

## Protective effect of FUT-175 on pulmonary function of xenografts in a guinea pig-to-rat lung perfusion model

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**Background:** FUT-175 (nafamostat mesilate) has a variety of pharmacological effects; in addition to its stable potent serine protease inhibitory activity, it exerts far stronger anti-complement activity than other protease inhibitors. Here, we evaluated the protective effect of FUT-175 on pulmonary function of xenografts in an ex vivo guinea pig-to-rat lung perfusion model, using a device for analyzing pulmonary function in small animals.

**Methods:** Animals were divided into three groups (n = 6 each), Isograft (Group I), Xenograft (Group X), and Xenograft with FUT-175 (Group XF). In the latter, 10 mg of FUT-175 was added to the extracorporeal circuit before perfusion with xenogeneic blood was started. The following parameters were serially measured in these three groups: complement activity causing 50% hemolysis (CH50 units) in the perfusion blood either before or during perfusion, pulmonary arterial pressure, dynamic pulmonary compliance, and airway resistance. In addition, Hematoxylin and Eosin staining of the lungs and assays of rat IgM, IgG, and anti-C3 deposition were carried out after perfusion.

**Results:** The duration of satisfactory pulmonary function after the start of perfusion was significantly increased in Group XF. CH50 in Group XF decreased significantly than in Group X. In addition, FUT-175 suppressed both the increase in pulmonary arterial pressure and airway resistance, and the decrease in dynamic pulmonary compliance. In Group XF, intraalveolar hemorrhage and the thickening of the arterial wall were not observed. Groups X and XF showed deposition of IgM, IgG, and C3 at the endothelium of the pulmonary arteries but less in Group I.

**Conclusions:** This study suggests that FUT-175 inhibited complement activation including the alternative pathway and improved lung xenograft pulmonary function. FUT-175 ameliorates hyperacute rejection in a guinea pig-to-rat ex vivo xenogeneic lung perfusion model.

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**Keywords:** xenogeneic lung transplantation, hyperacute rejection, complement, protease inhibitor, FUT-175

### Introduction

Clinical lung transplantation was first performed by Hardy et al. at Mississippi University in the United States in 1963<sup>1)</sup>, but at that time, all approaches were compromised by severe rejection problems. After the development of immunosuppressive drugs such as cyclosporine, the first long-term survival with right unilateral lung transplantation

was reported by Cooper et al. at Toronto University in Canada in 1983<sup>2)</sup>. Currently, either unilateral or bilateral lung transplantation is routinely performed world wide. However, because the lung is continuously exposed to the external environment through the airway, this often prevents potential donor organs from being candidates for allografting, due to infection, injury to either airway or lung parenchyma, and adhesion, even when other organs such as

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kidney, or liver from the same donor can be successfully transplanted. Lack of donor organs has thus been an even more serious problem in lung transplantation compared to other organs.

As a solution to the problem of limited organ availability, xenotransplantation has been a focus of attention recently. This utilizes animal organs for grafting, and has been studied extensively worldwide in an effort to translate experimental systems into clinical utility. Xenotransplantation is classified into two types. The first is concordant xenotransplantation between evolutionarily close species such as baboon to human, and the other is discordant xenotransplantation between more distant species, such as swine to human and guinea pig to rat. In discordant combinations, donor organs usually suffer hyperacute rejection within minutes to hours, caused by activation of complement induced by natural antibodies against donor cells already present in the recipient serum. It is mandatory to control this hyperacute rejection for successful xenotransplantation. Many different approaches to controlling complement activation have been explored, focusing either on the host natural antibody or the donor xenogeneic carbohydrate antigens. This has resulted in the development of transgenic pigs expressing complement inhibitory proteins, used in combination with immunosuppressive drugs. Recently, carbohydrate xenoantigen-knockout pigs have also been created and have attracted much attention.

An alternative, or possibly complementary, approach is described here. The serine protease inhibitor FUT-175 (nafamostat mesilate, Futhan<sup>®</sup>) exerts several pharmacological actions in addition to its protease inhibitory effects. FUT-175 has potent activity against the C1r and C1s subunits of the classical pathway, as well as factor B and D of the alternative pathway<sup>3,5</sup>. This drug prevents complement activation in various *in vivo* experimental models<sup>3,6,7</sup>. It has also been demonstrated to contribute to the prolongation of graft survival in either xenogeneic liver or heart transplant models<sup>8,9</sup>.

