



Inflammatory Response Induced in Pulmonary Embolic Lung: Evaluation Using a Reproducible Murine Pulmonary Embolism Model

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ABSTRACT

Background: To assess the pathophysiological response in pulmonary embolism, we established a novel model using a certain volume of relatively small thrombi in mice.

Methods: Thrombi with a maximum diameter of 100 μm or 500 Goudy Old Stylem were administered intravenously under anesthesia, and the survival ratio was evaluated at 4 hours. The thrombus location, hemodynamics and Computed Tomography (CT) angiography was assessed after thrombus administration. In addition, quantification of cytokine mRNAs and immunohistochemical analysis for interleukin (IL)-6 and CD68 as a macrophage marker were also performed in normal and embolized lungs at 4 hours.

Results: Mice with 100 μm clots showed a dose-dependent survival between 2.3 $\mu\text{L/g}$ and 3.0 $\mu\text{L/g}$ 4 hours after embolization. The thrombi were located at the peripheral region of the lung, which was consistent with the disruption of blood circulation. In CT angiography analysis, approximately 60% of vessels with a diameter of less than 100 μm was occluded in these mice. IL-6 and tumor necrosis factor alpha mRNA were significantly higher and lower, respectively, in embolized lungs than in normal lungs at 4 hours. In both the normal and embolized lungs, IL-6 was expressed in CD68-positive macrophages, and their numbers were comparable.

Conclusion: These results show that the pulmonary embolism model induced by a certain amount of small clot is highly reproducible and useful for evaluating pathophysiological responses in the embolized lung. Furthermore, it was found that inflammatory responses shown by IL-6 increase may contribute to the pathogenesis in early stage of pulmonary embolism.

Keywords: Pulmonary embolism; Inflammation; CT angiography; IL-6; Macrophage

INTRODUCTION

Pulmonary embolism is a disease in which a clot formed in the deep veins reaches the pulmonary artery and becomes an embolus. Various models of pulmonary embolism have been established via exogenous clot injection or endogenous clot induction by administration of coagulation factors, such as thrombin or tissue factor [1-5]. Although these models are useful for evaluating the effect of thrombolytic agents and endogenous fibrinolytic activity in dissolving pulmonary emboli [3-5], they are not suitable for assessing the pathophysiological responses of pulmonary emboli owing to the large variability in the pathogenesis associated with pulmonary emboli.

In this report, we established a novel murine pulmonary embolism model caused by injection of many relatively small pulmonary artery emboli with a fixed total volume of small clots, in which the pathogenesis was induced in a clot dose-dependent manner.

First, we studied the effect of clot size on pathological responses in the pulmonary embolism model and found that the survival rate 4 h after administration was dose independent with clots of a maximum diameter of 500 μm but was dose dependent with clots a maximum diameter of 100 μm between 2.0 $\mu\text{L/kg}$ and 3.0 $\mu\text{L/kg}$. Using the clots with diameters of up to 100 μm , we then investigated the hemodynamics and induction of proinflammatory cytokines in the lung after pulmonary embolization.

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MATERIALS AND METHODS

Animals

All animal experimental procedures were approved by the Committee on Animal Care and Use of the Nagahama Institute of Bio-Science and Technology (Permit Number: 017). Animal studies were performed in accordance with institutional and national guidelines and regulations and the ARRIVE (Animal Research: Reporting of In vivo Experiments) guidelines. Fifteen male C57BL/6J mice (CLEA Japan, Tokyo, Japan), aged 12-14 weeks and weighing 25-30 g, were used for plasma sampling. Fifty-eight female BALB/c mice (CLEA Japan), aged 12-16 weeks and weighing 20-28 g, were used for pulmonary embolism induction. The following were used for analysis: 26 for clot dose response, three for clot distribution with fluorescence-labeled clots, three for blood perfusion in the embolized lung, 14 for Computer Tomography (CT) angiography, and 12 for mRNA measurement.

