i.p.) administration of sodium pentobarbital (Dainabot Co., Osaka, Japan), and then had intratracheal instillation into airways of either physiological saline (normal group) or 0.2 units/100 μl/animal of bleomycin, using syringe with a 27-gauge needle (TERUMO, Tokyo, Japan) on day 0.

In prophylactic studies, mice received daily oral administration of dexamethasone, cyclosporin A, and tacrolimus (these were all prepared by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) and methylprednisolone (Pharmacia Upjohn Co., Tokyo, Japan) from 1 day before (day −1) bleomycin instillation to 14 days after instillation (day 14). Tacrolimus and methylprednisolone were suspended in a sterilized distilled water, cyclosporin A in olive oil, dexamethasone in 0.5% of methylcellulose. Doses administered were 0.32–1.0 mg/kg for dexamethasone, 10–100 mg/kg for methylprednisolone, 32–100 mg/kg for cyclosporin A, and 3.2–32 mg/kg for tacrolimus, respectively. On day 0, drugs were given 1 h before bleomycin instillation. In a therapeutic study with tacrolimus, 32 mg/kg of tacrolimus was administered, starting from day 1, day 3, and day 6 in relation to progression of disease, respectively. As a vehicle control, 0.5% methylcellulose or placebo was daily administered.

2.3. Bronchoalveolar lavxage

Animals were euthanized with an excess amount of sodium pentobarbital. The trachea was exposed by incising the skin and cannulated with an 18-gauge catheter (Terumo, Tokyo, Japan). Lavage of the whole lung was performed with a 0.5-ml aliquot of phosphate-buffered saline (PBS) and was repeated three times. The collected fluids were centrifuged at $250 \times g$ for 10 min to sediment cells. Supernatants were also collected and stored at −30 °C until analyses of cytokine and protein content in bronchoalveolar lavage fluids were performed. Total cell number was manually counted using a counting chamber, and dispersed on a slide glass by centrifugation using Cytospin 2 (Shandon, USA) at 75 g for 2 min. Cells were stained with Wright-Giemsa and 200 cells were differentially counted to determine populations of each cell fraction.

2.4. Analysis of chemokine and protein content in bronchoalveolar lavage fluid

Concentrations of monocyte chemoattractant protein-1 (MCP-1), macrophage inhibitory protein-1α, -2 (MIP-1α, MIP-2) and keratinocyte chemoattractant (KC) in bronchoalveolar lavage fluid were measured by enzyme-linked immunosorbent assay (ELISA) using OptEIA™ mouse MCP-1 kit (PharMingen, San Diego, CA, USA) and Quantikine® for mouse MIP-1α, MIP-2 and KC (R&D SYSTEMS, Inc., MN, USA), according to manufacturer's instructions. Concentration of protein in bronchoalveolar lavage fluid after bleomycin instillation was assayed using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) and compared to a bovine serum albumin standard curve.

2.5. Analysis of DNA fragmentation

Lungs were removed 1, 3 and 6 days post-bleomycin instillation. They were minced using surgical scissors, suspended in cell lysis buffer containing 100 mM Tris HCl (pH 8.0), 40 mM Na_2EDTA, 10 mM NaCl and 1%

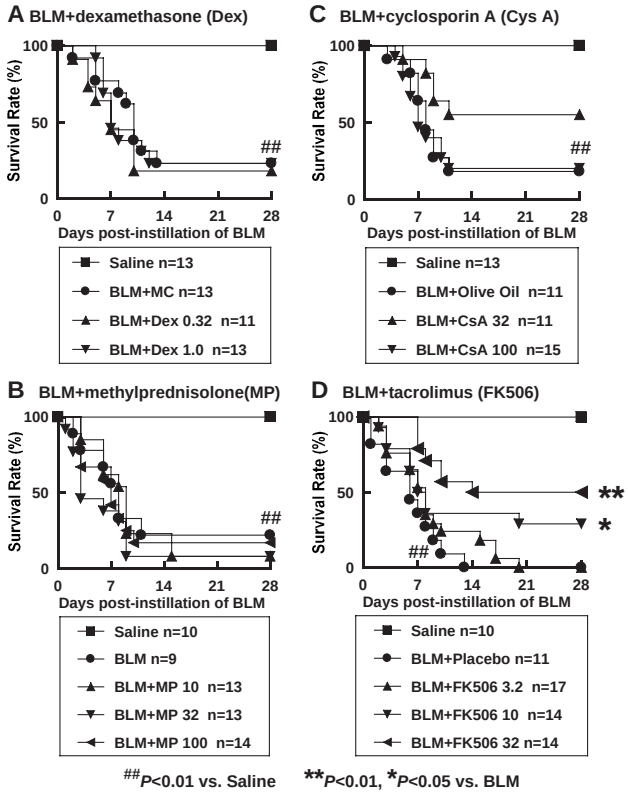

Fig. 1. Effects of dexamethasone (Dex), methylprednisolone (MP), cyclosporin A (Cys A), and tacrolimus (FK506) on cumulative survival rates in mice receiving intratracheal instillation of bleomycin (BLM). BLM (0.2 units/mouse) was instilled intratracheally to female C57BL/6J mice on day 0 and all animals were monitored daily throughout the experimental period (up to day 28). Each drug was administered daily via oral administration on days −1, 0, 1–14 (for 16 consecutive days) in a prophylactic regimen. Doses administered were 0.32–1.0 mg/kg for Dex (A), 10–100 mg/kg for MP (B), 32–100 mg/kg for Cys A (C), and 3.2–32 mg/kg for FK506 (D), respectively. $^{\#\#}P<0.01$, compared to saline-treated mice; $^{*}P<0.05$, $^{**}P<0.01$, compared to BLM group.

