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Protection from Lethal Apoptosis in Lipopolysaccharide-Induced Acute Lung Injury in Mice by a Caspase Inhibitor

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LPS (lipopolysaccharide) is one of the major factors that induce acute lung injury. Recently, it was reported that LPS induced disseminated endothelial apoptosis, preceding nonendothelial tissue damage. Caspases play important roles in apoptosis, including tumor necrosis factor-α-induced apoptosis, in several systems. We therefore investigated whether the injection of a caspase inhibitor prevents LPS-induced apoptosis and acute lung injury in mice. LPS (30 mg/kg) was administered intravenously to Institute for Cancer Research mice. Electron microscopic findings demonstrated characteristic features of apoptosis in endothelial cells and alveolar epithelial cells. The caspase-3 activity and the number of terminal dUTP nick-end labeling-positive cells in lung tissues were significantly increased after LPS administration. Benzyloxycarbonil-Val-Ala-Asp fluoromethylketone (Z-VAD.fmk), which is a broad-spectrum caspase inhibitor, was injected before and after the administration of LPS. The injection of Z-VAD.fmk suppressed the caspase-3 activity in lung tissues, and significantly decreased the number of terminal dUTP nick-end labeling-positive cells. Furthermore, the survival rate of mice was prolonged significantly by the injection of Z-VAD.fmk. These results indicate that apoptosis may play an important role in acute lung injury, and thus that inhibition of caspase activity may constitute a new therapeutic approach for treatment of this disease. ***(Am J Pathol 2000, 157:597–603)***

Although there have been many studies investigating the mechanism leading to acute lung injury, the mortality rate remains high in patients with acute respiratory distress syndrome.[1,2] Sequestration of neutrophils in lung tissues, intravascular coagulation, disruption of capillary integrity leading to pulmonary edema, and increased shunt function are major characteristics of this condition.[3] Although many therapeutic approaches directed at the control of inflammatory responses, such as inflammatory cytokines,[4] adhesion molecules,[5] the compliment system,[6] and oxygen radicals,[7] have been evaluated, these approaches have neither attenuated the severity nor decreased the mortality of the disease.

The alveolar epithelium is a key structural component for gas exchange in the lung. In addition, alveolar epithelial cells synthesize, secrete, and take up the surfactant, which is a key determinant of intra-alveolar pressure. The predominant pathological finding in acute lung injury is diffuse alveolar damage.[8] The severity of lung injury is closely associated with the structural and functional deficiency of epithelial cells.[9,10] Therefore, treatments aimed at diminishing the damage to epithelial cells might become a key element in accelerating recovery and decreasing the mortality of patients with lung injury.[11]

Lipopolysacharride (LPS) is one of the major factors that induce acute lung injury. Recently, it was reported that injection of LPS induced disseminated endothelial apoptosis, preceding nonendothelial tissue damage in mice,[12,13] and that tumor necrosis factor-α and ceramide generation indicated LPS-induced endothelial cell apoptosis. Guinee et al[14] reported that apoptosis of epithelial cells was detected in diffuse alveolar damage. It was also recently reported that apoptosis of epithelial cells and the Fas/Fas ligand system plays an important role in the pathogenesis of acute respiratory distress syndrome.[15] Furthermore, apoptosis of parenchymal cells might trigger widespread organ inflammation.[16–18]

Activation of caspases is one of the intracellular events required for cell death, including tumor necrosis factor-α-induced apoptosis,.[19] The tripeptide benzyloxycarbonil-Val-Ala-Asp fluoromethylketone (Z-VAD.fmk), a broad-spectrum caspase inhibitor, has been shown to inhibit the intracellular activation of caspase-like proteases *in vitro*.[20] Because apoptosis of endothelial and epithelial cells in lung tissues is an important event in acute lung injury, caspase inhibitors could be a novel therapy for this disease via protection of the parenchymal cells.

