Attenuation of bleomycin-induced pneumopathy in mice by a caspase inhibitor

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Received 17 January 2000; accepted in final form 19 September 2000

Kuwano, Kazuyoshi, Ritsuko Kunitake, Takashige Maeyama, Naoki Hagimoto, Masayuki Kawasaki, Tokujii Matsuba, Michihiro Yoshimi, Ichiro Inoshima, Koichiro Yoshida, and Nobuyuki Hara. Attenuation of bleomycin-induced pneumopathy in mice by a caspase inhibitor. Am J Physiol Lung Cell Mol Physiol 280: L316–L325, 2001.—Caspases have been implicated in the effector process of apoptosis in several systems including the Fas-Fas ligand pathway. We previously demonstrated that excessive apoptosis of lung epithelial cells and the Fas-Fas ligand pathway were essential in the pathogenesis of bleomycin-induced pneumopathy in mice. Therefore, the purpose of this study was to investigate whether a caspase inhibitor could prevent the development of this model. The expression of caspase-1 and caspase-3 was upregulated on lung epithelial cells, alveolar macrophages, and infiltrating inflammatory cells in this model. We previously demonstrated that excessive apoptosis of lung epithelial cells and the Fas-Fas ligand pathway were essential in the pathogenesis of bleomycin-induced pneumopathy in mice. Therefore, the purpose of this study was to investigate whether a caspase inhibitor could prevent the development of this model. The expression of caspase-1 and caspase-3 was upregulated on lung epithelial cells, alveolar macrophages, and infiltrating inflammatory cells in this model. We demonstrated that a broad-spectrum caspase inhibitor, N-benzylxycarbonyl-Val-Ala-Asp-fluoromethylketone, decreased the caspase-1- and caspase-3-like activity, the number of apoptotic cells, the pathological grade of lung inflammation and fibrosis, and the hydroxyproline content in lung tissues in this model. We conclude that caspase inhibitors could be a new therapeutic approach against lung injury and pulmonary fibrosis.

Apoptosis; lung injury

IDIOPATHIC PULMONARY FIBROSIS (IPF) has an aggressive course and is usually fatal, on average, 3–6 yr after the onset of symptoms. Patients commonly present with dyspnea on exertion, cough, or both. Although the etiology is unknown, it is thought that IPF begins with alveolitis. As the disease continues, the inflammation persists, and there is a gradual loss of normal lung parenchyma and interstitial fibrosis (6). Kuwano et al. (18) previously demonstrated that there was DNA damage and apoptosis in bronchiolar and alveolar epithelial cells in IPF using an in situ DNA nick end-labeling method (18). Damage to and apoptosis of epithelial cells in acute lung injury were also demonstrated (3, 11).

The administration of bleomycin has been extensively used as a model of pulmonary fibrosis. The acute pulmonary toxicity induced by bleomycin in vivo is DNA damage (13), which is known to induce apoptosis in vitro (26). Hagimoto et al. and Kuwano et al. previously demonstrated that excessive apoptosis of epithelial cells and upregulation of the Fas-Fas ligand (FasL) pathway in bleomycin-induced pneumopathy in mice (12) and the administration of a soluble form of Fas or anti-FasL antibody prevented apoptosis of epithelial cells and subsequently the development of fibrosis (17). Kuwano et al. (17) also found that Fas- or FasL-deficient mice were resistant against the induction of bleomycin-induced pulmonary fibrosis in mice. These results may indicate that excessive apoptosis of epithelial cells induced by the Fas-FasL pathway is essential in the development of pulmonary fibrosis.