Clot

Clots were obtained from mouse plasma of male C57BL/6J mice. Under anesthetization with a combination of 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol, blood taken from the heart was mixed with 10% sodium citrate for anticoagulation. After centrifugation, plasma from 15 mice was mixed and stored at -80°C until use. In the stock plasma, the fibrinogen concentration, measured using a commercial kit for humans (AssayPro, MO, USA) was 270 mg/dL. For clot formation, 100 µL of plasma was mixed with 2.5 µL of 1 M CaCl₂ (Nacalai Tesque, Kyoto, Japan) and 10 µL human thrombin (Nacalai Tesque) and incubated at 37°C for 1 h. Then, 400 µL of 4% sodium citrate was added to prevent further coagulation. The clot was dissected using small scissors and crushed using a sonicator (UD-201; Tomy, Tokyo, Japan) for a certain period of time. The size of clots was examined under a microscope. To visualize the clots, 0.67 mg/mL of human Fbg-TM488 (Invitrogen, MA, USA) was added upon clot formation.

Pulmonary embolism

Pulmonary embolism was induced via injection of clots into BALB/c mice. Mice were anesthetized with isoflurane and kept on a heat pad at 37°C. The skin of the neck was incised, and a catheter filled with clot solution was inserted into the jugular vein. A certain amount of clot solution was infused through the catheter for approximately 10 s, after which the catheter was withdrawn, and the skin was replaced and sutured. To evaluate the survival ratio, the animals were kept under isoflurane anesthesia for 4 h. If the breath stopped for more than 30 s, the animals were euthanized via intraperitoneal injection of 200 mg/kg sodium secobarbital.

Hemodynamics

To evaluate the perfusion region of the lung after clot injection, the mice were perfused with Evans blue. Under isoflurane anesthesia, the left atrium was incised and 10 mL of 4% Evans Blue in saline was perfused from the left ventricle of the heart in mice. Then, the lung vessels were ligated, and the lungs were taken and photographed.

CT imaging

The pulmonary arteries were imaged using CT angiography. Deeply anesthetized mice with sodium secobarbital were perfused with 2 mL contrast agent containing 10% titanium dioxide nanoparticle (10-100 nm diameter in size) followed by 10 mL saline (0.9% NaCl) from the right ventricle and drained into the trimmed left atrium with a 25 G winged needle at a rate of 2 mL/min, applying a 35 mmHg pressure to the cardiac wall. After perfusion, the lung was inflated by air from the trachea to a size that filled the pulmonary cavity. The lungs, heart, and ligated trachea were dissected and fixed in 10% buffered formalin for 72 h at room temperature, after which the fixed samples were analyzed using R_mCT2 (Rigaku, Tokyo, Japan). The parameters used for the CT scans were as follows: tube voltage, 90 kV; tube current, 200 µA; axial field of view, 30 mm; in-plane spatial resolution, 10 µm; and total scan time, approximately 3 min. The image data were stored in the Digital Imaging and Communications in Medicine format, and the number of branches was counted using the Aivia analysis system (version 8, DRVision, Bellevue, WA, USA) performed with the ORIENT SYSTEM, INC. (Tokyo, Japan). In this analysis, three-dimensional images were reconstructed for each animal, and all branches in the image were divided into two groups based on their diameters, namely over or below 100 µm. Then, the number of branches with diameters less than 100 µm was counted.

Quantitative Polymerase Chain Reaction (PCR)

The mRNA expression of cytokines was measured. Four hours after pulmonary embolism, the mice were euthanized via intraperitoneal injection of 200 mg/kg sodium secobarbital. The lung was taken, frozen in liquid nitrogen, and stored at -80°C until measurement. The lung was mixed with TRIzol (Nacalai Tesque) and homogenized using a bead homogenizer (MS-100, Tomy). Total RNA was isolated using an RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed using a cDNA Reverse Transcription Kit (Toyobo, Tokyo, Japan). Quantitative real-time PCR was performed using TB Green Fast qPCR Mix (Takara Bio Inc., Kusatsu, Japan) using a PCR system (Roche, Basel, Switzerland). The PCR primers used are listed in Table 1. The specific mRNA amplification of the target was determined as the Ct value followed by normalization to the GAPDH mRNA level. The value is represented by the ratio against that in the control lung.

Table 1: PCR primers.