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Materials and Methods

LPS and Caspase Inhibitor Injections

Mice used in this study were 5- to 6-week-old (20 to 22 g) ICR males (Kyudo, Tosu, Japan). Mice were injected with 30 mg/kg LPS from *E. coli* serotype O111:B4 (Difco Laboratories, Detroit, MI) through the tail vein. Z-VAD.fmk (Kamiya, Thousand Oaks, CA) was dissolved at 2 mg/ml in 1% dimethyl sulfoxide in sterile saline, and administered to mice by the method of Rodriguez et al.[21] A single intravenous injection of Z-VAD.fmk (0.25 mg) was made 15 minutes before LPS injection, followed by three intravenous injections of Z-VAD.fmk (0.1 mg each) per hour. Control mice were injected with the same volume of 1% DMSO in sterile saline.

Histological Examination

The mice were killed by exsanguination at 3, 6, 12, or 24 hours after the administration of LPS. After thoracotomy, the pulmonary circulation was flushed with saline, and the lungs were explored. After sacrifice, lung samples were inflated with 10% formalin solution instilled at 15 cm H_2O pressure through the trachea for 2 hours and fixed with buffered 10% formalin solution for 24 hours. After embedding in paraffin, samples were cut at 5-μm thickness and stained with hematoxylin and eosin (H&E).

For electron microscopy, LPS-treated mice were killed 24 hours after LPS injection, and lungs were fixed with 2.5% glutaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for 18 hours. The lungs were dissected into small pieces and postfixed for 1.5 hours in 1% OsO_4 dissolved in 0.1 mol/L phosphate buffer (pH 7.4), then dehydrated through a series of graded ethanol solutions and embedded in Epon. Ultrathin sections were cut, stained with uranyl acetate and lead nitrate, and examined under a JEM-1200 EX transmission electron microscope (Jeol, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL) Assay

Apoptosis *in vivo* was assessed by TUNEL. The lungs were fixed overnight at 4°C in 10% buffered formalin, and were embedded in paraffin. An *in situ* Apoptosis Detection Kit (Takara, Otau, Japan) was used to carry out TUNEL staining on sections of 5-μm thickness according to the manufacturer's instructions.[22] Color revelation was realized with diaminobenzidine. Ten fields at ×200 were randomly selected, and the number of TUNEL-positive cells was calculated.

Caspase Activity Analysis

The activity of caspase-1 and caspase-3 were determined using a fluorometric CaspACE Assay System (Promega, Madison, WI). In brief, lung protein extracts were prepared by the homogenization of frozen lung tissues in a hypotonic buffer (25 mmol/L Hepes, pH 7.5, 5 mmol/L $MgCl_2$, 1 mmol/L ethylen bis(oxyethylenenitrile)-tetraacetic acid (EGTA), 1 mmol/L phenylmethyl sulfonyl fluoride, 1 mg/ml leupeptin, and aprotinin). Homogenates were centrifuged (15,000 rpm, 10 minutes, 4°C), and the supernatants were used. Twenty μg of the extracted proteins were incubated with the fluorescent substrates YVAD-AMC (Ac-Tyr-Val-Ala-Asp-aminomethylcoumarin) for caspase-1 or DEVD-AMC (Ac-Asp-Gul-Val-Asp-aminomethylcoumarin) for caspase-3. The fluorescence of cleaved substrates was determined using a spectrofluorometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The caspase activity was expressed in picomoles per minute per milligram of protein.

Measurement of Myeloperoxidase (MPO) Activity

The lung tissue MPO activity was determined following a previously described method[23] with minor modifications. The lungs were removed from the thorax, blotted with gauze to remove blood, and frozen at −80°C until assay. Collected lungs were then homogenized for 30 seconds at 4°C in 1 ml phosphate-buffered saline (PBS). The corresponding extracts were centrifuged (15,000 rpm, 10 minutes, 4°C), and supernatants containing hemoglobin were discarded. The pellets were resuspended in 1 ml PBS supplemented with hexodecyl-trimethyl-ammonium bromide (HTAB, 0.5%) and ethylenediaminetetraacetic acid (5 mmol/L) and homogenized again. After centrifugation, 50 μl of each supernatant was placed in a test tube with 200 μl of PBS-HTAB-ethylenediaminetetraacetic acid, 2 ml PBS, 100 μl O-dianisidine dihydrochloride (1.25 mg/ml), and 100 μl H_2O_2 (0.4 mmol/L). The MPO activity was determined as the change in absorbance at 460 nm for 15 minutes.

