

Protective Effects of Imatinib on
Ischemia/Reperfusion Injury in Rat Lung

(イマチニブの肺虚血再灌流障害
に対する保護効果)

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Background. Ischemia/reperfusion injury (IRI) remains a significant complication after lung transplantation. Endothelial damage and inflammation contribute to its development. Imatinib has been reported to regulate vascular permeability by maintaining endothelial junctions and showing antiinflammatory effects through inhibition of the Abl kinases. We hypothesized that imatinib could have a protective effect against IRI.

Methods. Male Lewis rats were heparinized and underwent left thoracotomy, and the left hilum was clamped for 90 minutes followed by reperfusion for 120 minutes. Imatinib mesylate (50 mg/kg) and a solvent were administered intraperitoneally 20 minutes before ischemia in the imatinib group and the vehicle group, respectively ($n = 7$ in each group). After reperfusion, lung function, lung wet to dry weight (W/D) ratio, and histologic findings were obtained. The expression of vascular endothelial cadherin (VEC), the phosphorylation level of CrkL (pCrkL) (an exclusive target of Abl kinases),

and the cytokine level were evaluated using lung tissue lysate. The imatinib concentrations of plasma and lungs after reperfusion were measured in this hilar clamp model ($n = 7$).

Results. In the imatinib group, lung function was improved with a lower W/D ratio. Perivascular edema and neutrophil infiltration were ameliorated. The imatinib group demonstrated maintained expression of VEC, inhibition of pCrkL, and a significantly higher level of interleukin (IL)-10. The imatinib concentration in both lungs showed a strong correlation with plasma concentration.

Conclusions. In a rat IRI model, imatinib attenuated lung injury by an antipermeability and antiinflammatory effect. The delivery and function of imatinib in the lung was also confirmed in this model.

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Lung transplantation has been established as a life-saving procedure for patients with end-stage respiratory failure, and the number of operations has been increasing [1–3]. Perioperative management including organ preservation has been developed [4–7]; however, primary graft dysfunction, caused initially by pulmonary ischemia/reperfusion injury (IRI), remains a significant problem [2, 7]. Endothelial damage during ischemia and reperfusion contributes to the development of IRI [4, 6, 7]. In lung transplantation, it has become not only the cause of postoperative morbidity and mortality but also a risk factor for chronic rejection [6–8]. In addition, IRI is also a significant complication after trauma, cardiopulmonary bypass, cardiopulmonary resuscitation, and intervention for chronic thromboembolic pulmonary hypertension [9, 10]. Treatment strategies are generally empirical or supportive and are applied in patients with acute respiratory distress syndrome and include lung-protective

ventilation strategies; avoidance of excess fluid administration; administration of steroids, nitric oxide, and prostacyclin; and extracorporeal membrane oxygenation support [5–7]. A novel therapeutic strategy is needed.

Imatinib, 1 of the tyrosine kinase inhibitors, has recently been reported to attenuate vascular leakage [11]. Imatinib was developed as a molecular targeting therapeutic drug for patients with Bcr-Abl-positive chronic myelogenous leukemia. It has also been used in the management of gastrointestinal stromal tumor and some other types of leukemia [12, 13]. Its targets include Abl, Abl-related gene (Arg), platelet-derived growth factor receptors (PDGFRs), cKit, and discoidin domain receptor tyrosine kinase 1 [12]. Abl and Arg are nonreceptor tyrosine kinases, known to regulate cytoskeletal dynamics, adhesion, migration downstream of receptor tyrosine kinases, cadherin, and integrin [14]. Recent reports have shown that imatinib prevented edema formation under permeability-inducing conditions [11]. Against permeability-inducing factors such as thrombin, histamine, vascular endothelial growth factor, lipopolysaccharide (LPS), and oxidative stress [15–18], Abl/Arg inhibition protected the endothelium by maintaining vascular endothelial cadherin (VEC) specific for the

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endothelial adherens junction [19], as well as activating antiinflammatory signals [11, 15-17].

Considering the reported antipermeability and antiinflammatory effects of imatinib, it was suggested that imatinib could be a novel treatment for IRI; however, to date it has not been evaluated in an in vivo model. This study was aimed at testing the hypothesis that imatinib could have a protective effect against IRI by using a rat model.