Primers	Sequences
GAPDH forward	5'-TTTGATGTTAGTGGGGTCTCT-3'
GAPDH reverse	5'-AGCTTGTCATCAACGGGAAG-3'
IL-6 forward	5'-TTCCATCCAGTTGCCTTCT-3'
IL-6 reverse	5'-CAGAATTGCCATTGCACAAC-3'
TNF-α forward	5'-TTGAGATCCATGCCGTTG-3'
TNF-α reverse	5'-CTGTAGCCCACGTCGTAGC-3'
IL-1β forward	5'-AGCTGGATGCTCTCATCAGG-3'
IL-1β reverse	5'-AGTTGACGGACCCAAAAG-3'
IL-8 forward	5'-CAAGGCTGGTCCATGCTCC-3'
IL-8 reverse	5'-TCGTATCACTTCCTTTCTGTTGC-3'

Immunohistochemistry

Four hours after the induction of pulmonary embolism, the mice were perfused with 4% paraformaldehyde under sebo-barbital anesthesia. Their lungs were removed, embedded in paraffin, and sliced into 5 mm thick sections, which were then immunostained as described previously [6]. Briefly, the sections and adjacent sections were treated with anti-interleukin (IL)-6 and anti-CD68, which is a macrophage marker. After treatment with the appropriate secondary antibody conjugated with peroxidase, immunoreactivity was visualized by diaminobenzidine coloration. The sections were then counterstained with hematoxylin. Microphotographs were taken under a microscope.

Statistical analysis

Statistical analysis was performed using Student's *t*-test. Statistical significance was set at $p < 0.05$.

RESULTS

Effect of clot size and clot dose on survival ratio

First, we studied the clot-dose response on the survival ratio by using clots of two different sizes. When clots with a maximum size of approximately 500 μm were used, the survival ratio was not associated with clot dose. In contrast, when clots with a maximum size of approximately 100 μm were used, the survival ratio was associated with clot dose, that is, the survival ratio increased in a dose-dependent manner from 2.3 $\mu\text{L/g}$ to 3.0 $\mu\text{L/g}$ (Table 2). Based on these results, we chose clots with a maximum size of approximately 100 μm for subsequent experiments.

Clot distribution in the embolized lung

In the embolized lung with a 2.5 $\mu\text{L/g}$ clot solution mixed with human Fbg-TM488, several fluorescent signals were observed, which were distributed relatively near the edges of the lung leaves (Figure 1).

Perfused region in the embolized lung

In normal lungs, the whole tissue showed positive staining with Evans blue (Figure 2A), indicating that blood perfusion was normally maintained in all areas of the lung. However, in lungs with pulmonary embolization of 2.5 $\mu\text{L/g}$ clot solution, the edges of the major lung leaves with the whole area of the right subleaf were not perfused (Figure 2B). Similar results were observed in the other two animals either without or with pulmonary embolization (data not shown).

CT angiography

We visualized the vessels with diameters of approximately 50 μm using the titanium contrast. The number of vessels was less in the embolized lung with a 2.5 $\mu\text{L/g}$ clot solution (Figure 3A) than in normal lung (Figure 3B). In addition, the vessel branch of the right subleaf was shorter compared to the normal lung (Figures 3A and 3B). The number of vessel branches with diameters less than 100 μm was significantly lower in the embolized lung than in the normal lung (Figure 3C).

mRNA expression of cytokines

To evaluate the effect of embolism on gene expression related to hypoxia response and cytokines, we measured the mRNA levels of IL-1 β , IL-6, IL-8, and tumor necrosis factor alpha (TNF- α) as shown in Figure 4. Four hours after clot injection, the mRNA levels of IL-1 and IL-8 were comparable in both the normal and embolized lungs. However, the mRNA level of IL-6 increased while that of TNF- α decreased, both of which were significant.

Immunohistochemistry of IL-6 and CD68

In the immunohistochemical analysis of IL-6, positive cells were observed in both the normal and embolized lungs (Figures 5A and 5B). In the immunohistochemical analysis of CD68, a marker of macrophages, showed positive cells distributed consistently with IL-6 immunostained cells in adjacent sections (Figures 5C and 5D). The numbers of both IL-6 (Figure 5E) and CD68 (Figure 5F) immunostained cells were comparable between the normal and embolized lungs.

Table 2: Clot dose response.