One of the intracellular events required for cell death in several systems including the Fas-FasL pathway is the activation of caspases. Activation of initiator caspase-8 is triggered by ligation of death receptors through the adapter molecule Fas-associated death domain (FADD). Active caspase-8 activates effector caspases such as caspase-3. Active effector caspases mediate the cleavage of protein substrates, resulting in the morphological features of apoptosis. The tripeptide N-benzylxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK), a broad-spectrum caspase inhibitor, inhibited the intracellular activation of caspase-like proteases in vivo (4, 5, 7, 27, 30), protected mice against Fas-mediated fulminating liver destruction and death (27), prevented neuronal cell loss in acute bacterial meningitis in rabbits (4), and attenuated myocardial ischemia-reperfusion injury in rats (30). Because excessive apoptosis of lung epithelial cells induced by the Fas-FasL pathway seems to be essential in bleomycin-induced pulmonary fibrosis in mice, we examined the hypothesis that caspase inhibitors may prevent the development of this model.

MATERIALS AND METHODS

Model of bleomycin-induced pulmonary fibrosis in mice. Six-week-old ICR mice were used in this study. After measurement of their body weight, the animals were anesthe-
tized with an intraperitoneal injection of pentobarbital sodium (Dinabot, Osaka, Japan). Body weight of the mice was almost 30 g. The anesthetized animals intratracheally received 50 µl of a bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan) solution containing 1 U bleomycin/kg body wt dissolved in a sterile saline solution. After recovery from anesthesia, the mice were returned to their cages and allowed food and water ad libitum.

Inhalation of Z-VD-FMK with an ultrasonic nebulizer. Z-VD-FMK (100 µg/ml in 1% DMSO-saline; Kamiya, Seattle, WA) was inhaled by mice in the glass cage with an ultrasonic nebulizer for 30 min every day, starting 2 h after bleomycin instillation, for 14 days (group A). Because inflammatory cell infiltration into lung tissues started to occur 5 days after bleomycin instillation, Z-VD-FMK was inhaled every day starting at 5 days until 14 days (group B).

Control animals, 100 µg/ml of N-benzoyloxycarbonyl-Phe-Ala-FMK (Z-FA-FMK) (Kamiya) in 1% DMSO-saline was inhaled in the same manner as the mice in group A. The mice were anesthetized with ether 7 and 14 days after bleomycin instillation. After a thoracotomy, the pulmonary circulation was flushed with saline, and the lungs were explored. Samples of the right lung were excised to be processed for light microscopy. Samples of the left lung tissue were frozen in liquid nitrogen for the measurement of caspase activity and hydroxyproline content in lung tissues.

Histological evaluation. After death, the right lungs tissues were fixed by inflation with a buffered 10% Formalin solution for 24 h. After being embedded in paraffin, a midsagittal section of each lung was cut at 3 µm thickness and stained with hematoxylin and eosin. The pathological grade of inflammation and fibrosis in the whole area of a midsagittal section was evaluated with ×40 magnification by light microscopy. A pathological grade was analyzed blindly by one observer (R. Kunitake) who used pathological analysis. A pathological grade was determined according to the following criteria: 0 = no lung abnormality, 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.

Quantification of apoptosis in lung tissues by terminal deoxynucleotidyltransferase dUTP nick end labeling. Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) was performed with an in situ apoptosis detection kit (Takara, Ohtsu, Japan) according to the manufacturer's protocol. For histological evaluation, a midsagittal section of Formalin-fixed, paraffin-embedded lung tissue was examined by TUNEL in each mouse. The number of positive signals in the whole area of a midsagittal section was counted blindly by one observer (T. Maeyama) under light microscopy with ×400 magnification. The area (in mm²) of each midsagittal section was measured with NIH Image version 1.61 (National Institutes of Health).

Analysis of DNA fragmentation in lung tissue. The lungs 14 days after bleomycin instillation and with and without Z-VD-FMK treatment were minced with a razor blade and suspended in a mixture of 100 mM Tris·HCl (pH 8.0), 40 mM Na-EDTA, 10 mM NaCl, 1% SDS, and 500 ng/ml of proteinase K (AMRESCO, Solon, OH). The samples were incubated at 50°C overnight and then subjected to phenol-chloroform (1:1) extraction. After ethanol precipitation, the DNA was dried. The pellet was resuspended in 30 µl of 10 mM Tris·HCl (pH 7.5) and 1 mM EDTA, and DNA concentrations were ascertained with an ultraviolet spectrophotometer at 260 nm. Ten micrograms of DNA were electrophoresed on a 1% agarose gel, stained briefly with ethidium bromide, and photographed under ultraviolet transillumination.