Material and Methods

Animals

Male Lewis rats weighing 270 to 320 g (Japan SLC, Hamamatsu, Japan) were used in the experiments. The experimental protocol was approved by the Ethical Committee of the Graduate School of Medicine at Kyoto University, Japan. All animals received humane care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals, prepared by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

Experiment 1

RAT HILAR CLAMP MODEL EVALUATING IRI. Each rat was anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and was then tracheotomized and intubated with a plastic catheter and mechanically ventilated (Model SN-480-7, Shinano Seisakusyo, Tokyo, Japan). The fraction of inspired oxygen was kept at 1.0 and positive end-expiratory pressure was maintained at 2 cm H₂O throughout the procedure. In bilateral lung ventilation, tidal volume (TV) and respiratory rate (RR) were set to 10 mL/kg and 70 breaths/min, respectively. Heparin (50 IU) was injected through the jugular vein, and atropine (0.25 mg) was administered intramuscularly. Imatinib mesylate (50 mg/kg) dissolved with 0.5 mL of 20% dimethyl sulfoxide was injected intraperitoneally in the imatinib group (n = 7), and 0.5 mL of 20% dimethyl sulfoxide without imatinib was administered in the vehicle group (n = 7). We preliminarily tested the dose of 25 mg/kg, and it produced a little improvement in lung function without statistical significance (data not shown). The dose of 50 mg/kg and intraperitoneal administration were adopted based on this result and past reports [15, 16]. The animals underwent left thoracotomy, and the left hilum was occluded with a small metallic clamp. The occlusion was performed 20 minutes after imatinib or vehicle administration. During clamping, the TV and RR were adjusted to 8 mL/kg and 80 breaths/min, respectively. After 90 minutes of ischemia, the clamp was removed and reperfusion was maintained for 120 minutes. During reperfusion, blood flow and ventilation were restored in the bilateral lung. In the sham group (n = 6), the animals were heparinized, thoracotomized, and ventilated for 210 minutes.

ARTERIAL BLOOD GAS ANALYSIS AND LUNG MECHANICS MEASUREMENTS. After 120 minutes of reperfusion, a median sternotomy was performed to occlude the right hilum for the

evaluation of the left injured lung. TV and RR were set to 6 mL/kg and 100 breaths/min, respectively. Three minutes after occlusion of the right hilum (including the accessory lobe), a blood sample was obtained through the ascending aorta for arterial blood gas analysis. The animals were then connected to a rodent ventilator (flex-iVent, SCIREQ, Montreal, Quebec, Canada). After a stabilizing period, airway pressure and dynamic compliance were measured [20].

LUNG WET TO DRY WEIGHT RATIO. The upper part of the left lung was used to calculate the lung wet to dry weight (W/D) ratio. The wet weight was measured soon after the harvest, and the dry weight was measured after the specimen had been dried overnight at 100°C. The ratio was calculated as wet weight divided by dry weight.

HISTOLOGIC ANALYSIS. The middle part of the left lung was fixed in 10% formalin and stained with hematoxylin-eosin. Naphthol AS-D chloroacetate esterase stain was used for counting neutrophils infiltrating into the lung, as previously reported [21]. Three separate investigators (ST, KO, and TK) performed the evaluation in a blinded manner. The number of neutrophils was expressed by the average number of extravascular neutrophils in 4 randomly chosen high-power fields (HPFs) per section at a magnification of $\times 400$.

ENZYME-LINKED IMMUNOSORBENT ASSAY. The same quantities of the lower part of the left lung (30 mg) were homogenized with 500 μ L of phosphate-buffered saline with protease inhibitors (Wako, Osaka, Japan) and then centrifuged ($13,000 \times g$ for 10 minutes) to obtain lung tissue lysates for enzyme-linked immunoassay (ELISA). The concentrations of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , CXCL1, IL-6, and IL-10 in lung tissue lysates were measured using an ELISA kit according to the manufacturer's instructions.

WESTERN BLOT ANALYSIS. The lower part of the left lung, which had been stored at -80°C , was homogenized in a radioimmunoprecipitation assay buffer containing a cocktail of protease and phosphatase inhibitors (Nacalai Tesque, Kyoto, Japan) and centrifuged ($13,000 \times g$ for 10 minutes). Tissue lysates were obtained and the protein concentration was determined by a Bradford protein assay. The protein samples (15 μ g) were loaded on sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with primary and secondary antibodies using the standard procedure. The blots were then investigated with a chemiluminescence agent. The same membrane was used for repeated probing for nonphosphorylated molecules or β -actin. The procedures were performed in triplicate, and the image was analyzed by densitometry (CS Analyzer 3, ATTO, Tokyo, Japan) to calculate the relative intensity of the band.