of sodium dodecyl sulfate (SDS), then incubated for 30 min at 4 °C before centrifugation (18 000 × *g*, for 20 min). RNase A (Roche) was then added to the supernatant fluids before 60-min incubation at 37 °C, followed by addition of proteinase K (Boehringer Manheim Biochemicals, Indianapolis, IN, USA) and further 60-min incubation at the same temperature. Samples were incubated with 5 M NaCl and 2-propanol at −20°C overnight, then subject to centrifugation (18 000 × *g*, for 20 min). Supernatant fluids were removed and pellets were dissolved in water, then again incubated with 5 M NaCl and 2-propanol prior to centrifugation. After centrifugation, supernatant fluids were removed again and DNA pellets were dried. Pellets were resuspended in 10 mM Tris HCl and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer. DNA concentrations were ascertained by ultraviolet (UV) spectrophotometry at 280 nm. 10 μg of DNA was electrophoresed on 1% agarose gel, stained with ethidium bromide, and photographed under UV transilluminator (ATTO Co., Tokyo, Japan).

2.6. Histological analysis

After instillation of bleomycin, 10% neutralized-buffered formaldehyde was instilled into the lungs of mice treated with methylprednisolone, dexamethasone or tacrolimus, through intratracheal cannula, and whole lungs were excised and fixed in 10% buffered formaldehyde prior to preparation for histological analysis. The fixed lungs were embedded in paraffin and stained with hematoxylin and eosin.

2.7. DNA nick end labeling of tissue sections

Terminal deoxynucleotidyltransferase (TdT)-mediated deoxyribouridine triphosphate nick end labeling (TUNEL) assay was performed with an In Situ Apoptosis Detection Kit (TAKARA, Otsu, Japan), according to manufacturer's instructions. In this kit, fluorescent isothyocyanate-labeled nucleotides are incorporated at sites of DNA strand breaks by TdT. This assay was performed on each section from the paraffin-embedded blocks of lung (on day 6) described above. Almost all of total fields of each section were studied at an original magnification of 140-fold. In each field, we counted the number of positive cells in each field and calculated the means of all the numbers per field in each group.

2.8. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA in whole lung tissue was extracted using an acid guanidinium-phenol-chloroform method (TRIZOL® Reagent; GIBCO BRL®, Life Technologies™). 1 μg of total RNA was reverse-transcribed into complementary DNA, using TaqMan reverse transcription reagents containing MultiScribe reverse transcriptase (PE Applied Biosystems, Foster City, CA, USA).

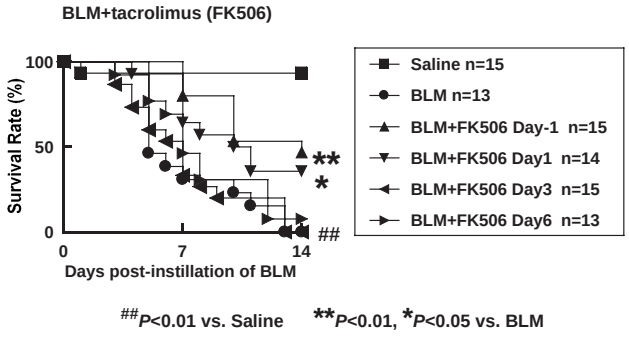

Fig. 2. Effect of tacrolimus (FK506) on survival rate in a therapeutic regimen. Bleomycin (0.2 units/mouse) was instilled intratracheally to female C57BL/6J mice on day 0. FK506 (32 mg/kg) was administered daily at the indicated time points up to day 14. As a positive control, FK506 was prophylactically administered, as described in Fig. 1 (referred to as day −1). Mice received physiological saline instead of bleomycin (BLM) (Saline group). $^{\#\#}P<0.01$, compared to saline-treated mice; $^{*}P<0.05$, $^{**}P<0.01$, compared to corresponding BLM group.