In the context of lung transplantation, measures of pulmonary function during acute rejection in allogeneic models have employed either air content assessment by chest x-ray or perfusion indices by blood flow scintigraphy. However, the kinetics of deterioration of xenograft pulmonary function during hyperacute rejection have yet to be clarified. Here, we have developed an *ex vivo* guinea pig-to-rat xenogeneic lung perfusion model using a special small animal device to analyze pulmonary function. We have used this model to evaluate the protective effect of FUT-175 on xenograft pulmonary function during hyperacute rejection.

## Materials and Methods

### Animals

Inbred male Lewis rats (Charles river laboratories Japan Inc., Yokohama, Japan) and outbred male Hartley guinea pigs (Tagawa laboratory animals Inc., Nagasaki, Japan) weighing 300 to 350 grams were used in all experiments. All animals in the study were maintained in accordance with the guidelines of the Committee of Animal Care of Nagasaki University School of Medicine, Nagasaki Japan. The research protocol for the present study was approved by the Animal Research Studies Committee of Nagasaki University School of Medicine, Nagasaki Japan (No. 646).

### FUT-175

The serine protease inhibitor FUT-175 (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) could be purchased commercially.

### Experimental Design

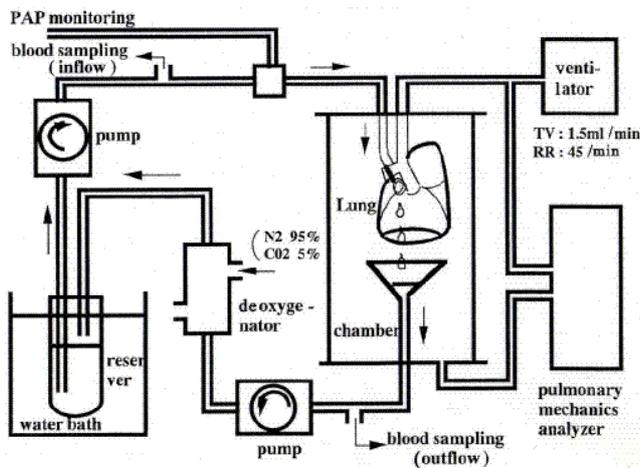
In this study, animals were divided into three groups ( $n = 6$  each). In the isograft group (Group I), the left lungs isolated from Lewis rats were used as donor grafts, while in the xenograft group (Group X), the left lungs isolated from guinea pigs were used. In the xenograft + FUT-175 group (Group XF), 10 mg of FUT-175 was added to the extracorporeal circulation before perfusion of the xenograft was started. Ten mg of FUT-175 mixed with 50 mL of blood, yielded an estimated concentration of  $3.7 \times 10^{-4}$  mol, which is about 6 times the  $IC_{50}$  of complement factor B ( $6.2 \times 10^{-5}$  mol), or about 2.6 times the  $IC_{50}$  of factor D ( $1.4 \times 10^{-4}$  mol), and corresponds to about 100-fold the suggested clinical dose.

### Donor Lung Procurement

Lewis rats and Hartley guinea pigs were anesthetized with intraperitoneal pentobarbital (60 mg/kg), mechanically ventilated using a rodent respirator (Harvard 683) with room air (stroke frequency, 90/min, tidal volume, 3 mL/min). The anterior chest wall was dissected and removed and the donor animal was heparinized (500 units/kg). The left lung of the donor was isolated following insertion of a flexible cannula into the left pulmonary artery and opening of the left pulmonary vein. Then the lung was connected to the circuit.

### Design of the lung perfusion circuit (Figure 1)

After priming with heparinized rat blood, the donor graft was suspended in a sealed chamber, and ventilated with room air with a tidal volume of 1.5 mL and respiratory rate of 45 breaths/min. The pulmonary function-analyzing device, which was connected to the chamber, detected signals of either airway pressure or flow rate of the ventilated air, and serially calculated dynamic pulmonary compliance and airway resistance. For ex vivo perfusion, a flexible tube was inserted into the left pulmonary artery and connected to the extracorporeal circulation. The graft was perfused with 50 ml of heparinized (1000 units/kg) whole blood harvested from male Lewis rats in all groups at a flow rate of 5 mL/min, at which rate blood dripped from the free open end of the left pulmonary vein and was collected in the reservoir by a roller pump. It was then warmed and recirculated from the left pulmonary artery by a pump. A deoxygenator was also set into this circuit, to diffuse a gas mixture of 95% N<sub>2</sub> and 5% CO<sub>2</sub> at a rate of 1 L/min for deoxygenation of the blood. Sampling ports were set at both the afferent and efferent loops of the circuit for blood analysis.