Experiment 2

RAT HILAR CLAMP MODEL EVALUATING CONCENTRATION OF IMATINIB. Imatinib administration (50 mg/kg), 90-minute clamping of the left hilum, and 120 minutes of reperfusion were performed as described in experiment 1 (n = 7).

To confirm the concentration of imatinib in this model, a blood sample was collected through the inferior vena cava, and the lungs were then flushed with 10 mL of normal saline through the pulmonary artery after reperfusion. Rat plasma and lung homogenate samples were diluted 10 and 200 times with saline, respectively. After a roscovitine solution was added to the diluted samples (100 ng/mL final concentration), samples were deproteinized with a double volume of acetonitrile. After centrifugation ($20,238 \times g$ for 15 minutes), the upper clear solution was diluted with an equal amount of 0.2% formic acid and filtered thereafter (Cosmonice Filter S (Solvent) 4 mm, Nacalai Tesque, Kyoto, Japan). The high-performance liquid chromatography system was an Eksigent Technologies ultra performance liquid chromatograph (AB SCIEX, Framingham, MA). Separation was carried out using an Inertsil ODS-3 (GL Sciences, Tokyo, Japan) with a mobile phase consisting of 0.1% formic acid and acetonitrile containing 0.1% formic acid at a flow rate of 0.2 mL/min using gradient separation. The injection volume of the sample was 1 μ L. Imatinib (m/z 494.171 $\rightarrow$ 393.900) and roscovitine (internal standard, m/z 355.047 $\rightarrow$ 233.100) were monitored with a QTRAP4500 triple-quadrupole tandem mass spectrometer (AB SCIEX, Framingham, MA).

Reagents and Antibodies

Imatinib mesylate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Antibodies used in this experiment included phospho-CrkL (Tyr207), phospho-Src (Tyr416), CrkL (32H4), and Src (L4A1) from Cell Signaling Technology (Beverly, MA); VEC (C-19)

from Santa Cruz Biotechnology (Dallas, TX); and β -actin (A5441) from Sigma-Aldrich (St. Louis, MO). Rat ELISA kits used in this experiment included TNF- α , IL-1 β , and CXCL1 from R&D Systems (Minneapolis, MN), and IL-6 and IL-10 from RayBiotech (Norcross, GA).

Statistical Analysis

Data were presented as the mean $\pm$ SE of the mean. Statistical analysis was performed by JMP Pro 11.2.0 (SAS Institute, Cary, NC). One-way analysis of variance followed by Tukey-Kramer HSD tests and the Student's *t* test were used for comparing 3 groups and 2 groups, respectively. Pearson's coefficient of correlation described relationships between variables. The differences were considered to be significant when the *p* value was less than 0.05.

Results

The Effect of Imatinib on Lung Function

Maximum and mean airway pressures were lower and dynamic compliance was higher in the imatinib group than in the vehicle group ($p < 0.01$, $p < 0.01$, and $p = 0.02$, respectively) (Figs 1A–1C). The imatinib group also showed better lung oxygenation ($p = 0.01$) (Fig 1D).

Effect of Imatinib on Lung Edema and Neutrophil Infiltration

On histologic examination, imatinib administration prevented the formation of perivascular edema and hemorrhage (Figs 2A–2C). Lung edema evaluated by W/D ratio was ameliorated in the imatinib group ($p = 0.01$) (Fig 2D).