Table 1
Time-course study of differential leukocytes in bronchoalveolar lavage fluid of mice after receiving bleomycin

Time	$n=$	Total	Macrophages	Neutrophils	Lymphocytes
0 day	5	0.7±0.1	0.7±0.1	0.0±0.0	0.1±0.1
Days after instillation of bleomycin					
1 day	9	0.4±0.1	0.3±0.1[a]	0.1±0.1	0.1±0.1
3 days	10	1.0±0.1	0.3±0.1	0.6±0.1[b]	0.1±0.1
6 days	6	1.4±0.1[a]	0.5±0.1	0.5±0.1[a]	0.4±0.1[a]

Bronchoalveolar lavage fluid from bleomycin-instilled mice was studied for determination of differential populations of leukocytes. Values are mean± S.E.M. (Unit: × 10^6 cells).
[a] $P<0.05$ vs. day 0 for each leukocyte population.
[b] $P<0.01$ vs. day 0 for each leukocyte population.

RT-PCR with fluorogenic amplification of first strand complementary DNA was performed using an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). TaqMan Universal PCR

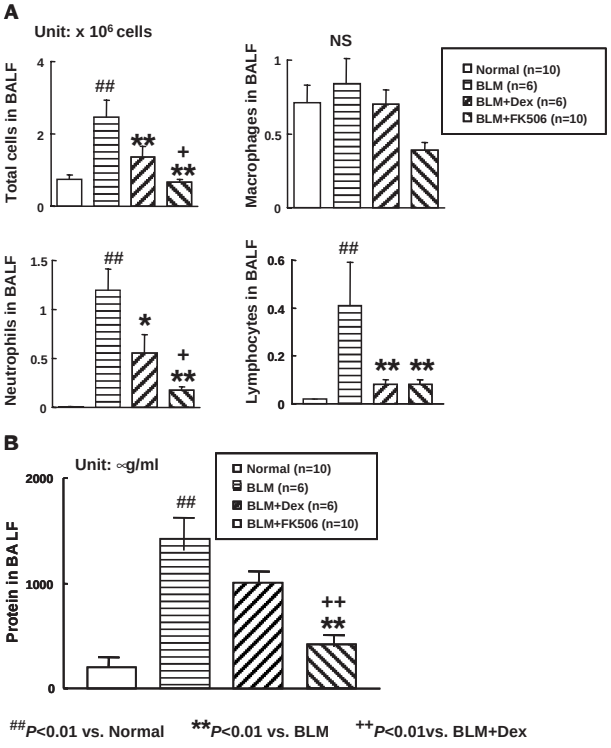

Fig. 3. Effects of dexamethasone (Dex) and tacrolimus (FK506) on cellular influx (A) and protein leakage (B) into the bronchoalveolar lavage fluid of mice after instillation of bleomycin (BLM). BLM (0.2 units/animal) was intratracheally instilled into lungs of C57BL/6J mice. Bronchoalveolar lavage fluids were collected 6 days after instillation of bleomycin (BLM). Two hundred cells from bronchoalveolar lavage fluid were counted and classified, using standard morphological criteria. The number of each cell population is expressed as mean±S.E.M (×10^6 cells). Concentration of protein in bronchoalveolar lavage fluid after bleomycin instillation was assayed using a bicinchoninic acid protein assay kit and compared to a bovine serum albumin standard curve. Dexamethasone (Dex) and tacrolimus (FK506) were administered daily at 1.0 mg/kg and 32 mg/kg, respectively. $^{\#\#}P<0.01$, compared to normal group; $^{**}P<0.01$ vs. BLM group; $^{+}P<0.05$, $^{++}P<0.01$, compared to BLM+Dex group. NS means "not significant" ($P>0.05$).