**Figure 1.** Schematic diagram of lung perfusion circuit. TV; tidal volume, RR; ventilated stroke rate, PAP; pulmonary arterial pressure

### Measured parameters

We serially measured complement activity in the perfusion blood causing 50% hemolysis (CH50 units) before and during perfusion using CH50 methods reported by Mayer et al<sup>10</sup>. Pulmonary arterial pressure (mmHg), dynamic pulmonary compliance (tidal volume/end-inspiratory airway pressure), and airway resistance (mean airway pressure/flow rate) in each group were also measured. In addition,

hematoxylin and eosin staining and FITC-labeled anti-rat IgM, IgG, and anti-rat C3 immunofluorescence staining of the lungs after perfusion were also performed.

### Antibodies of immunofluorescence staining

Immunofluorescence staining of rat IgM, IgG and C3 was performed using FITC labeled goat anti-rat IgM, IgG and C3 F(ab')<sub>2</sub>-fragments (American Qualex Inc., San Clemente, CA).

### Statistical analysis

Statistical analyses were performed on a Macintosh computer using Stat View software, version 5.01 (SAS Institute, NC, USA). Data were shown as mean ± standard deviation. Differences between two groups were analyzed with the Mann-Whitney U test, and considered to be significant when probability less than 0.05.

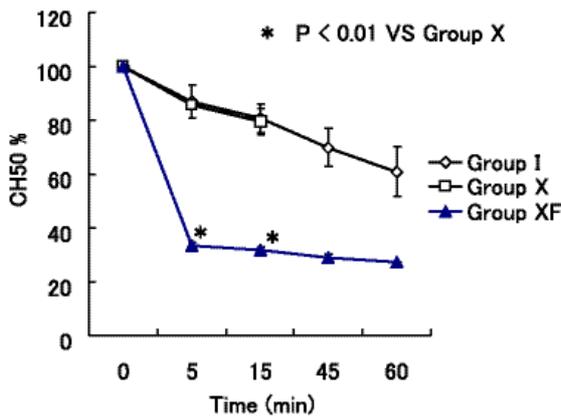
## Results

### Perfusion time

In both Groups I and XF, the grafts could be perfused for 60 minutes without any increase of mucus secretion in the airway, while, due to a rapid increase in mucus secretion in the airway and pulmonary congestion after the initiation of perfusion, measurements of pulmonary function could not be continued for more than 15 minutes in Group X. The measurement periods were thus defined as 60 minutes in Groups I and XF, but 20 minutes in Group X.

### CH50

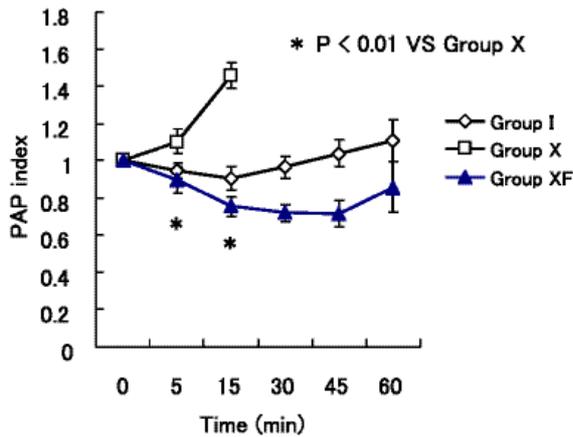
The time course of CH50, representing total hemolytic complement activity in the perfusion blood, is shown in Figure 2. In Groups I and X, it decreased to about 80% of the preperfusion level after perfusion, and continued to decrease gradually thereafter in Group I. On the other hand, in Group XF, in which FUT-175 was used, there was a more marked decrease in CH50 to as low as 30% of the preperfusion level 5 minutes after starting the perfusion, which was statistically significantly lower than in Group X. This low level of CH50 was maintained for the 60 minutes experimental period.