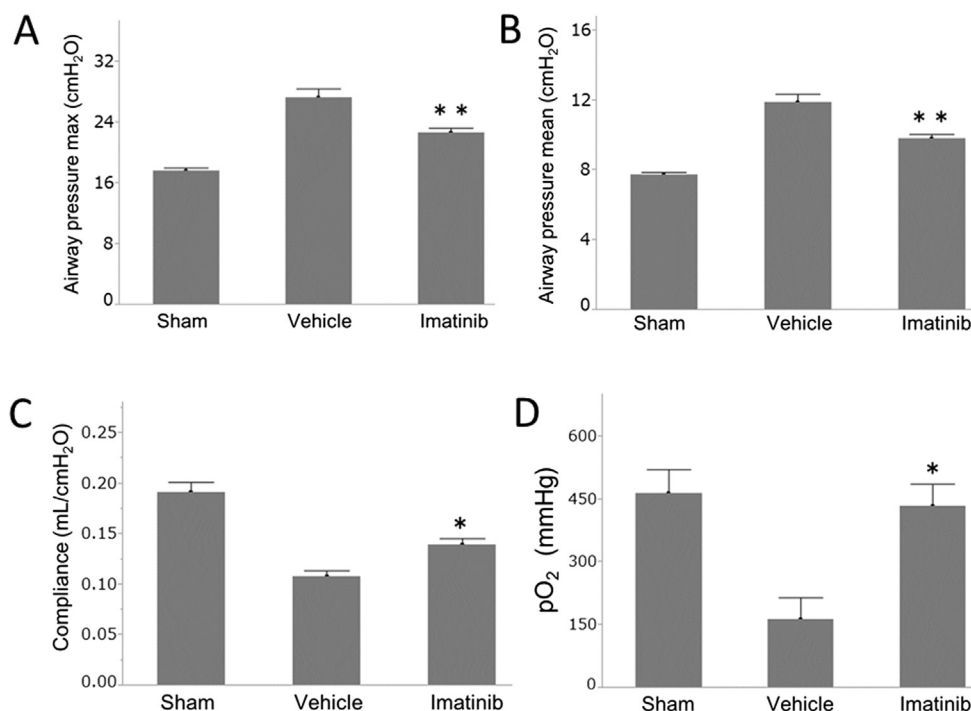
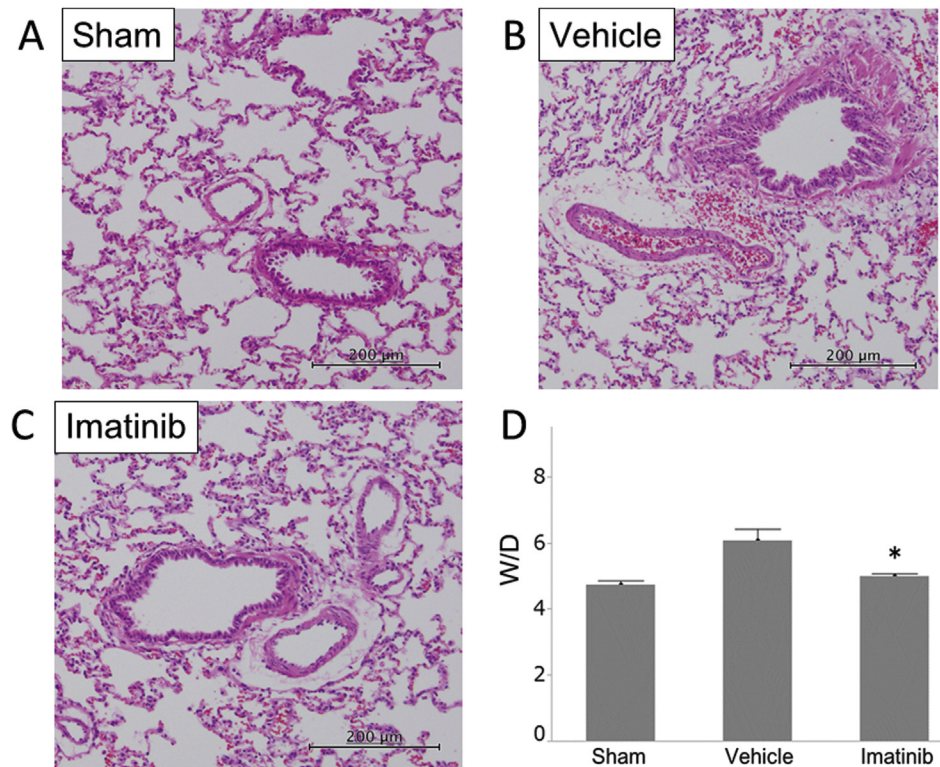


Fig 1. Lung mechanics and oxygenation after reperfusion. (A) Maximum airway pressure. (B) Mean airway pressure. (C) Dynamic compliance. (D) Arterial partial pressure of oxygen. All were improved in imatinib group. Bars and the error bars show mean and SE of mean; * $p < 0.05$; ** $p < 0.01$.

Fig 2. Histologic findings (hematoxylin-eosin stain $\times 100$) and lung wet to dry weight (W/D) ratio. (A) Sham group. (B) Vehicle group. (C) Imatinib group. (D) W/D ratio. Perivascular edema and hemorrhage were attenuated with lower W/D in imatinib group. Bars and error bars show mean and SE of mean; $*p < 0.05$.