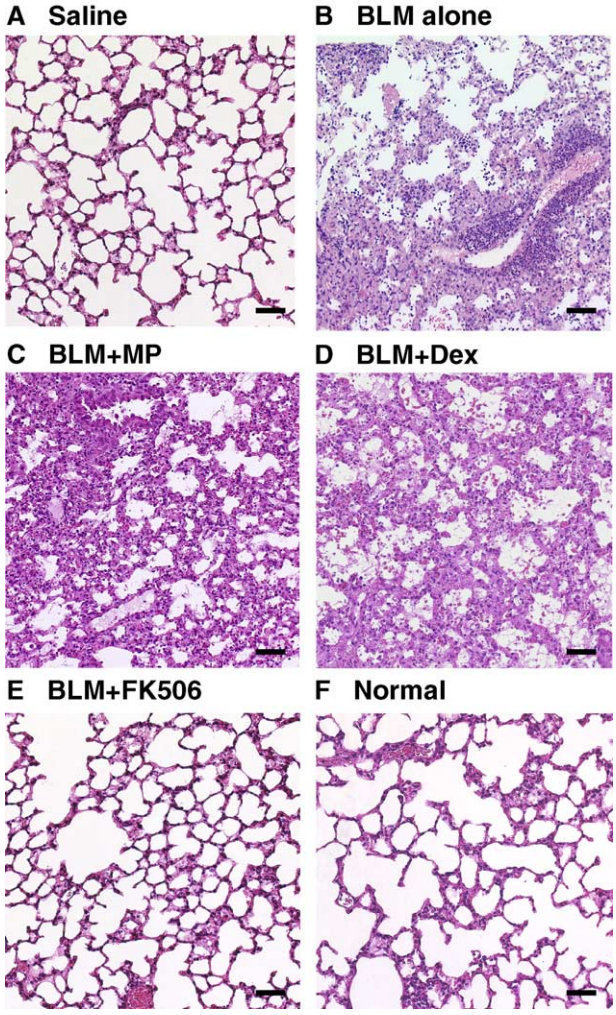

Fig. 4. Typical example of bleomycin (BLM)-induced histological changes in lungs after treatment with corticosteroids and tacrolimus (FK506). BLM was administered intratracheally to C57BL/6J mice. Lungs were removed on day 6 for histological examination. Whole lungs were excised and fixed in 10% buffered formaldehyde. Fixed lungs were embedded in paraffin, then stained with hematoxylin and eosin as described in Materials and methods. (A) Saline negative control group in which physiological saline was instilled instead of bleomycin (BLM); (B) Bleomycin (BLM)-treated group with vehicle administration; (C) Bleomycin (BLM)-treated group with 100 mg/kg methylprednisolone (MP) administration; (D) Bleomycin (BLM)-treated group with 1.0 mg/kg dexamethasone (Dex) administration; (E) Bleomycin (BLM)-treated group with 32 mg/kg tacrolimus (FK506) administration; (F) Normal Lung. Bars=30 μm.

Master Mix, containing murine MCP-1 or 18S-ribosomal RNA primers and fluorophore-labeled probes (murine MCP-1:4312869P, 18S-ribosomal RNA:4310893E, PE Biosystems) were purchased. Real-time fluorescence measurements were used to determine the threshold cycle (C_T) for each amplification curve by calculating the number of cycles required to reach a linear-scale fluorescence intensity greater than the baseline intensity. C_T obtained from murine 18S-ribosomal RNA primer was subtracted from C_T from MCP-1 primer (=ΔC_T). $\Delta\Delta C_T$ was further calculated to produce relative abundance of each messenger RNA (mRNA) level, based on each value

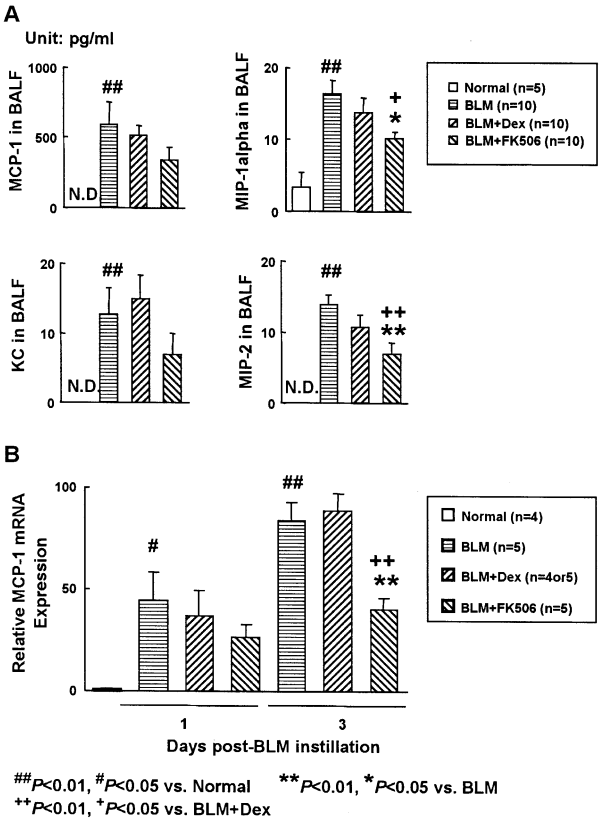

Fig. 5. Effects of dexamethasone (Dex) and tacrolimus (FK506) on expression of chemokines. (A) Effects of dexamethasone (Dex) and tacrolimus (FK506) on monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α, -2 (MIP-1α, MIP-2) and keratinocyte chemoattractant (KC) in bronchoalveolar lavage fluid of mice receiving intratracheal instillation of bleomycin (0.2 units/animal) on day 3. Dex and tacrolimus were administered at 1.0 mg/kg and 32 mg/kg, respectively. Chemokines were measured by specific enzyme-linked immunosorbent assay (ELISA) as described in Materials and methods. (B) Effects of dexamethasone (Dex) and tacrolimus (FK506) on elevation of monocyte chemoattractant protein-1 (MCP-1) messenger RNA (mRNA) expression in bleomycin (BLM)-instilled lung. Dex and tacrolimus were administered daily by oral route, as described in (A). Lungs were prepared prior to quantitative real-time polymerase chain reaction (PCR) as described in Materials and methods. Lungs from normal mice were used as negative control. Data are expressed as mean±R.E.M. $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared to normal group; $^{*}P<0.05$, $^{**}P<0.01$, compared to BLM group; $^{+}P<0.05$, $^{++}P<0.01$, compared to BLM+Dex group. N.D. means "Not detected".

for ΔC_T. The relative abundance of 18S-ribosomal mRNA in each sample was used to normalize MCP-1 mRNA level.