Dose (mL/g)	Clot size: max 500 mm			Dose (mL/g)	Clot size: max 100 mm		
	Live	Dead	% Live		Live	Dead	% Live
2.0	2	2	50	2.3	3	0	100
2.3	1	3	25	2.5	3	1	75
3.0	2	2	50	2.8	1	2	33
				3.0	1	3	25

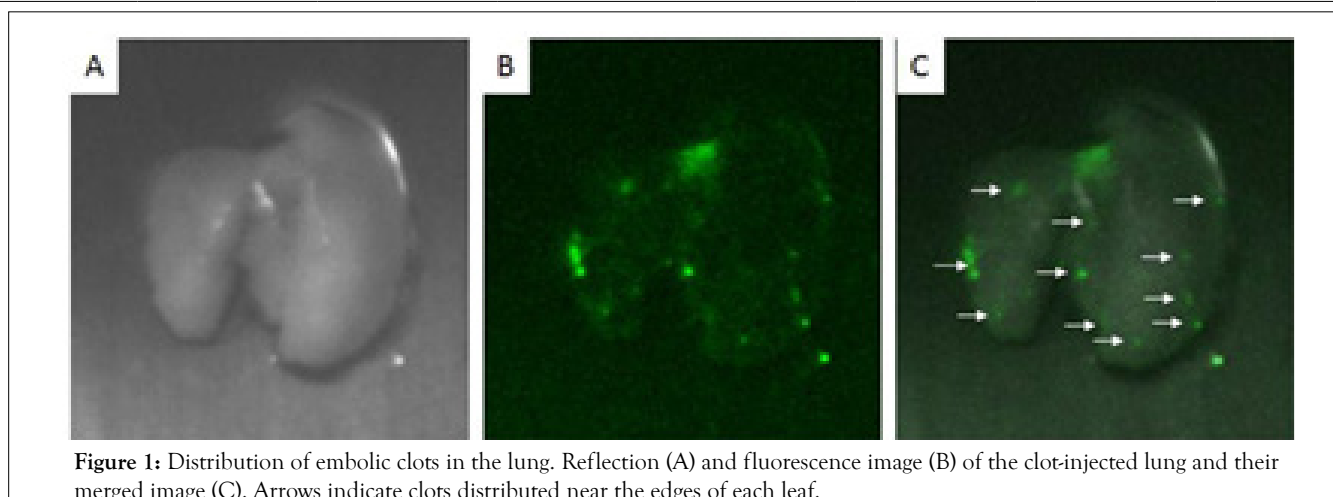


Figure 1: Distribution of embolic clots in the lung. Reflection (A) and fluorescence image (B) of the clot-injected lung and their merged image (C). Arrows indicate clots distributed near the edges of each leaf.

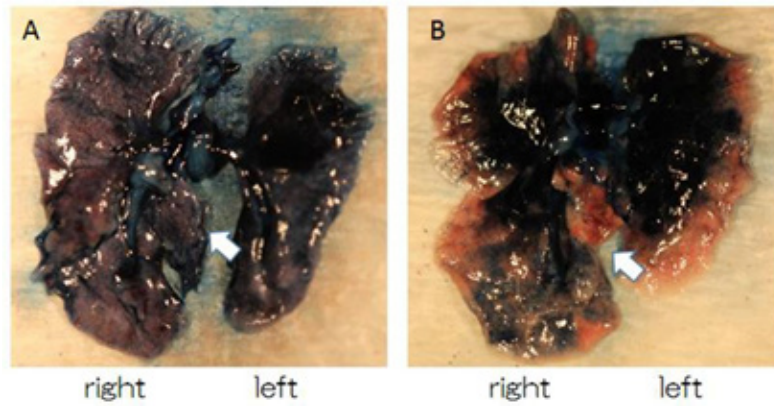


Figure 2: Perfusion in the lung. Photographs of lungs without (A) and with clot-injection (B) after perfusion of Evans blue from the right ventricle to the left atrium. Arrows indicate the right subleaf. The non-perfusion area was widely distributed at the edge of all lung leaves in clot-injected mice.