Serum Interleukin (IL)-1β Levels

The concentration of IL-1β in the serum was measured using a mouse IL-1β enzyme-linked immunosorbent assay kit (Biosource International, Camarillo, CA) according to the manufacturer's protocol.

Statistical Analysis

Statistical analysis was performed by Student's *t*-test. Survival curves (Kaplan-Meier plots) were compared using a log rank test. A P value <0.05 was considered statistically significant.

Results

Apoptosis and Acute Lung Injury Induced by LPS Injection

H&E staining of lung tissue from LPS-treated mice revealed thickening of the alveolar septa and infiltration of

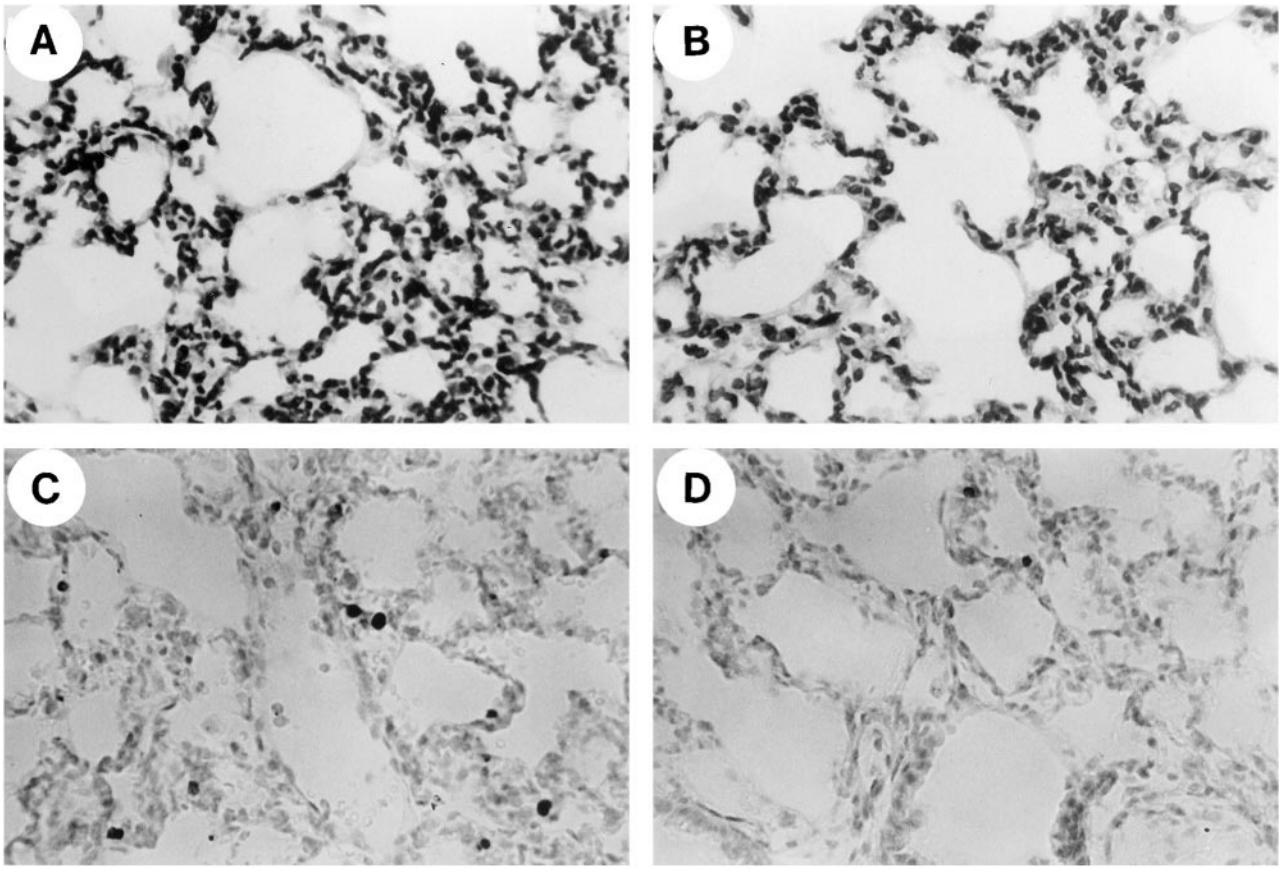


Figure 1. Histological appearance of lung tissues from LPS-administered mice with or without Z-VAD.fmk at 24 hours after LPS injection. H&E staining (**A** and **B**) **A:** The alveolar interstitium from a LPS-treated animal showed marked sequestration of inflammatory cells, which were predominantly neutrophils. **B:** Neutrophil sequestration induced by administration of LPS was not changed by the treatment with Z-VAD.fmk. Detection of cellular DNA fragmentation using the TUNEL method as described in Methods (**C** and **D**). **C:** The lung section of a mouse 24 hours after treatment with LPS showed numerous TUNEL-positive cells. **D:** A lung section that was treated with LPS plus Z-VAD.fmk showed a marked reduction in TUNEL-positive cells.

inflammatory cells at 24 hours after LPS injection (Figure 1A). The inflammatory cells in lung tissues consisted of a large number of neutrophils and some lymphocytes. These findings were consistent with those of other investigators.[24,25] Positive signals for TUNEL were found at 41.8 ± 9.8/×200 field in lung tissues 24 hours after LPS administration (Figure 1C). The number of TUNEL-positive cells was significantly ($P < 0.01$) and time-dependently increased after LPS administration (Figure 2). Electron microscopy on lung tissues isolated from mice at 24 hours after LPS administration demonstrated the condensation of chromatin and the cell shrinkage in type I and II alveolar epithelial cells, and large vacuoles in endothelial cells (Figure 3). These findings were consistent with the characteristic features of apoptosis.