2.9. Statistical analysis

All data are presented as the mean±S.E.M. Comparisons among multiple groups were made using Dunnett's multiple comparison and Log-rank test. Two-tailed Student's *t*-test or Aspin–Welch test was applied to assess statistically significant differences between two groups, depending on whether S.D. was equal or not.

3. Results

3.1. Survivals

As shown in Fig. 1, intratracheal administration of bleomycin (0.2 units/mouse) decreased the survival rate in mice. At first, we evaluated the effect of tacrolimus on this model in a prophylactic regimen, compared with those of corticosteroids (i.e. dexamethasone and methylprednisolone), and that of cyclosporin A, another calcineurin inhibitor. Neither dexamethasone (at doses up to 1 mg/kg) nor methylprednisolone (at doses up to 100 mg/kg) improved survival rate (Fig. 1A and B). In addition, 32 mg/kg of cyclosporin A showed a tendency to improve survival rate, but it was not statistically significant. At 100 mg/kg, cyclosporin A failed to improve survival. On the contrary, tacrolimus at more than 10 mg/kg significantly improved survival rate in a dose-dependent fashion (Fig. 1D). Since most bleomycin-exposed mice died until day 14, we next tested the possible therapeutic effect of tacrolimus on survivals through 14-day period. Tacrolimus (32 mg/kg) given from 1 day after instillation of bleomycin significantly improved survival as in a prophylactic regimen (Fig. 2). Tacrolimus given from later than day 3 did not show any beneficial effect on survival rate.

3.2. Bronchoalveolar lavage fluid analysis

We assessed the time-course of the leukocyte infiltration in bronchoalveolar lavage fluid of mice exposed to bleomycin (Table 1). The number of neutrophils in bronchoalveolar lavage fluid peaked on day 3 and reached plateau thereafter. The number of lymphocytes increased on day 6. Both neutrophils and total cells in bronchoalveolar lavage fluid observed on day 6 were

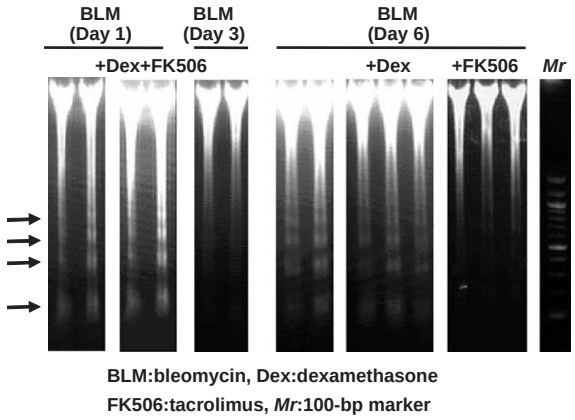

Fig. 6. Effects of dexamethasone (Dex) and tacrolimus (FK506) on DNA ladder formation in whole lungs of mice receiving Bleomycin (BLM). BLM (0.2 units/animal) was intratracheally instilled into lungs of C57BL/6J mice. Dex and tacrolimus were administered for indicated periods at 1.0 and 32 mg/kg, respectively. Whole lungs were prepared prior to analysis of DNA fragmentation as described in Materials and methods. Arrows show a series of ladders. Mr indicates 100-bp marker.

significantly reduced by both dexamethasone and tacrolimus (Fig. 3A). Moreover, the inhibitory effects of tacrolimus on migrations of total cells and neutrophils were significantly greater than those of dexamethasone. Interestingly, increased number of lymphocytes induced by bleomycin was inhibited by both tacrolimus and dexamethasone.

The protein content in bronchoalveolar lavage fluid significantly increased 6 days after intratracheal instillation of bleomycin, compared to that in normal mice (Fig. 3B). No significant increase in the protein content was observed until day 3 (data not shown). Tacrolimus significantly attenuated this protein leakage into alveolar spaces, but dexamethasone had no effect.

3.3. Histology of lungs

In lungs from saline-instilled mice, there was slight interstitial wall thickening, compared to those from normal one on day 6 (Fig. 4A and F). Lungs from bleomycin-