Caspase activity assay. To confirm that the protection afforded by Z-VD-FMK inhalation was correlated with its ability to inhibit caspase activity, the activities of caspase-1 and caspase-3 were measured. Lung protein extracts were prepared by homogenization of lung tissue in a hypotonic buffer containing 25 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml each of leupeptin and aprotonin. Homogenates were centrifuged at 15,000 rpm for 10 min, and the supernatants were assayed for caspase activity with the CaspACE assay system (Promega, Madison, WI). Briefly, 100 µg of the extracted proteins were incubated with the fluorescent substrate N-acetyl-Tyr-Val-Ala-7-amino-4-methylcoumarin (Ac-YVAD-AMC) for caspase-1 or N-acetyl-Asp-Glu-Val-Asp-AMC (Ac-DEVDA-AMC) for caspase-3 at a concentration of 50 µM, 20 mM DMSO, 10 mM dithiothreitol, interleukin-1β-converting enzyme-like enzyme assay buffer (100 mM HEPES (pH 7.5), 10% sucrose, and 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfate (CHAPS)) at 30°C for 60 min. The fluorescence of the cleaved substrates was determined with a spectrofluorometer set at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The fluorescence intensity was calibrated with standard concentrations of AMC, and caspase activity is expressed in picomoles of AMC liberated per minute per microgram of protein.

Western blotting. Lung protein extracts were prepared by homogenization of lung tissue in the same hypotonic buffer as for the caspase activity assay. One hundred thirty micrograms of the extracted proteins were separated by 12% SDS-PAGE, blotted onto a nitrocellulose membrane, and blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; 10 mM Tris·HCl, pH 7.5, and 50 mM NaCl) and 0.1% Tween 20. After washes with TBS-Tween, the blots were incubated for 16 h with goat polyclonal anti-caspase-8 p20 antibody, goat polyclonal anti-caspase-1 p20 antibody, or goat polyclonal anti-caspase-3 p11 antibody at 4°C. These antibodies detect both procaspases and cleaved fragments. The blots were washed again with TBS-Tween, incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies (1: 500) for 1 h at room temperature, washed again, and developed with enhanced chemiluminescence Western blotting detection reagents (Amersham). Pictures of the membranes were taken and scanned. The images were analyzed with NIH Image version 1.61.

Hydroxyproline assay. The left lung tissues obtained from mice were used for a hydroxyproline assay. The lungs were frozen in liquid nitrogen, lyophilized with a LYPH LOCK 12 (LABCONCO, Kansas City, MO), weighed, and minced into a fine homogeneous mixture. The lung tissue was hydrolyzed in 6 N HCl for 16 h at 120°C. The hydroxyproline content of each sample was determined according to the protocols of Woessner (34).

Immunohistochemistry for caspase-1 and caspase-3. Immunohistochemistry was performed on Formalin-fixed, paraffin-embedded lung tissues. After deparaffinization, immunohistochemistry was performed with a modified streptavidin-biotinylated peroxidase technique with a Histofine SAB-PO kit from Nichirei. The sections were incubated at 4°C overnight with goat anti-mouse caspase-1 polyclonal antibody and goat anti-mouse caspase-3 polyclonal antibody, which were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These antibodies react with the caspase-1 p20 subunit and caspase-3 p20 subunit, respectively, and do not cross-react with other known cysteine proteases. The sections were rinsed with PBS and incubated with biotinylated
Fig. 1. Expression of caspase-1 and caspase-3 in bleomycin-induced pulmonary fibrosis in mice. The results of immunohistochemistry demonstrated that caspase-1 and caspase-3 proteins (Fig. 1, A and C, respectively) were barely detected in the cytoplasm of bronchiolar epithelial cells and alveolar macrophages in untreated mice, whereas caspase-1 (B) and caspase-3 (D) protein expression was detected in bronchiolar and alveolar epithelial cells, infiltrating inflammatory cells, and macrophages. The immunoreactivity was detected in both nucleus and cytoplasm.

anti-goat IgG for 30 min. For control incubations, anti-mouse antibodies were replaced by goat serum.