The Effect of Z-VAD.fmk on LPS-Induced Acute Lung Injury

Z-VAD.fmk treatment did not affect the pathological findings on H&E staining at 24 hours of LPS-induced lung injury (Figure 1B). To access the neutrophil sequestration in lung tissues, we investigated the MPO activity in lung tissues. The MPO activity in lung tissues 24 hours after LPS administration was about eight times higher than that in control mice (control, 1.9 ± 1.2; LPS, 15.9 ± 2.2 δOD460/gram of lung). Z-VAD.fmk did not affect the MPO activity in lung tissues (LPS + Z-VAD.fmk, 13.1 ± 0.3; $P = 0.19$ compared with LPS alone). We also examined the lung weight/body weight ratio. Although the ratio was significantly elevated from 6 hours to 24 hours after LPS administration, Z-VAD.fmk treatment did not affect the lung weight/body weight ratio. However, the Z-VAD.fmk treatment significantly decreased the number of TUNEL-positive cells from 12 to 24 hours after LPS administration (LPS and LPS plus Z-VAD.fmk-treated mice at 12 hours, 1.8 ± 0.4 and 0.1 ± 0.3; at 24 hours, 41.8 ± 9.8 and 12.0 ± 2.2/×200 field, respectively) (Figures 1D and 2).

The Caspase-1 and Caspase-3 Activity in Lung Tissues

Accumulating evidence indicates that the activation of caspases is critical for many forms of apoptotic cell death. We examined whether the caspase activity was increased after LPS administration. Lung extracts were incubated with the tetrapeptide substrates YVAD-AMC or DEVD-AMC. YVAD-AMC is a preferred substrate for caspase-1 and DEVD-AMC is a preferred substrate for caspase-3. Figure 4 demonstrates the change of caspase-1 and caspase-3 ac-

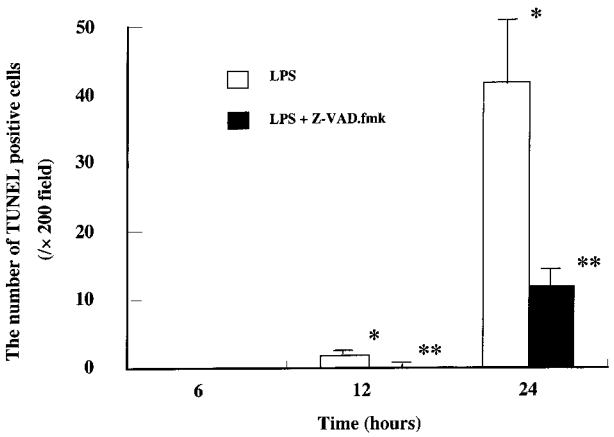


Figure 2. Quantitative analysis of apoptotic cells. TUNEL-stained lung sections were quantitated for apoptotic cells. Cells were counted in 10 random fields (×200) of two slides and expressed as the number of apoptotic cells per field. Data represent means ± SE of three animals. *, $P < 0.01$ compared with LPS alone at 6 hours; **, $P < 0.01$ compared with LPS alone.