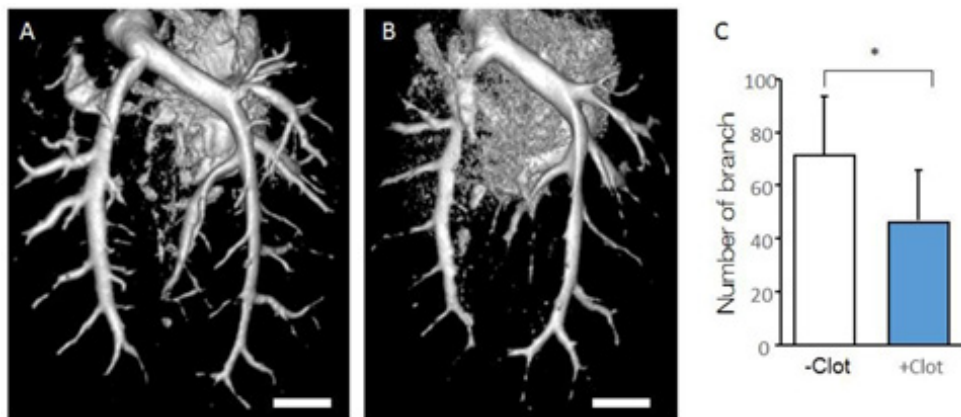


Figure 3: CT angiography of the lung. CT angiography of lungs from mice without (A) and with clot injection (B). Number of branches with diameters less than 100 μm (C). Each group consisted of seven mice. Data represent the mean ± standard deviation (SD). Note: *p<0.05.

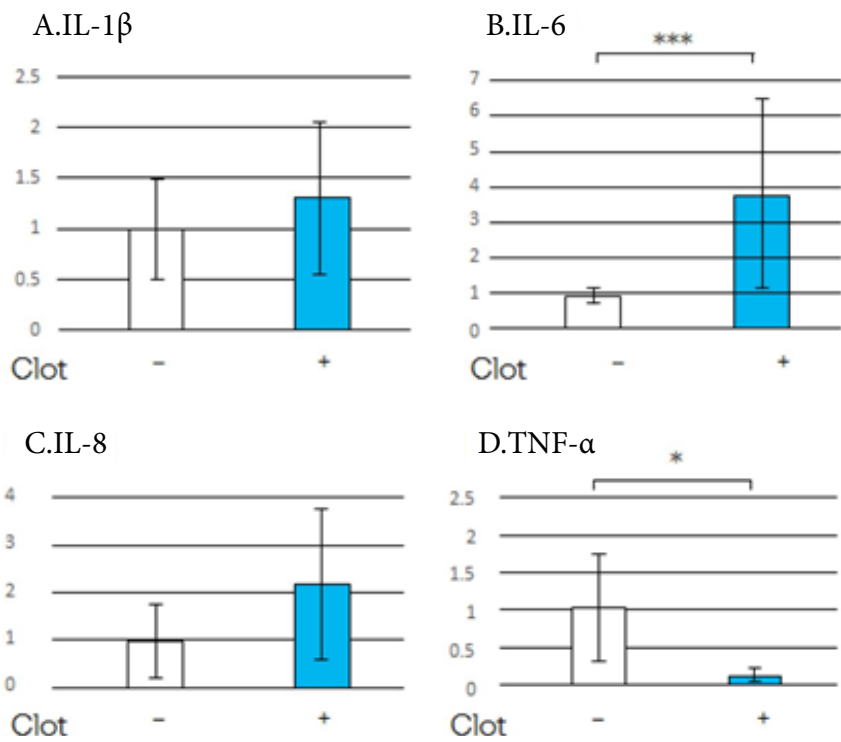


Figure 4: Effect of clot injection on mRNA expression of cytokines. mRNA levels of *IL-1β* (A), *IL-6* (B), *IL-8* (C), and *TNF-α* (D) 4 h after clot injection. Data represent the mean ± SD of relative values against mice without clot injection (-Clot). Each group consisted of six mice. *:p<0.05, ***:p<0.001.