**RNA preparation and analysis.** Total RNA was prepared from lung tissues with the ISOGEN RNA extraction kit (Nippon Gene, Tokyo, Japan) 1 h, 6 h, 1 day, 7 days, and 14 days after bleomycin treatment. For polymerase chain reaction (PCR) analysis of RNA, cDNA was prepared by reverse transcription (RT) of 2 μg of each RNA sample in a 20-μl reaction volume containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 4 mM MgCl₂, 0.1% Triton X-100, 1 mM dithiothreitol, 0.25 mM deoxynucleotide triphosphates (dNTPs), 5 μM random hexamer primers, 0.1 U/μl of ribonuclease inhibitor (Promega), and 10 U/μl of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). The reaction mixture was incubated at 42°C for 1 h and at 95°C for 5 min. The cDNAs were then diluted to 100 μl and used in all PCRs. The PCR amplifications were performed in a 50-μl reaction volume containing 5 μl of each cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM dNTPs, and 1.25 U of Taq polymerase (Takara Biochemicals, Tokyo, Japan). The primers and probes used were β-actin sense, 5′-TCCTGTGGGATCATCATAGAAACT-3′, and antisense, 5′-CTTCGTGAACGCCACGTGCTA-3′; caspase-1 sense, 5′-GGGACCTATGTGATCTGTCTC-3′, and antisense, 5′-CAGTCAGTCCTGGAATGTGCC-3′; and caspase-3 sense, 5′-CTGACTGGAAAGCCGAAA-3′, and antisense 5′-GCAAAGGCGACTGGATGAA-3′. The conditions for amplification were 93°C for 3 min for 1 cycle; 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles; and 72°C for 7 min for 1 cycle. Cycle curve studies confirmed that for the amounts of cDNA being amplified, the reactions had not reached the plateau of the amplification curve at 30 cycles for any primer pair. Negative controls performed with no RT yielded no detectable fragments with either primer pair.

**Statistical analysis.** To determine significance, the pathological grade was compared with Mann-Whitney U-test with Abacus Concepts Statview J4.11 package. Caspase-1 and caspase-3 activities, optical density of the Western blotting, number of TUNEL-positive cells, and lung hydroxyproline content were compared with one-way ANOVA combined with post hoc testing with the Microsoft Excel version 5.0a package. P values of <0.05 were considered significant.

**RESULTS**

**Expression of caspase-1 and caspase-3 in bleomycin-induced pulmonary fibrosis in mice.** The results of immunohistochemistry demonstrated that caspase-1 and caspase-3 proteins (Fig. 1, A and C, respectively) were barely detected in the cytoplasm of bronchiolar epithelial cells and alveolar macrophages in untreated mice. In bleomycin-treated mice, both caspase-1 and caspase-3 protein expression (Fig. 1, B and D, respectively) were detected in bronchiolar and alveolar epithelial cells, lymphocytes, neutrophils, and macrophages in which immunoreactivity was detected in both the nucleus and the cytoplasm. As shown by RT-PCR analysis for caspase-1 and caspase-3 mRNAs, these expressions were upregulated in the lungs of the bleomycin-treated mice compared with those of saline-treated mice (Fig. 2).