**Figure 2.** The time course of CH50. CH50 continued to decrease gradually after perfusion in Groups I and X. It decreased to as low as 30% of the preperfusion level 5 minutes after starting the perfusion in Group XF. This low level of CH50 was maintained for the 60 minutes.

*PAP index*

Pulmonary arterial pressure was analyzed and expressed as pulmonary arterial pressure index (PAP index; the ratio relative to the preperfusion value). The PAP index remained the same during the 60 minutes period in Group I, but increased after perfusion in Group X (1.17 at 5 minutes and 1.50 at 15 minutes), while it was 0.85 at 5 minutes and 0.73 at 15 minutes in Group XF. This difference was statistically significant (Figure 3).

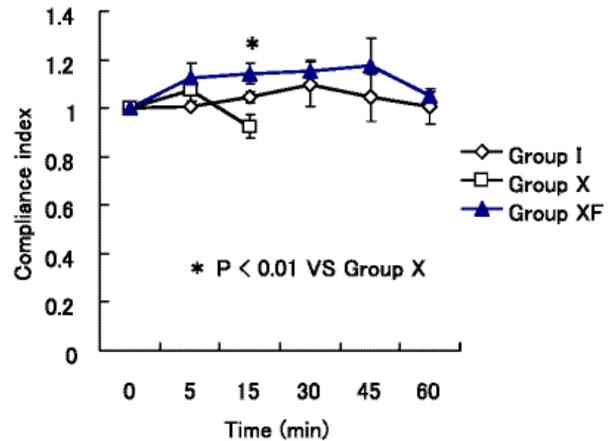


**Figure 3.** The time course of PAP (pulmonary arterial pressure) index; the ratio relative to the preperfusion value. It remained the same during the 60 minutes period in Group I, but increased after perfusion in Group X. In Group XF, the PAP index expressed significantly lower level compared with the values at 5 and 15 minutes in Group X.

*Compliance index*

Additionally, dynamic pulmonary compliance or airway

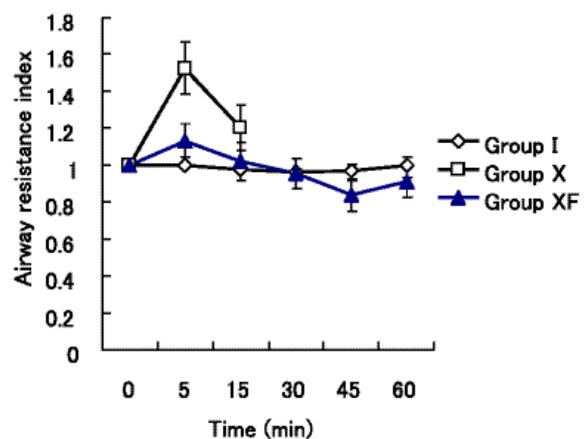
resistance was also evaluated as indices relative to preperfusion values. The compliance index decreased 5 minutes after the initiation of perfusion and was 0.87 at 15 minutes in Group X, while it showed no decrease in Group XF (1.17 at 15 minutes). This difference was also statistically significant (Figure 4).



**Figure 4.** The time course of compliance index; the ratio relative to the preperfusion value. It decreased to 0.87 at 15 minutes after starting the perfusion and the perfusion stopped in Group X. It showed no decrease and ventilated 60 minutes in Group XF (1.17 at 15 minutes). This difference was statistically significant.

*Airway resistance index*

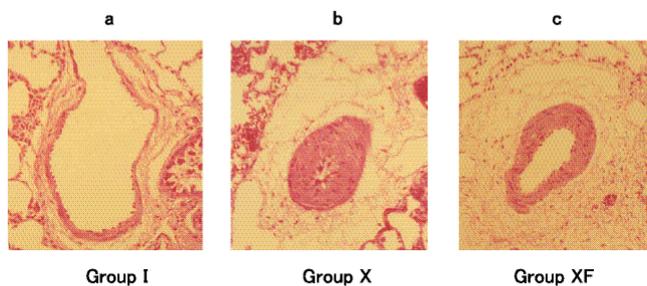
The resistance index increased immediately after perfusion in Group X, but did not change either in Group XF or Group I (Figure 5). However, these differences did not reach statistical significance.