tivity in lung tissues. The enzyme activity was assayed spectrophotometrically by measuring the extent of cleavage of the peptide substrate by the extracts. Although no change was observed in caspase-1 activity, caspase-3 activity was significantly increased in a time-dependent manner. The caspase-3 activity was about fourfold that of controls at 12 hours, and 10-fold that of controls at 24 hours after LPS administration. The Z-VAD.fmk treatment suppressed caspase-3 activity in lung tissues from 6 hours to 24 hours after LPS administration (LPS and LPS plus Z-VAD.fmk-treated mice at 6 hours, 1703 ± 399 and 941 ± 218, $P = 0.07$; at 12 hours, 3533 ± 476 and 1392 ± 207, $P = 0.02$; at 24 hours: 7601 ± 1472 and 5382 ± 819, $P = 0.18$, respectively, unit: pmol/minutes/mg protein).

Serum IL-1β Levels

To determine the effect of Z-VAD.fmk on caspase activation of pro-IL-1β, we measured the concentration of IL-1β in serum (Table 1). The concentration of IL-1β in serum from mice injected with LPS was increased from 6 to 24 hours. The Z-VAD.fmk treatment did not affect the elevation of serum IL-1β levels.

The Effect of Z-VAD.fmk on Survival

Survival data are presented in Figure 5. All mice succumbed to LPS within 30 hours. By contrast, the mice treated with Z-VAD.fmk survived significantly ($P < 0.05$) longer and 27% of the mice survived more than 7 days. Histological examination of the lungs of mice surviving at 7 days revealed normal lung architecture (data not shown).

No Effect of LPS on the Liver

We also examined liver tissues, because LPS might induce other organ failure. LPS neither induced inflammatory changes nor increased the number of TUNEL-positive cells in the liver. In addition, caspase activity in liver tissues was not affected by the administration of LPS with or without Z-VAD.fmk (data not shown).

Discussion

Apoptosis, or programmed cell death, plays a major role in cellular homeostasis, maintaining the delicate balance between cell proliferation and cell death.[26] Recent data indicate that apoptosis plays an important role in several diseases. One of the intracellular events required for cell death is the activation of caspases. In animal models, the suppression of apoptosis by caspase inhibitors might have therapeutic efficacy in hypoxia/reperfusion brain injury,[27] hypoxia/reperfusion myocardial injury,[28] sepsis-induced hepatic injury,[16] and bacterial meningitis.[29] We first demonstrated here that the suppression of apoptosis