**Effect of Z-VAD-FMK on caspase activity in lung tissues.** The activities of caspase-1 and caspase-3 in lung tissues of untreated mice were 0.24 ± 0.17 and 0.19 ± 0.09 (SD) pM·min⁻¹·μg⁻¹, respectively (n = 3). These activities were significantly increased to 1.10 ± 0.26 and 0.99 ± 0.17 pM·min⁻¹·μg⁻¹, respectively, at 7 days and 0.70 ± 0.15 and 0.61 ± 0.13 pM·min⁻¹·μg⁻¹, respectively (n = 3 mice), after bleomycin instillation. There was no significant difference in caspase-1 or caspase-3 activity between bleomycin treatment alone and bleomycin with Z-FA-FMK treatment. The activity of caspase-1 in lung tissues of mice 7 days after bleomycin instillation was significantly suppressed by inhalation of Z-VAD-FMK starting at...
2 h \( (n = 5) \) but not at 5 days \( (n = 7) \) compared with that in mice that inhaled Z-FA-FMK \( (n = 12; \text{Fig. 3B}) \). Fourteen days after bleomycin instillation, the activity of both caspase-1 and caspase-3 (Fig. 3, C and D, respectively) in lung tissues of mice was significantly suppressed by the inhalation of Z-VAD-FMK starting at 2 h \( (n = 5) \) or 5 days \( (n = 5) \) compared with that in mice that inhaled Z-FA-FMK \( (n = 8) \).

Western blot analysis. Figure 4A shows representative results of Western blot analysis for the cleaved forms of caspase-1, caspase-3, and caspase-8. These antibodies detect both procaspases and their cleaved forms. There were no differences among saline-, bleomycin-, and bleomycin plus Z-VAD-FMK-treated mice in the density of procaspases (data not shown). Quantitative analysis for the Western blot shows that the cleaved forms of these caspases was significantly increased after bleomycin treatment (Fig. 4B). The increase in the cleaved form of caspase-1 was significantly suppressed by Z-VAD-FMK treatment. The cleaved forms of caspase-3 and caspase-8 appeared to be decreased by Z-VAD-FMK treatment; however, it was not significant.

DNA fragmentation analysis on lung tissue. We previously demonstrated (12) that DNA fragmentation was detected during the acute phase from 1 to 12 h after bleomycin administration and that DNA smear-
ing was evident from day 7 to day 14. After DNA was end labeled with digoxigenin-11-dideoxy-UTP, DNA fragmentation was clearly detected both at 6 h and on day 14 after bleomycin instillation. These results show that DNA smearing covered the laddering pattern of DNA (11). We detected DNA smearing that was suppressed by Z-VAD-FMK treatment in lung tissues 14 days after bleomycin treatment. DNA extracted from saline-treated mice did not show DNA smearing (Fig. 5).

Effect of Z-VAD-FMK on the results of TUNEL. Although the type of cells was not clearly identified, some bronchiolar and alveolar epithelial cells or inflammatory cells in an inflammatory lesion showed evidence of apoptosis as estimated by the TUNEL method 7 and 14 days after bleomycin instillation (Fig. 6, F and G, respectively) but not in untreated mice (Fig. 6E). The positive signals for TUNEL at 14 days were abrogated in the lung tissues of mice treated with inhalation of Z-VAD-FMK starting 2 h or 5 days after bleomycin instillation (Fig. 6H). Figure 7 shows a summary of the TUNEL results. The number of positive signals for TUNEL in lung tissues at 7 and 14 days was significantly decreased in lung tissues of mice treated with Z-VAD-FMK starting at 2 h (n = 5 and 13, respectively) or 5 days (n = 7 and 9, respectively) compared with that in mice treated with Z-FA-FMK (n = 5 and 14, respectively).