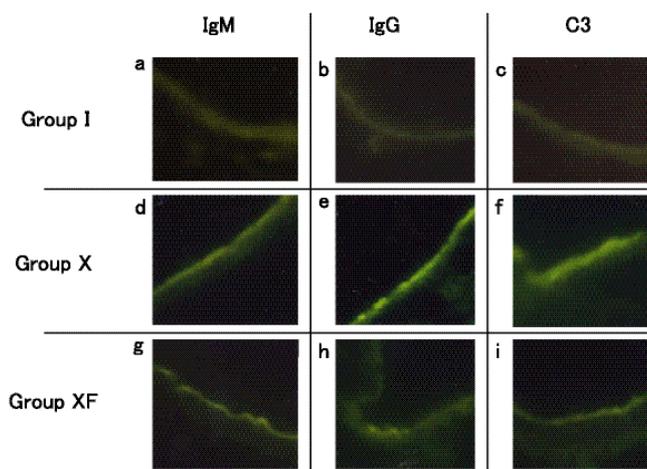
**Figure 5.** The time course of airway resistance index; the ratio relative to the preperfusion value. It increased immediately after perfusion in Group X, but did not change either in Group XF or Group I during the 60 minutes. These differences did not reach statistical significance.

### Histological and immunopathological findings

By hematoxylin and eosin and immunofluorescence staining of the lungs after perfusion, Group I showed no marked changes in either the alveolus or the arterial lumen (Figure 6a), while in Group X there was either intraalveolar hemorrhage or luminal narrowing of arteries due to thickening of the vascular smooth muscle (Figure 6b), which probably showing the edema and vasoconstriction. This finding is consistent with previous reports on pulmonary histology during hyperacute rejection from Tavakoli et al<sup>11</sup>. In contrast, in Group XF, intraalveolar hemorrhage was not observed, and the thickening of the arterial wall was less marked (Figure 6c). However, both Groups X and XF showed deposition of IgM, IgG, and C3 at the endothelium of the pulmonary arteries but Group I showed less (Figure 7).



**Figure 6.** Hematoxylin and eosin staining of the lung after perfusion (x 100). a; Group I showed no marked changes in either the alveolus or the arteriolar lumen. b; Group X showed either intraalveolar hemorrhage or luminal narrowing of arterioles due to thickening of the vascular smooth muscle, which probably showing the edema and vasoconstriction. c; Intraalveolar hemorrhage was not observed, and the thickening of the arteriolar wall was less marked in Group XF.



**Figure 7.** Immunofluorescence study revealed the presence of IgM, IgG, and C3 deposits on the arteriolar endothelium in both Group X and XF, but less in Group I (x 400).

### Discussion

In allogeneic transplantation, the acute phase problem consists of ischemia-reperfusion injury, in which excessive production of inflammatory cytokines by either neutrophils or macrophages increases tissue factor activity, which in turn triggers extrinsic coagulation reactions and decreases the expression of anticoagulants such as thrombomodulin. The resulting microcirculation insufficiency of transplanted organs causes graft insufficiency. It was therefore thought that pulmonary injury after ischemia-reperfusion might be ameliorated by exploiting the anticoagulation and anti-inflammatory effects of protease inhibitors. This has been investigated using gabexelate mesilate (FOY®) and ONO-5456, a leukocyte elastase inhibitor, in combination with inhaled nitric oxide, in an experimental model of cadaveric allogeneic lung transplantation in pigs<sup>12,13</sup>.