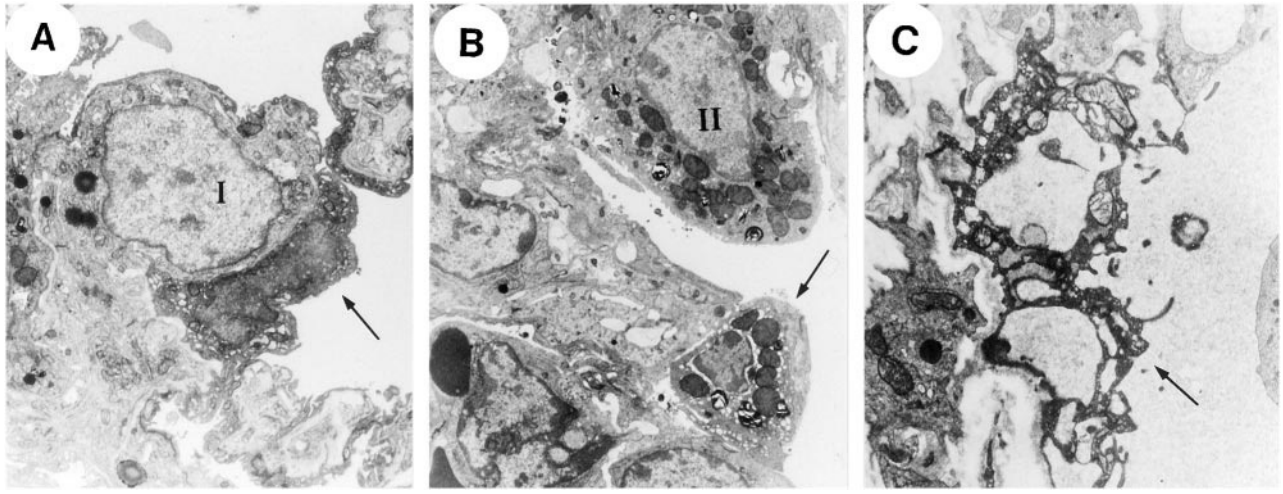


Figure 3. Electron microscopic findings on lung parenchymal cells 24 hours after injection of LPS. **Arrows** indicate the typical apoptotic change of individual cells. "I" and "II" show normal type I and II alveolar epithelial cells, respectively. **A:** Remarkable condensation of the chromatin and cell shrinkage were observed on type I alveolar epithelial cell. **B:** Cell shrinkage and detachment from the basement membrane were observed on type II alveolar epithelial cells, which also contained intact cytoplasmic organelles such as mitochondria and rough endoplasmic reticulum. **C:** Endothelial cells showed the condensation of cytoplasm and had large vacuoles.