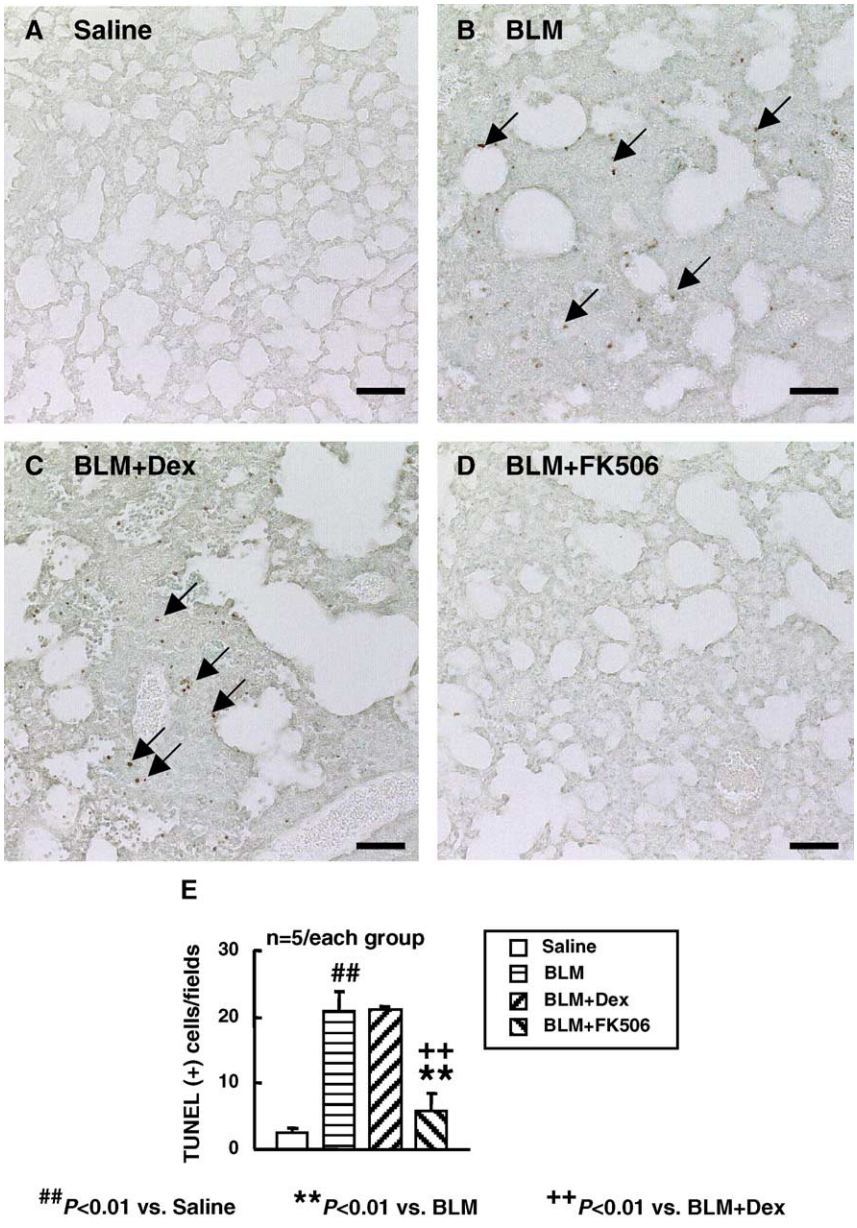

Fig. 7. Terminal deoxynucleotidyltransferase-mediated deoxyribouridine triphosphate nick end labeling (TUNEL) analysis of murine lungs exposed to bleomycin (BLM). BLM-instilled lungs were removed 6 days after instillation, followed by fixation with 10% formaldehyde and paraffin-embedding prior to TUNEL staining. (A) Saline negative control group in which saline was instilled instead of bleomycin (BLM); (B) Bleomycin (BLM)-treated group with vehicle administration; (C) Bleomycin (BLM)-treated group with 1.0 mg/kg dexamethasone (Dex) administration; (D) Bleomycin (BLM)-treated group with 32 mg of tacrolimus (FK506) administration. (E) Effects of dexamethasone (Dex) and tacrolimus (FK506) on TUNEL-positive cells subject to apoptosis by bleomycin (BLM). Positive cells are expressed as mean±S.E.M. Arrows depict positive signals. $^{\#\#}P<0.01$, compared to normal group; $^{**}P<0.01$, compared to BLM-treated group; $^{++}P<0.01$, compared to BLM+Dex group. Bars=30 μm.

instilled mice demonstrated marked morphological changes on day 6 (Fig. 4B), although no prominent histological change was seen until day 3 (our unpublished observation). These histological changes included infiltrations of various inflammatory and red blood cells associated with architectural collapses, especially in the alveolar regions. Tacrolimus diminished these changes, but slight interstitial wall thickening was observed (Fig. 4E). Regarding corticosteroids, methylprednisolone and dexamethasone failed to reverse these histological changes (Fig. 4C and D).

3.4. Effect of dexamethasone and tacrolimus on chemokine content in bronchoalveolar lavage fluid

In a preliminary experiment, we found that MCP-1 increased in bronchoalveolar lavage fluid, peaked 3 days post-instillation of bleomycin and declined thereafter (data not shown). Thus, we evaluated the effects of dexamethasone and tacrolimus on production of several chemokines on day 3. As shown in Fig. 5, mice exposed to bleomycin had significant increases in MIP-1α and MIP-2 content in bronchoalveolar lavage fluid. Tacrolimus significantly inhibited the increases in these chemokines. With regard to MCP-1 and KC, tacrolimus showed a tendency to suppress their increases, but it was not statistically significant. Dexamethasone did not significantly suppress increases of these chemokines.