Effect of Z-VAD-FMK on histological findings. The alveolar wall was beginning to thicken with an infiltration of neutrophils and lymphocytes 7 days after bleomycin instillation (Fig. 6B) compared with that in untreated mice (Fig. 6A). After 14 days, a large number of lymphocytes infiltrated into the lung interstitium, and thickening of alveolar septa, collapse of alveolar spaces, and proliferation of fibroblasts were observed.
(Fig. 6C). Inhalation of Z-VAD-FMK starting 2 h after bleomycin instillation abrogated the inflammatory cell infiltration and fibrosis in lung tissues at 14 days (Fig. 6D). Figure 8 shows the summary of the histological evaluation. The pathological grade at 7 days was significantly decreased in mice treated with inhalation of Z-VAD-FMK starting at 2 h ($n = 5$) but not at 5 days ($n = 7$) compared with mice treated with Z-FA-FMK ($n = 5$). The pathological grade at 14 days was significantly decreased in mice treated with inhalation of Z-VAD-FMK starting at 2 h ($n = 13$) or 5 days ($n = 9$) compared with mice treated with Z-FA-FMK ($n = 14$).

**Effect of Z-VAD-FMK on hydroxyproline content in lung tissues.** The hydroxyproline content in lung tissues of bleomycin-untreated mice was $109 \pm 24$ (SD) mg/lung ($n = 3$). Figure 9 shows the effect of Z-VAD-FMK on hydroxyproline content in lung tissues, which represents the extent of fibrosis, 14 days after bleomy-
cin instillation. There was a significant decrease in lung hydroxyproline content in mice treated with inhalation of Z-VAD-FMK starting 2 h (n = 5) or 5 days (n = 5) after bleomycin instillation compared with that in mice treated with Z-FA-FMK (n = 5). The hydroxyproline content in lung tissues of mice that inhaled Z-VAD-FMK after bleomycin instillation was almost the same as that in the lungs of bleomycin-untreated mice.

DISCUSSION

We demonstrated that the expression of caspase-1 and caspase-3 was detected in lung epithelial cells, alveolar macrophages, and infiltrating inflammatory cells after bleomycin instillation by immunohistochemistry, whereas few positive signals were localized in the cytoplasm of bronchiolar epithelial cells and alveolar macrophages in untreated mice. Caspase-1 and caspase-3 proteins were detected in the cytoplasm and nucleus of these cells after bleomycin instillation. It was reported that both caspase-1 and caspase-3 proteins, which were associated with apoptosis of neuroblastoma cells (25) and HeLa cells (22), were expressed at high levels, localizing in the nucleus as well as in the cytoplasm. Therefore, the upregulation and nuclear localization of caspase-1 and caspase-3 in these cells may reflect the activation of these caspases. We also examined caspase-1 and caspase-3 mRNAs in lung tissues with RT-PCR. Caspase-1 and caspase-3 mRNAs were not detectable in the untreated lung, whereas they were upregulated after bleomycin challenge. These results support that these caspases, at least in part, were newly synthesized.

The inhalation of Z-VAD-FMK significantly suppressed the caspase-1- and caspase-3-like activities in lung tissues 7 and 14 days after bleomycin instillation except for caspase-1 activity at 7 days in mice treated with Z-VAD-FMK starting at 5 days. In this group of mice, the pathological grade at 7 days was not decreased, whereas the caspase-3 activity and the number of TUNEL-positive cells were significantly decreased at 7 days. These results imply that caspase-1 plays a proinflammatory role rather than a role as an executioner of apoptosis.

Some caspases show overlapping specificity for some substrates [caspase-3 and caspase-7 can cleave poly-(ADP-ribose) polymerase]. A common feature of these substrates is the presence of a D-X-X-D motif. Because caspase-7 cleaves Ac-DEVD-AMC and this reaction is potently inhibited by Ac-DEVD aldehyde (Ac-DEVD-CHO), it is likely that many substrates described as being cleaved by caspase-3 will also be cleaved by caspase-7. Although DEVD-based reagents are caspase-3 specific and YVAD-based reagents are caspase-1 specific, YVAD chloromethylketone (YVAD-CMK) can inhibit caspase-3 and caspase-6 and DEVD-CHO can inhibit caspase-1 at concentrations used to inhibit caspase-3 and caspase-6 to caspase-10 (2). Caspase
activity assays with incubation of crude samples do not cleave individual caspases. Therefore, we also examined Western blot analysis for caspase-1, caspase-3, and caspase-8. These caspases were cleaved after bleomycin instillation, and the cleavage appeared to be suppressed by Z-VAD-FMK. The expression and activity of these caspases are variable from cell type to cell type. Antiapoptotic molecules may inhibit the activity of cleaved caspases. However, the suppression of cleavage of procaspase did not reach significance.