The nucleus and cytoplasmic granule of the neutrophils were stained clear blue by naphthol AS-D chloroacetate esterase stain, and the number of neutrophils that had invaded in the lung was easily counted (Figs 3A–3C). The average number of neutrophils per high-power field (HPF) was 19.1 ± 0.38 , 53.4 ± 11.1 , and 28.6 ± 2.3 , in the sham, vehicle, and imatinib groups, respectively ($p = 0.01$) (Fig 3D).

Effect of Imatinib on Cytokine and Chemokine Levels

ELISA of lung tissue lysates revealed that the levels of IL-1 β and CXCL1 (rat homologue of IL-8) tended to be low in the imatinib group but did not reach statistical significance ($p = 0.76$ and $p = 0.13$, respectively) (Figs 4A, 4B). The level of IL-10, an antiinflammatory cytokine, was significantly increased in the imatinib group ($p = 0.02$) (Fig 4C). TNF- α and IL-6 were not detected in these tissue lysates.

Effect of Imatinib on Phosphorylation of CrkL and Src, and VEC Expression

CrkL phosphorylation at Tyr207 (pCrkL), an exclusive target for Abl and Arg [15, 16], was inhibited in the imatinib group (Fig 5A). Src family kinases were not the direct targets of imatinib but were reported to have interaction with Abl [14]. The Src activation was shown to contribute to the development of IRI through proinflammatory effects [10]. Src phosphorylation at Tyr416 was inhibited, which meant that Src activity was also reduced (Fig 5B). The expression of VEC, a cell-to-cell

adhesion molecule specific for endothelium-regulating vascular permeability [19], was maintained in the imatinib group (Fig 5C).

Concentration of Imatinib in Plasma and Lung Tissue

In experiment 1, intraperitoneal administration of imatinib was adopted as described previously [15, 17] because of the simplicity of the procedure, which was different from the administration methods in the clinics. Therefore the evaluation of drug delivery into the blood and lung was considered to be necessary. In the similar hilar clamp model, the plasma concentration of imatinib was 5.6 ± 2.3 $\mu\text{g/mL}$ at the end of reperfusion. The concentrations of the left lung and right lung were 28.3 ± 6.9 $\mu\text{g/g}$ for the left lung and 78.5 ± 1.5 $\mu\text{g/g}$ for the right lung ($p = 0.01$), respectively. The concentration in bilateral lung tissue showed a strong correlation with plasma concentration (right lung, $r = 0.971$; left lung, $r = 0.903$), with statistical significance ($p < 0.01$) (Figs 6A, 6B).

Comment

This was the first study showing that imatinib improved lung injury in a simple and validated in vivo lung IRI model and inhibited Abl kinases in lung tissue. Abl kinases are nonreceptor tyrosine kinases that are important in various cell dynamics such as cytoskeletal changes, adhesion, and migration [14, 22]. In epithelial cells and cancer cells, Abl kinases were demonstrated to be necessary in the formation and maintenance of cell-cell