The expression of MCP-1 mRNA in whole lung increased by more than 40-fold 1 day post-bleomycin instillation. A further increase in the mRNA level was observed (more than 80-fold compared to normal group) 3 days post-instillation (Fig. 5). Tacrolimus did not inhibit the increase in MCP-1 mRNA on day 1, but inhibited the further increase in MCP-1 mRNA expression on day 3 significantly. Dexamethsone did not inhibit the increase in MCP-1 mRNA.

3.5. DNA ladder and immunohistochemical analysis of whole lung tissues exposed to bleomycin

Electrophoretic analysis of DNA extracted from whole lung tissue exposed to bleomycin showed biphasic DNA fragmentation. One day after bleomycin instillation, DNA fragmentation was initially observed, but disappeared on day 3 (Fig. 6). Thereafter, the second phase of DNA fragmentation reappeared on day 6. Interestingly, tacrolimus only inhibited reappearance of DNA fragmentation at the second phase. In contrast, dexamethasone failed to diminish DNA fragmentation at both phases.

To assess further the possible effect of tacrolimus on apoptosis at the second phase, we performed TUNEL staining on the sections of the lungs. Immunohistochemical analysis demonstrated that TUNEL-positive cells were observed in lung cells of bleomycin-treated mice on day 6 (Fig. 7B). Tacrolimus abolished the appearance of positive cells while dexamethasone did not (Fig. 7C and D). The intratracheal instillation of saline-treated group, which is a negative control group, had few positive apoptotic cells (Fig. 7A). In a semi-quantitative analysis, tacrolimus also significantly inhibited bleomycin-induced increase in positive cells (Fig. 7E).

4. Discussion

In this study, we showed that tacrolimus significantly improved survival rate in a bleomycin-evoked acute lung injury model, while treatment with dexamethasone failed. Some reports have shown that dexamethasone treatment significantly inhibits lung injury induced by several kinds of toxins including bleomycin (Grunze et al., 1988; Viviano et al., 1995), while other did not inhibit lung injury (Khalil et al., 1993). One possible explanation for this discrepancy may be that the dose of dexamethasone used in this study was not high enough to observe such a beneficial effect. We have evaluated the effect of dexamethasone at doses of up to 1 mg/kg. In our model, however, higher doses of dexamethasone were not tolerable for bleomycin-injected mice because of its toxic effects (e.g. severe loss of body weight). On the contrary, tacrolimus (at doses up to 32 mg/kg) did not show such an apparent toxic effect (data not shown). To evaluate further whether there could be possible effects of corticosteroid in our model, we next investigated the effect of methylprednisolone ranging from 10 to 100 mg/kg, and it failed to show beneficial effects. Thus, we conclude that corticosteroids at maximal doses without intolerable side effects are not effective in our bleomycin-induced acute lung injury model. Therefore, tacrolimus may have some beneficial advantages over corticosteroids.

There is an extensive literature on actions of cyclosporin A against acute lung injury (Lossos et al., 1996; Corbel et al., 1999; Naidu et al., 2002; Pagano et al., 2004). Some have shown that cyclosporin A protected acute lung injury (Naidu et al., 2002; Pagano et al., 2004). Thus, to elucidate whether calcineurin could be involved in our bleomycin-evoked acute lung injury, we also have investigated the effect of cyclosporin A. Cyclosporin A at 32 mg/kg showed a trend to improve the survival, but it was not statistically significant. On the other hand, 100 mg/kg of cyclosporin A did not improve survival at all. Thus, the underlying protective mechanisms of tacrolimus might involve a calcineurin-dependent one as discussed below. However, we could not get a clue from these ambiguous results with cyclosporin A to determine whether the protective mechanism of tacrolimus was solely dependent on calcineurin. In this respect, tacrolimus appeared to have more potential to improve survival than cyclosporin A at least in our model.

Although macrophages, lymphocytes, neutrophils, eosinophils and their chemoattractants clearly participate in the pathogenesis of acute lung injury and fibrosis, it is still widely debated as to which type of leukocytes plays the

pivotal role (Sharma et al., 1996; Helene et al., 1999). Dexamethasone as well as tacrolimus suppressed accumulation of total cells, neutrophils, and lymphocytes in bronchoalveolar lavage fluid, in comparison to bleomycin alone. Furthermore, tacrolimus significantly inhibited infiltration of neutrophils and total leukocytes greater than dexamethasone. On the other hand, it has been shown that transepithelial efflux of neutrophils in bleomycin-evoked murine lung injury results in higher mortality significantly, suggesting that decreases in efflux of neutrophils into alveolar spaces are protective against fatal lung injury (Li et al., 2002). Therefore, it is suggested that survival difference observed between dexamethasone and tacrolimus therapy may partly depend on their difference of inhibitory effect of neutrophils' migration. Meanwhile, it has been shown that T-lymphocytes primarily participate in establishment of lung injury and fibrosis (Kuwano et al., 1999). Thus, we could not rule out the possibility that an inhibition of function of lymphocyte alone or in combination to neutrophil which involves in the pathophysiology of pulmonary acute lung injury may be also important for the improvement of the survival.