The caspase family comprises 13 cysteine proteases. The activation of caspase-3 reflects various forms of apoptosis, whereas that of caspase-1 appears to be restricted to a few apoptosis pathways (20, 28). Caspase-1 is activated by caspase-11 and plays a prominent role in inflammation through converting proinflammatory cytokines (IL-1β and pro-IL-18) to the active form (10, 32). Caspase-1-deficient mice have less IL-1β, tumor necrosis factor-α, and IL-6 (16). Macrophages and lymphocytes release a variety of inflammatory cytokines including mature IL-1β. Similar to caspase-1, other caspases have been described to have a capacity to activate IL-1β (24). Recently, Tsutsui et al. (33) demonstrated that a caspase-1-independent pathway of IL-18 secretion from FasL-stimulated macrophages, its critical involvement in FasL-induced liver injury, and the secretion of IL-18 were inhibited by caspase inhibitors. In addition to the roles in apoptosis, activated caspases may play proinflammatory functions associated with the pathophysiology of bleomycin-induced pneumopathy in mice. Therefore, the inhibitory effect of Z-VAD-FMK on the development of this model may be, at least in part, due to the anti-inflammatory functions by inhibiting broad-spectrum caspases.

Fig. 8. Effect of Z-VAD-FMK on the pathological grade in bleomycin-induced pneumopathy in mice. Each symbol represents a different mouse; n, no. of mice. The pathological grade was significantly decreased at 7 (A) and 14 (B) days by the inhalation of Z-VAD-FMK starting at 2 h or 5 days compared with that in mice treated with Z-FA-FMK except for the pathological grade of mice treated with Z-VAD-FMK starting at 5 days.

Fig. 9. Effect of Z-VAD-FMK on lung hydroxyproline content in bleomycin-induced pneumopathy in mice. Data are means ± SD; n, no. of mice. The lung hydroxyproline content at 14 days was significantly decreased by the inhalation of Z-VAD-FMK starting at 2 h or 5 days compared with that in mice treated with Z-FA-FMK.
TUNEL-positive cells were primarily detected in the core of the inflammatory lesion. Although the type of cells positively stained by TUNEL was not clearly identified, Hagimoto et al. (12) previously found that apoptotic cells were predominantly bronchial and alveolar epithelial cells using electron microscopy in this model. The number of TUNEL-positive cells at 7 and 14 days was significantly decreased by the inhalation of Z-VAD-FMK starting at 2 h or 5 days. The inhalation of Z-VAD-FMK starting at 2 h or 5 days significantly attenuated inflammation and fibrosis histologically and also decreased the hydroxyproline content in lung tissues at 14 days. These results suggest that the cell death pathway carried by caspases in epithelial cells may have important roles in the development of this model and also suggest that a caspase inhibitor given in a delayed fashion resulted in significant prevention of apoptosis.