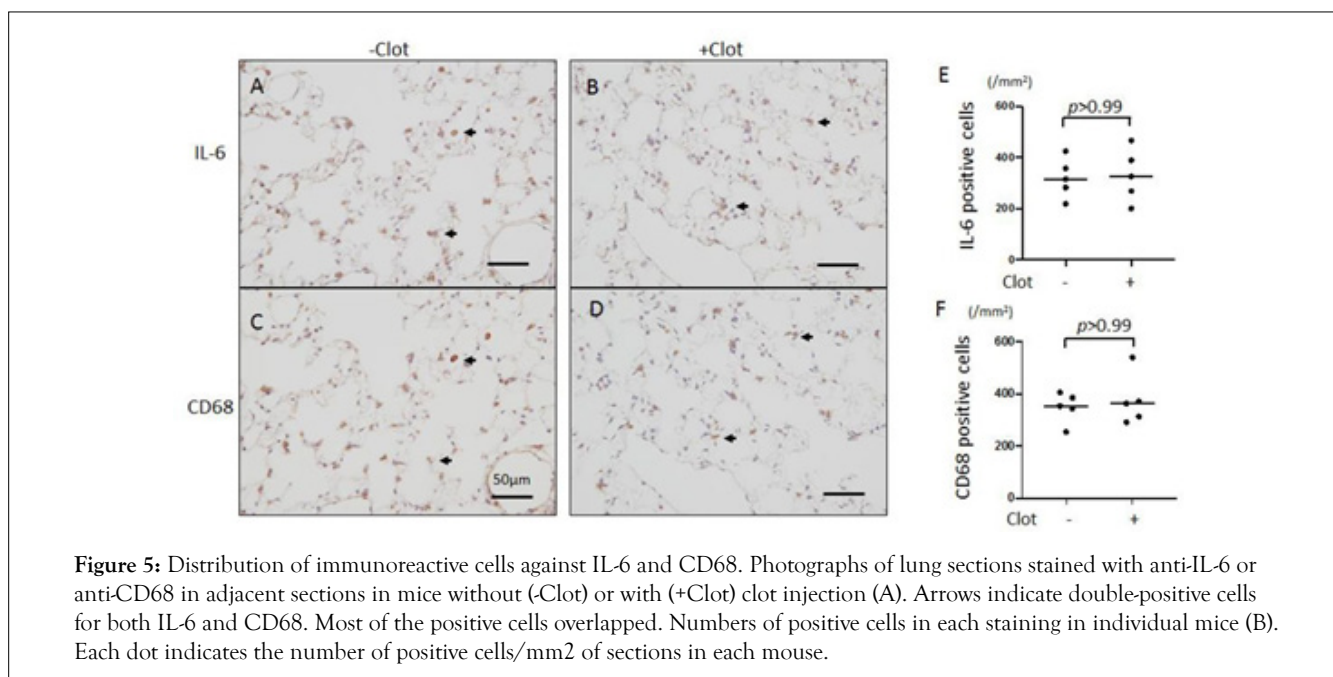


Figure 5: Distribution of immunoreactive cells against IL-6 and CD68. Photographs of lung sections stained with anti-IL-6 or anti-CD68 in adjacent sections in mice without (-Clot) or with (+Clot) clot injection (A). Arrows indicate double-positive cells for both IL-6 and CD68. Most of the positive cells overlapped. Numbers of positive cells in each staining in individual mice (B). Each dot indicates the number of positive cells/mm² of sections in each mouse.

DISCUSSION

In this study, we established a pulmonary embolism model by administering a fixed amount of small clots, in which the survival rate was reproducible and increased in a dose-dependent manner. Using this model, we successfully maintained the animals with pulmonary embolism with near maximum ischemic damage, and measured the expression of cytokine mRNAs 4 h after pulmonary embolism. The results demonstrate the usefulness of this model for analyzing pathophysiological responses after pulmonary embolism.

Several pulmonary embolism models have been established, in which embolization was induced by administration of a couple of large clots or generated clots in the body [1-5]. These models are useful for evaluating the effects of thrombolytic agents and endogenous fibrinolytic activity in dissolving pulmonary emboli. However, since their ischemic tendency varies greatly, they are not useful for the analysis of the pathological responses after pulmonary embolism. In the present study, we also found that there was no correlation between survival rate and dose in pulmonary embolism caused by relatively large clots with a maximum diameter of 500 μm . In contrast, for pulmonary embolism caused by clots with a maximum diameter of 100 μm , the survival rate decreased in a dose-dependent manner between 2.3 $\mu\text{L/g}$ and 3.0 $\mu\text{L/g}$. This result suggests that the pathophysiological effects induced by clots with a maximum diameter of 100 μm is reproducible, and therefore, the clots with this size should be used in order to investigate the quantitative of pulmonary embolization. Since oxygen and nutrients are supplied to the lung tissue via the bronchial arteries, obstruction of the pulmonary arteries does not affect blood supply to the lungs. Therefore, the effects of ischemia in the lung caused by pulmonary emboli are expected to depend on the volume of the occluded region of the lung rather than the location of the pulmonary embolus. In addition, since the downstream area of the large branches of the pulmonary artery varies per branch, the size of the occluded area of the lung will also vary depending on the branch being occluded by several large thrombi; therefore, the survival ratio will be independent of the total volume of clot. However, in the case of pulmonary embolism caused by many small thrombi, the number of occluded small branches increases depending on the amount of clot, resulting in an increase in the