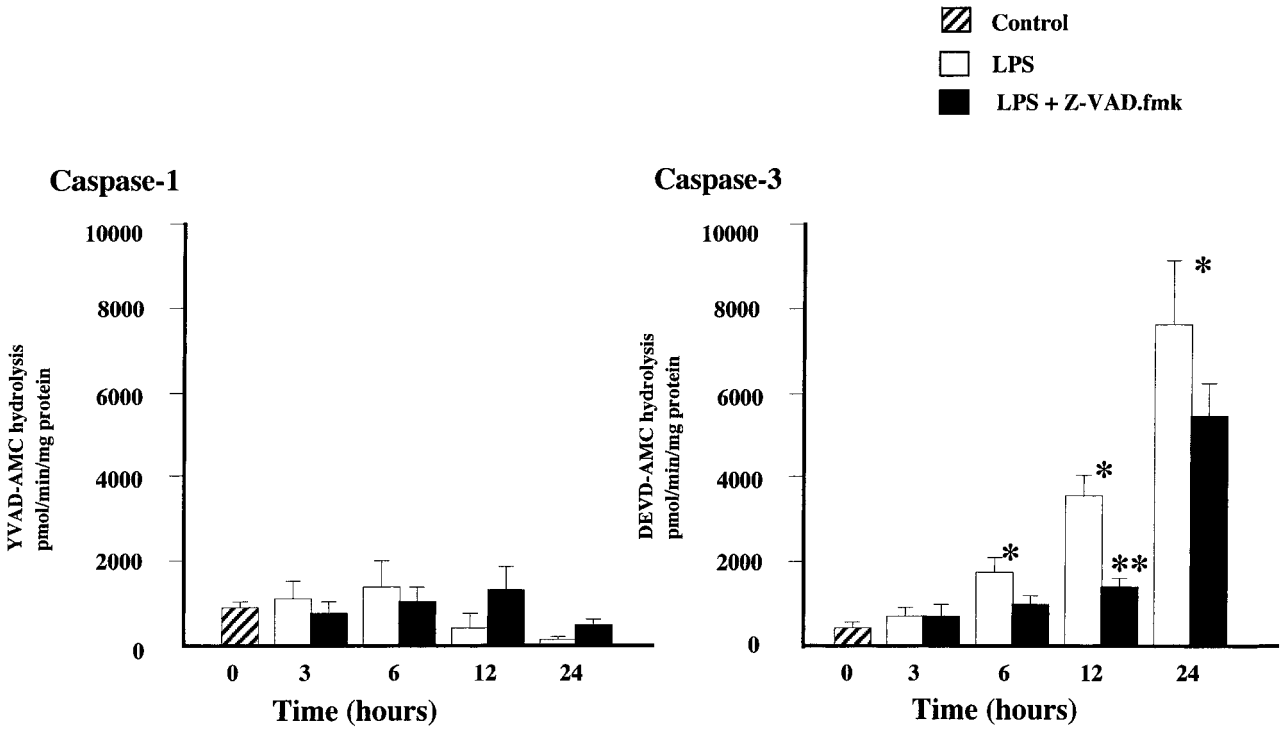


Figure 4. Change of caspase activity before and after intravenous administration of LPS with or without Z-VAD.fmk. Caspase activity was determined by measuring the cleavage assays of the fluorescent substrates YVAD-AMC for caspase-1 and DEVD-AMC for caspase-3. The caspase activity was expressed in picomoles per minute per milligram of protein. Results are means ± SE; $n = 3$ mice/group. $^*P < 0.05$ compared with the caspase-3 activity in control mice; $^{**}P < 0.05$ compared with the caspase-3 activity in mice treated with LPS alone.

using Z-VAD.fmk reduced apoptosis of endothelial and epithelial cells in the lung and improved the survival rate in a mouse model of acute lung injury.

Initially, Z-VAD.fmk was used as an inhibitor of caspase-1 (ICE: IL-1β converting enzyme)-like activity *in vitro*.[20] More recently, Z-VAD.fmk was reported to suppress broad-spectrum caspases, particularly in an *in vivo* model. Further, it has been reported that Z-VAD.fmk inhibits the caspase-3-like activity more than the caspase-1-like activity.[16] A recent study has revealed two main pathways of caspase activation.[19] In the first pathway, the activation of initiator caspase-8 is triggered by the ligation of death receptors, including Fas and tumor necrosis factor type I receptors. In the second pathway, a variety of extracellular and intracellular death stimuli trigger the release of cytochrome c from mitochondria. Cytosolic cytochrome c binds to Apaf-1 (apoptotic protease activating factor 1), and Apaf-1 promotes the activation of caspase-9. Active caspase-8 or caspase-9 activates the effector caspase-3. The active caspase-3 mediates the cleavage of apoptotic regulators, resulting in morphological features of apoptosis and demise of the cell. We have shown here that the caspase-3 activity and the number of TUNEL-positive cells in the lung dramatically increased in an LPS-induced acute lung injury model. Administration of Z-VAD.fmk *in vivo* inhibited the caspase-3 activity and prevented LPS-mediated apoptosis. Because Z-VAD.fmk inhibits the activation of caspase-3, it can prevent both pathways of caspase-mediated apoptosis. Therefore, it is considered that Z-VAD.fmk could prevent death-receptor

Table 1. Serum IL-1β Levels in Mice

Time after LPS stimulation	LPS	LPS with Z-VAD.fmk
0 hours	<20	
6 hours	29.2 ± 6.5	55.6 ± 12.5
12 hours	41.2 ± 5.0	43.2 ± 11.5
24 hours	46.0 ± 3.3	34.1 ± 18.3

Serum levels of IL-1β (pg/ml) in mice injected with LPS with or without Z-VAD.fmk. The serum from three mice of each group was pooled for estimation of IL-1β by ELISA. The data are means ± SE of two replicates from one pooled sample from each group. No significant differences were detected with or without Z-VAD.fmk. $n = 3$ mice/group.

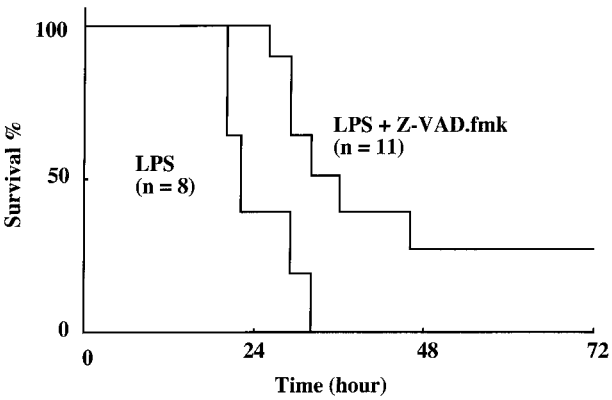


Figure 5. Survival of mice after injection of LPS with or without Z-VAD.fmk. The data shown are for the total LPS group ($n = 8$) and LPS with Z-VAD.fmk group ($n = 11$). The survival of mice treated with Z-VAD.fmk after LPS administration was significantly improved ($P < 0.05$ by log rank test) compared with that of mice treated with LPS alone.

mediated apoptosis and another apoptosis pathway triggered by a variety of stimuli, such as inflammatory cytokines, growth factors, reactive oxygen, and others, which are also involved in this model.