There are two major mechanisms of activating the caspase cascade that results in apoptosis: one is receptor-mediated apoptosis and the other is chemical-induced apoptosis. Caspase-8 and caspase-9 are the most apical caspases in receptor-mediated and chemical-induced apoptosis, respectively. Caspase-8 directly activates effector caspases, which leads to biochemical and morphological apoptosis. Alternatively, caspase-8 cleaves BID, leading to the release of cytochrome c from mitochondria and the activation of caspase-9 (19, 21). Mitochondrial cytochrome c release, which subsequently leads to the activation of caspase-9, in chemical-induced apoptosis, is caspase independent in contrast to receptor-mediated apoptosis because it is not inhibited by Z-VAD-FMK (29). It was demonstrated that the primary target of Z-VAD-FMK in receptor-mediated apoptosis was inhibition of the activation of caspase-8 before any effect on mitochondria and before the activation of downstream caspases, whereas the primary target in chemical-induced apoptosis was inhibition of the processing or activity of caspase-9 (29). The epithelial cell death induced by the Fas-FasL pathway seems to be the chief mechanism of this model as Kawan et al. (17) previously described. Therefore, Z-VAD-FMK could inhibit epithelial cell death mainly by inhibiting the upstream activator caspase-8, which resulted in inhibition of the processing of downstream caspases such as caspase-1 and caspase-3 and also in the inhibition of mitochondrial cytochrome c release.

Z-FA-FMK inhibits various cysteine proteases but not caspases. Various studies (4, 8, 9, 23) used Z-FA-FMK as a negative control for Z-VAD-FMK and demonstrated that Z-FA-FMK does not inhibit caspase activity and apoptosis. Therefore, Z-FA-FMK is usually used as a negative control peptide in the study of Z-VAD-FMK. Because it is possible that Z-VAD-FMK inhibits other proteases, Z-FA-FMK seems to be a more specific negative control than no administration of peptide in the study of caspase inhibitors. Furthermore, there was no difference in the pathological grade, the number of TUNEL-positive cells, and the hydroxyproline content in the lung tissues between mice that inhaled Z-FA-FMK after bleomycin instillation and mice administered bleomycin alone. There was no pathological change or survival in mice after administration of Z-FA-FMK or Z-VAD-FMK alone. Therefore, we used Z-FA-FMK-treated mice as a negative control.

Although Fas- or FasL-deficient mice are resistant to the development of this model, some mice develop mild disease (17). Although excessive apoptosis induced by the Fas-FasL pathway may be essential in bleomycin-induced pneumopathy in mice, it seems that other death receptors or ligands, death signals such as reactive oxygen or nitrogen species and proinflammatory cytokines or chemokines are also involved in this model. In other animal models of lung injury or human diseases such as acute respiratory distress syndrome and IPF, various inflammatory mediators and death factors induce epithelial cell damage. Therefore, it is unlikely that a single treatment is sufficiently effective in patients with severe lung injury. Although the severity of lung injury is mainly dependent on the degree of inflammation and most treatment has been targeted against the inflammatory reaction, the mortality of acute respiratory distress syndrome patients or acute exacerbation of IPF remains high (14, 15). The survival and recovery of epithelial cells and the prevention of pulmonary fibrosis appear to be the key in the prognosis of patients. Because epithelial control of mesenchymal cell proliferation has been suggested, it is possible that epithelial cell loss may result in the overgrowth of mesenchymal cells after lung injury (1, 31). Therefore, protecting epithelial cells from apoptosis and maintaining their function may be effective therapeutic strategies to decrease the mortality of severe lung injury. Because it is now thought that many forms of cell death are finally dependent on caspase activation, it may be an effective treatment to block the downstream executioner of apoptosis. Furthermore, caspases may also play proinflammatory roles in this model in addition to those as an executioner of apoptosis. We speculate that a caspase inhibitor also attenuates inflammation as well as apoptosis. Although we did not think that an inflammatory reaction is necessary for lung injury, we believe that the suppression of apoptosis may lead to amelioration of lung injury that subsequently leads to a resolution of inflammation. Because we demonstrated that a caspase inhibitor could inhibit apoptosis of lung epithelial cells and prevent inflammation and pulmonary fibrosis in bleomycin-induced pneumopathy in mice, caspase inhibitors could be one of great therapeutic tools in lung injury and pulmonary fibrosis.

This work was supported by Grant-in-Aid for Scientific Research 09670620 from the Ministry of Education, Science, and Culture of Japan.

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