occlusion volume and a decrease in the survival rate.

To characterize the pulmonary embolization by a clot with a maximum diameter of 100 μm , the distribution of clot, blood perfusion area in the lung, and CT angiography were performed. Several thrombi were found to be localized in the marginal region of the lung lobes. Blood perfusion in the lung lobes was also inhibited in the marginal region, which was consistent with the distribution of thrombi. Furthermore, the number of vessel branches with diameters less than 100 μm was significantly decreased in the embolized lung to approximately 60% of that in the normal lung when 2.5 $\mu\text{L/g}$ of clot was administered. Considering that the survival rate with 2.5 $\mu\text{L/g}$ of clot was 75%, it is suggested that the survival rate decreases when the number of vessel branches with diameters less than 100 μm falls below 60%.

It was found that the mRNA expression level of IL-6 was significantly increased. IL-6 is a cytokine that plays an important role in inflammatory reactions, which induces chemokine production in various cells and enhances the expression of adhesion molecules in vascular endothelial cells. In the lung, it has been shown to be increased significantly during the acute inflammatory response, including bronchoscopic alveolar lavage and acute pneumonia caused by lipopolysaccharide within 3 h [7,8], which are thought to exacerbate the lung damage. In addition, it has been reported that ischemia also increases IL-6 in the brain, heart, and kidney in the early stages [9-11], indicating that IL-6 contributes to the acute inflammatory response associated with ischemia. The current finding that IL-6 mRNA was upregulated in the embolized lung also suggests that IL-6 is expressed in the embolized lung and may be involved in the pathogenic response in the acute phase of pulmonary embolism. Immunohistological studies revealed that macrophages expressed IL-6, which was increased in the lungs 4 h after pulmonary embolization. IL-6 induction in alveolar macrophages has also been reported in systemic sclerosis and pneumonia [12,13], being involved in these pathogenesis. In contrast, it was found that the number of both IL-6 immunoreactive cells and macrophages in the lung was similar to that in the normal lung 4 h after pulmonary embolization. These results suggest that the increase in IL-6 mRNA levels may not be due to an increase in the number of macrophages expressing IL-6, but increase in

the level of IL-6 in individual macrophage. The function of IL-6 in pathogenesis varies depending on the organ and disease; IL-6 exacerbates the pathology in the acute phase of cerebral infarction [14], whereas it plays a protective role in renal ischemia-reperfusion injury [15]. The contribution of IL-6 to the pathogenesis of pulmonary embolism is subject for further investigation.

We also found that TNF- α mRNA levels were significantly lower in embolized lungs than in normal lungs. It is known that TNF- α is mainly expressed in macrophages and is upregulated by hypoxia [16,17]. Since alveolar macrophages have a higher TNF- α production capacity than peripheral blood monocytes in human alveolar macrophages are thought to express relatively high levels of TNF- α even under normal conditions [18]. Therefore, the decrease in TNF- α in the embolized lung may be due to the effects of pulmonary embolization other than hypoxia on alveolar macrophage function. The pathophysiological role of the decrease in TNF- α expression in the acute phase of embolized lungs is also subject for further investigation.

CONCLUSION

We established a novel murine pulmonary embolism model using relatively small clots, in which pathological damage was reproducible and the survival ratio was clot dose dependent. Using this model, it was shown that inflammatory responses associated with an increase in IL-6 mRNA expression and a decrease in TNF- α mRNA expression was induced in the embolized lung 4 h after induction of embolism.

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