Caspase-1 is considered not to be important in apoptotic processes, but to be the key factor in generating the bioactive form of the proinflammatory cytokine IL-1β from its biologically inactive precursor.[30] In contrast to caspase-3, no increase in caspase-1 activity was observed in the lung tissues of LPS-administered mice, in the present study. An increase of caspase-3 activity without any change of caspase-1 activity has also been observed in LPS-stimulated liver tissues in D-galactosamine-sensitized mice.[16,31] Although caspase-1 activity was not increased in lung tissues, IL-1β concentration in the serum was increased after LPS administration. Furthermore, this increase was not suppressed by Z-VAD.fmk. It has been reported that ICE-deficient mice can still generate mature IL-1β in response to local inflammatory stimulation.[32] Recently, it was also suggested that IL-1β could be activated by other proteases, such as other caspases, matrix metalloproteinases, and proteinase 3.[33–35] Therefore, it is considered that IL-1β can be released *in vivo* in a caspase-1-independent manner without affecting Z-VAD.fmk. The lack of a significant increase of caspase-1 activity under this condition was attributed to the fact that the protective effect of Z-VAD.fmk on LPS-induced apoptosis of endothelial cells and epithelial cells was mainly mediated by the inhibition of caspase-3 activity.

Apoptosis of neutrophils is generally considered to be important in the resolution of inflammation.[36] Apoptosis provides a way to remove neutrophils from an area of inflammation with minimal damage to the surrounding tissue. It has been reported that bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome prolongs the survival of normal human neutrophils *in vitro*.[37] Initially, we expected that Z-VAD.fmk might suppress the neutrophil apoptosis, leading to the observed accumulation of neutrophils in the lung tissue. However, the number of accumulated neutrophils in lung tissues was not increased by Z-VAD.fmk treatment. Although the serum IL-1β level was not decreased by Z-VAD.fmk administration, Z-VAD.fmk inhibited epithelial and endothelial cell apoptosis without any effect on the number of neutrophils in lung tissues. It is possible that apoptosis of epithelial and endothelial cells is primarily dependent on the caspase cascade, whereas neutrophil apoptosis is not.[38] Another possibility is that Z-VAD.fmk suppresses the sequestration of neutrophils, because of decreased parenchymal cell damage, and also suppresses the apoptosis of those neutrophils that lived longer. Although we did not address the mechanism by which Z-VAD.fmk affects the function of neutrophils in acute lung injury in this study, it is considered to be important for the treatment of acute lung injury that Z-VAD.fmk does not augment neutrophil accumulation.

In a previous study using the present model, it was reported that injection of LPS induced disseminated endothelial apoptosis in the intestine, lung, fat tissue, and thymus.[12] We also reported that apoptosis of endothelial cells occurred in LPS-induced lung injury in mice.[13] In the present study, we evaluated the lung and liver tissue, because they were considered to be very important organs in sepsis. As there were no histological changes or apoptosis in the liver, apoptosis in the lung tissue was considered to be critical in this model.

In summary, we have shown that apoptotic cells and caspase-3 activity were significantly increased in the lung tissues of mice sustaining LPS-induced lung injury. Electron microscopic findings demonstrated the morphological characteristics of apoptosis on the pulmonary endothelial and epithelial cells. Apoptosis of these cells was considered to be important in lung injury because of the loss of normal functional cells. The injection of Z-VAD.fmk decreased the caspase-3 activity and the number of TUNEL-positive cells, and also improved the survival of mice. Because the survival of mice was only improved from 0 to 27% by Z-VAD.fmk treatment, it is possible that the dose of caspase inhibitor was insufficient or that factors other than apoptosis affected the survival of mice in this model. However, our results indicate that apoptosis of lung parenchymal cells may play an important role in the pathophysiology of lung injury, and that inhibition of caspase activity could be a new therapeutic approach against lung injury. As the murine endotoxin model is not always an accurate indication of responses in humans, the study might need to be repeated with a tissue model of infection.

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