It has been reported that several chemokines, including MCP-1, MIP-1α, MIP-2 and KC, are involved in murine bleomycin-induced lung injury and fibrosis (Tokuda et al., 2000; Moore et al., 2001). In bleomycin-evoked models, MCP-1 facilitates the recruitment of inflammatory cells into inflamed sites, contributing to acute lung injury and fibrosis (Brieland et al., 1992). It has been shown that the degree of lung fibrosis induced by bleomycin was reduced in MCP-1 receptor-deficient mice, but not in MIP-1α receptor-knockout mice (Moore et al., 2001), while another study has shown that MIP-1α receptor signaling may be crucial for the establishment of inflammation during the establishment of acute lung injury and fibrosis (Tokuda et al., 2000). MIP-2 and KC are also reported to enhance migration of neutrophils into alveolar spaces, leading to lethal responses (Li et al., 2002; Michael et al., 1999). In our study, tacrolimus, but not dexamethasone, significantly suppressed production of MIP-1α and MIP-2 protein in bronchoalveolar lavage fluid. Tacrolimus also showed a strong trend toward inhibition against production of MCP-1 and KC but it was not statistically significant. These data indicate that suppressive effects of tacrolimus on chemokine production may contribute to its more inhibition of neutrophilic infiltration than dexamethasone, as shown in Fig. 3A. Interestingly, tacrolimus significantly suppressed mRNA expression of MCP-1 observed on day 3 but not day 1 (Fig. 4). In the therapeutical regimen (Fig. 2), tacrolimus starting 1 day, but not 3 days, after instillation of bleomycin significantly enhanced survival, strongly suggesting that some critical subcellular changes which seem to be sensitive to tacrolimus occurred between day 1 and day 3 after bleomycin instillation, affecting subsequent animal survival. Thus, the elevation of chemokine mRNA expression during this period could be closely associated with such a kind of change which tacrolimus but not dexamethasone was able to suppress. Recently, on the other hand, evidence has been presented that tacrolimus may upregulate renal cortical gene expression of MCP-1 by promoting nuclear factor-kappa B translocation in experimental tacrolimus-induced chronic nephropathy (Tamada et al., 2003). These apparently opposing findings imply that the exaggerating or protective actions of tacrolimus may depend on the conditions of study, such as cells or tissue types examined.

When excessive apoptosis occurs, it may contribute to pathogenesis in some diseases. In bleomycin-evoked lung injury, excess apoptosis may make a significant contribution to its pathogenesis (Hagimoto et al., 1997; Kuwano et al., 2000; Matsuoka et al., 2002). Therefore, we evaluated the possible involvement of cell death in development of acute lung injury, plus effects of tacrolimus and dexamethasone on excessive cell death, in our model. Electrophoretic analysis of DNA extracted from whole lung tissue demonstrated biphasic appearances of DNA fragmentation induced by bleomycin exposure, initially occurring on day 1 and then reappearing 6 days post-bleomycin instillation, consistent with the previous report (Hagimoto et al., 1997). Interestingly, tacrolimus, but not dexamethasone, suppressed the reappearance of DNA laddering occurring on day 6, which has been reported to be confirmed to be under an apoptotic process (Hagimoto et al., 1997), whereas neither tacrolimus nor dexamethasone affected the first phase of DNA fragmentation. We found that TUNEL-positive cells in bleomycin-exposed lungs at 6 days were detected and tacrolimus inhibited the second phase of apoptosis of lung cells, which should closely be associated with the improvement of animal survival.

It has been shown that tacrolimus prevented apoptosis and caspase-3 activation caused by stimuli-induced cell death (Springer et al., 2000; Muramoto et al., 2003), suggesting that tacrolimus has a potential to suppress apoptosis, which may also account for a protective mechanism of action of tacrolimus on DNA laddering in this study. Kuwano et al. reported that apoptosis-inducing factors may greatly participate in bleomycin-induced biphasic apoptosis of epithelial cells, rather than anti-apoptotic proteins such as Bcl-2 (Kuwano et al., 2000). Nevertheless, it is still possible that anti-apoptotic proteins were involved in the inhibitory mechanisms of tacrolimus on the second phase of DNA fragmentation. However, further study is required to confirm this.

The first phase of DNA fragmentation was possibly due to a direct toxic action of bleomycin itself and the second phase may be due to an indirect one, probably caused by exaggerated inflammatory responses following the first phase of cell death. The protective action of tacrolimus on apoptosis could be partly due to its anti-inflammatory action, but its anti-inflammatory activity alone was an incomplete one. Therefore, we speculate that tacrolimus has a protective action against apoptosis, independent of its anti-inflammatory action and both types of activities of

tacrolimus may contribute to the ameliorative effects on lung injury.

Overall, the results of this study suggest both anti-inflammatory and anti-apoptotic actions of tacrolimus contribute to improvement of bleomycin-induced acute lung injury. Therapies directed against both inflammatory responses and excessive apoptosis may provide a beneficial option for clinical intervention in diseases accompanying lung injury.

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