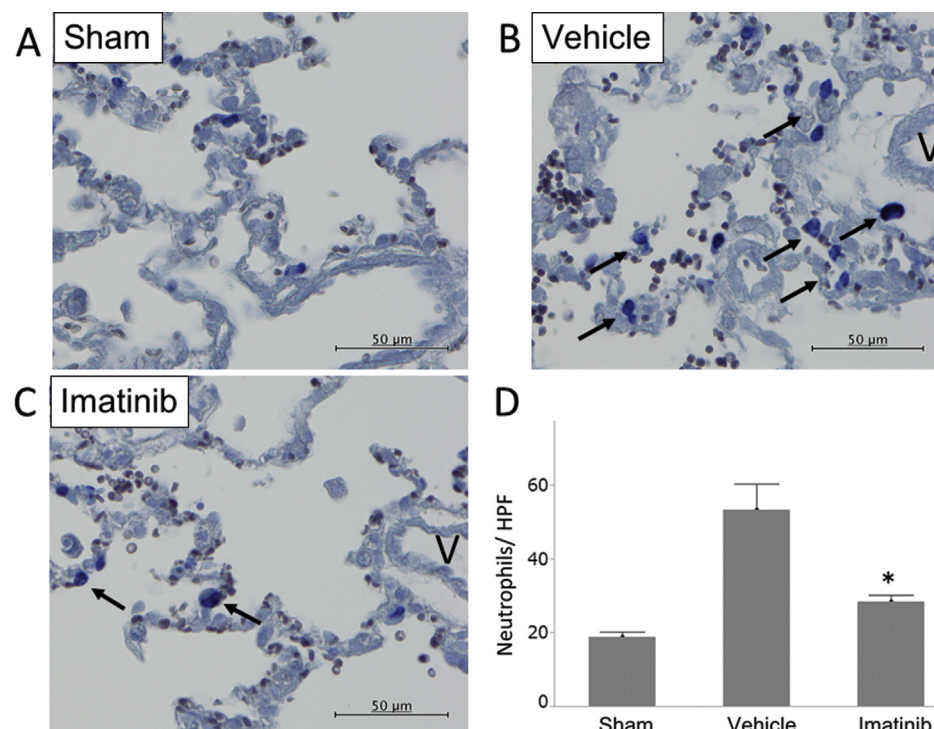


Fig 3. Neutrophil infiltration detected by naphthol AS-D chloroacetate esterase stain ($\times 400$). (A) Sham group. (B) Vehicle group. (C) Imatinib group. (D) Average number of neutrophils per high-power field (HPF). Neutrophil infiltration (arrows in B and C) was significantly decreased in the imatinib group. Bars and error bars show mean and SE of mean; * $p < 0.05$. (V = vessel.)

junctions [22]. However, recent reports showed that Abl/Arg inhibition was protective of the endothelium against various permeability-inducing stimuli in vitro, and imatinib mitigated vascular leakage in vivo [11]. Against

thrombin, histamine, and vascular endothelial growth factor, Abl/Arg inhibition was reported to activate Rac1 and Rap1 (GTPases positively regulating the endothelial barrier), impair the mobilization of calcium in the

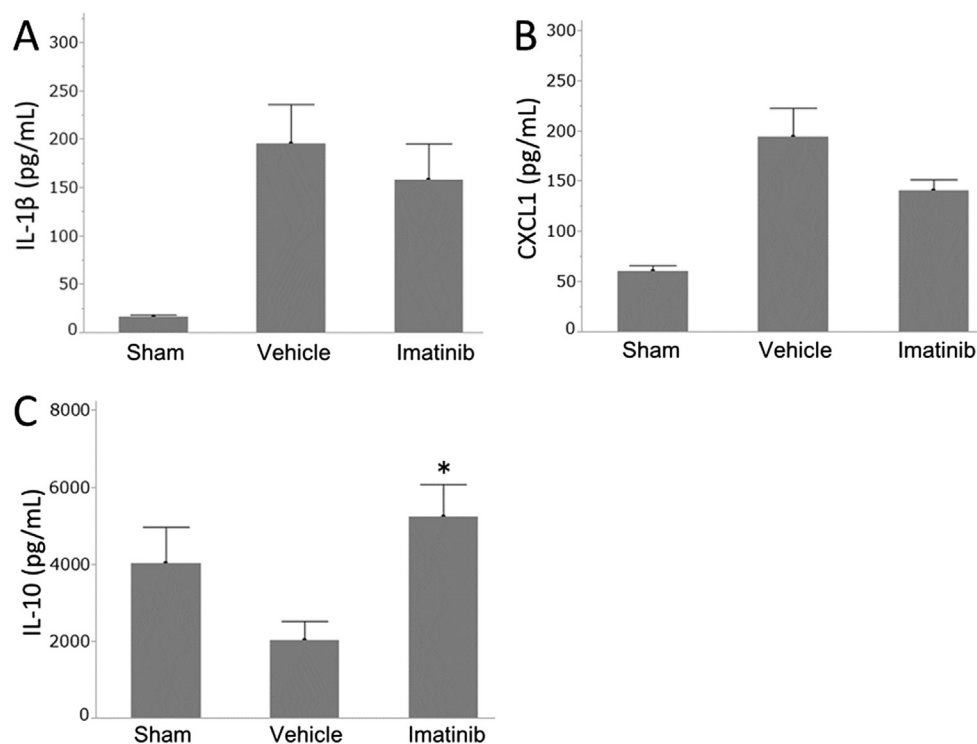
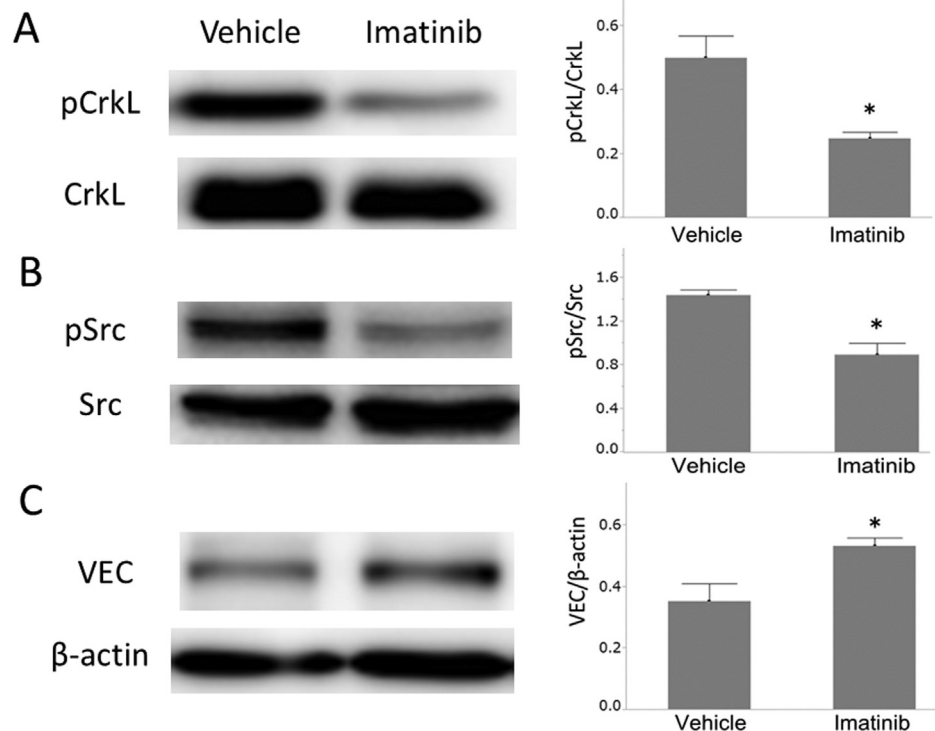


Fig 4. Level of cytokines and chemokine in lung tissue lysates. (A) Interleukin (IL)-1 β . (B) CXCL1. (C) IL-10. IL-10 level was significantly higher in the imatinib group. Bars and error bars show mean and SE of mean; * $p < 0.05$.

Fig 5. Western blot analysis of lung tissue lysates evaluating phosphorylation of CrkL and Src and vascular endothelial cadherin (VEC) expression. (A) pCrkL and CrkL. (B) pSrc and Src. (C) VEC and β -actin. Phosphorylation of both CrkL and Src in lung tissue was inhibited by imatinib administration. Imatinib maintained VEC expression. Bars and error bars show mean and SE of mean; * $p < 0.05$.

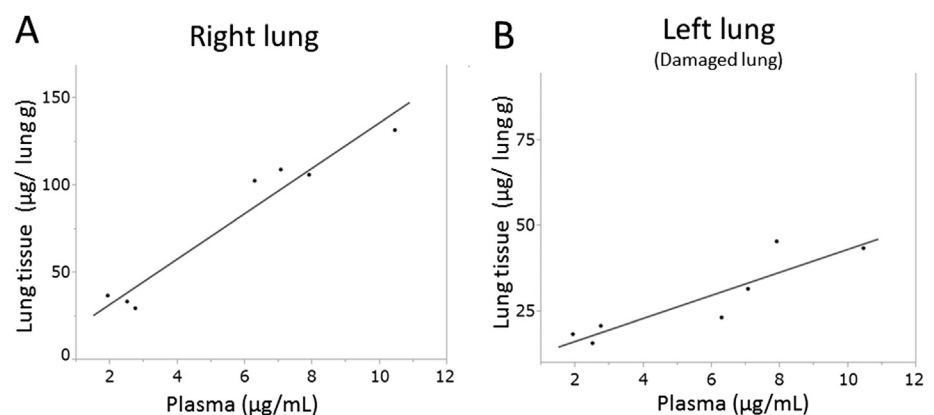


endothelium, and attenuate the generation of actomyosin contractility in vitro. Imatinib administration in vivo showed decreased edema formation against such stimuli [15]. In addition, endothelial Abl knockout mice developed less edema [16]. Against LPS, Abl/Arg inhibition contributed to the upregulation of VEC and the inhibition of nuclear factor- κ B activation in vitro, showing barrier-protective and antiinflammatory effects. Imatinib ameliorated LPS-induced lung injury with decreased inflammatory cytokines in mice [17, 23]. Against oxidative stress by H_2O_2 , Abl inhibition protected against endothelial damage by activating antioxidant enzymes in vitro. Imatinib attenuated oxidative stress-induced lung injury in mice [18]. These observations lead to the possibility that imatinib could be a potential therapeutic agent in patients

with acute respiratory distress syndrome characterized by persistent pulmonary inflammation and vascular leakage [11, 17]. Based on these reports, it was hypothesized that imatinib could attenuate lung injury through its anti-permeability and antiinflammatory effects in IRI.

In experiment 1, we showed that various pulmonary physiologic measurements were improved by preconditioning with imatinib. VEC is a well-known adherens junction molecule, playing a pivotal role in regulating vascular permeability. The positive effect of imatinib on VEC expression with attenuation of lung edema was confirmed in this in vivo IRI model. Concerning cytokine levels, the increased level of IL-10 in the lung was characteristic in this study. IL-10 is an important antiinflammatory cytokine that attenuates IRI, and gene

Fig 6. Correlation of lung tissue concentration and plasma concentration of imatinib and comparison of lung tissue concentration between right and left lungs. (A) Correlation of plasma and right lung. (B) Correlation of plasma and left lung. Concentration of bilateral lungs showed strong correlation with plasma concentration. Left (damaged) lung concentration was significantly lower than that of right lung.



therapy using it was experimentally effective for the treatment of injured donor lungs [24]. Without reaching statistical significance, IL-1 β and CXCL1 (rat homologue of IL-8) levels were also decreased. Local production of IL-8, the major neutrophil chemotactic factor, was shown to be important in IRI experimentally and clinically [6, 25]. IL-6 and TNF- α are intriguing; however, they were undetectable in the tissue lysates used for ELISA. Neutrophil infiltration into the lung is also important in inducing IRI and after lung graft rejection [6, 26], which was decreased by the imatinib administration. Taken together, the antipermeability and antiinflammatory effects of imatinib were observed in this in vivo IRI model.

In experiment 2, we focused on imatinib delivery into the lung tissue. To our knowledge, this is the first report evaluating drug delivery into the lung in an IRI model. Plasma concentration at the end of this experiment was 5.6 ± 2.3 $\mu\text{g/mL}$, which was about 1.6-fold greater than the maximum blood concentration in humans, when the standard amount of imatinib (600 mg/body) was administered orally once [13]. Our results showed that the concentration of lung tissue strongly correlated with the plasma concentration in bilateral lungs. Combined with the inhibition of pCrkL in the imatinib group, it was confirmed that the drug was definitely transferred into the lung through blood flow and functioned there accordingly. However, it was noticed that the left (damaged) lung showed lower imatinib concentrations than the right lung. This was considered to be caused by multiple factors, such as the interruption of blood flow during hilum clamping and edema formation in the damaged lung. Another possibility was that the damaged lung had a relative malperfusion with platelet activation and microthrombi in IRI [27]. When we consider the treatment strategy for IRI, it is important to take into account that drug delivery into the lung through blood flow was possibly hindered by IRI.

Limitations were as follows. First, the exact molecular mechanism was not determined in this model. Targets other than Abl/Arg were not evaluated. PDGFR inhibition showed antiinflammatory and antifibrotic effects in some experimental models [28, 29]. cKit was related to the development, activation, and maturation of dendritic cells and lymphocytes; thus, imatinib is considered to have an immunomodulating effect [30, 31]. Although mostly shown in models of chronic disease, the inhibition of PDGFR and cKit could be an alternative mechanism of imatinib's effect in this model. Second, the possible function of imatinib as a pulmonary artery vasodilator was not considered. Imatinib has been reported to be an arterial vasodilator in an experimental model of pulmonary arterial hypertension [32]. Pulmonary vascular resistance is 1 of the important contributing factors in IRI [7]. Third, the optimal timing and duration of imatinib administration were not determined. Imatinib was administered once before clamping the hilum in this study. In some experimental models, imatinib showed its effect by administration after injury [15, 17, 33]. In lung transplantation, imatinib can be used at various times, such as adding it to the preservation solution or during

ex vivo lung perfusion before transplantation, and administering to the recipient. In addition, it is important to note that imatinib is a multikinase inhibitor, and long-term use may produce other effects [15, 17]. Further investigations, including a lung transplantation model, are needed to address this issue. To begin, we are planning transplantation in rats to investigate the effects of imatinib for IRI in a setting of transplantation.

In conclusion, imatinib administration improved lung function with attenuation of lung edema in a rat IRI model. It maintained VEC expression, increased the antiinflammatory cytokine level, and decreased neutrophil infiltration. Furthermore, imatinib delivery into the lung through blood flow was confirmed in this model.

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