FUT-175, as well as gabexelate mesilate, was developed for the therapy of acute pancreatitis and disseminated intravascular coagulation syndrome, and is protease inhibitors showing anti-complement activity. FUT-175 was shown to have by far the stronger anti-complement effect compared to gabexelate mesilate, in both the classical and alternative pathways. FUT-175 inhibits both C1r and C1s in the classical pathway, and factor B, factor D, and C3 convertase in the alternative pathway in a dose-dependent manner. The IC<sub>50</sub> (inhibitory concentration 50%) of each enzyme, as given by the manufacturer, is  $1.8 \times 10^{-7}$  mol for C1r,  $2.4 \times 10^{-8}$  mol for C1s,  $6.2 \times 10^{-5}$  mol for factor B, and  $1.4 \times 10^{-4}$  mol for factor D. Here, we used a concentration of  $3.7 \times 10^{-4}$  mol, which should inhibit even factor D. However, FUT-175 exists in an ester form with a molecular weight of about 540, and is easily hydrolyzed in vivo by elastases in either blood or the liver. It shows biphasic pharmacokinetics with a serum half-life of about 23 minutes, with an initial rapid distribution from blood to organs for about 1 minute (distribution phase), and elimination from the body by either metabolism or excretion over about 23 minutes (elimination phase). The serum concentration thus rapidly decreases if administered by bolus rather than continuous infusion. On the other hand, the concentration of FUT-175 in tissues is higher than in the blood. The elimination half-life of the unmetabolized form is shortest in the liver (about 7 minutes), but is more than 1 hour in the kidney and more than 3 hours in the lung. Miyagawa et al. reported that in the guinea pig-to-rat heterotopic cardiac xenograft model, intraperitoneal administration of 20 mg/kg of FUT-175 to rats suppressed about 80% of C3 activity and almost 100% of factor B activity, but only about 40% of factor D

activity, within 1 hour of administration. It was also reported that FUT-175 abrogated ACH50 (reflecting the potency of the alternative pathway) from one to 6 hours after administration, and prolonged the beating time of heterotopic heart grafts about 3-fold. This suggests that FUT-175 directly inhibits serine protease factor B in vivo, which contributes to the complete abrogation of complement activity and the prolongation of xenograft survival<sup>9</sup>.

In the present study, we established a guinea pig-to-rat ex vivo xenogeneic lung perfusion model, in which we clearly demonstrated that the perfusion time during which pulmonary function remained satisfactory was extended from 15 minutes to more than 60 minutes by FUT-175 in the perfusion blood. In this ex vivo experimental model, it cannot be formally excluded that the pharmacokinetics, including serum half-life of administered FUT-175, may be somewhat different from in vivo, and that the results may not completely reflect the in vivo situation. Nonetheless, in this model, FUT-175 suppressed both increases in pulmonary arterial pressure and airway resistance, as well as the decrease in dynamic pulmonary compliance, that were the results of severe congestion, pulmonary edema and intratracheal secretions of the rejected lung grafts. These effects may therefore result not only from suppression of complement activation (including the alternative pathway), but also the activation of the coagulation cascade. Kirk et al. reported in the ex-vivo pig-to-human cardiac xenograft model that xenoperfused hearts stopped beating by 24 to 34 minutes, that deposition of IgM, IgG, and C3, as well as cellular infiltration by neutrophils, macrophages, and lymphocytes, was observed in the rejected hearts<sup>14</sup>. Tavakoli et al. also reported deposition of IgM, IgG, and C3, as well as cellular infiltration of lymphocytes, in a guinea pig-to-rat pulmonary xenograft model in which the lungs were rejected at a mean of 53.3 minutes<sup>11</sup>. These results imply a contribution of endothelial injury mediated by leukocytes to the hyperacute rejection which occurs within minutes. Therefore, the protective effects of FUT-175 on graft pulmonary functions observed in the present study are probably also at least partly mediated via suppressive effects on microcirculation injury resulting in acute pulmonary injury caused by either inflammatory cytokines or activation of leukocyte-endothelial adhesion, or leukocyte elastases.

In conclusion, in an ex vivo xenogeneic lung perfusion model, administration of FUT-175 to the recipient could protect the pulmonary functions of donor grafts for a certain period. In addition to the action of FUT-175 on complement activation during hyperacute rejection, a possible association with a variety of other pharmacologic functions

was also suggested. Protease inhibitors such as FUT-175 may thus prove useful for effective anti-rejection treatment in xenogeneic lung transplantation.

## Acknowledgments

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## References

- 1) Hardy JD, Webb WR, Dalton ML Jr, Walker GR Jr. Lung homotransplantations in man. *JAMA* 186: 1065-1074, 1963
- 2) Unilateral lung transplantation for pulmonary fibrosis. Toronto Lung Transplant Group. *N Engl J Med* 314 :1140-1145, 1986
- 3) Aoyama T, Ino Y, Ozeki M, et al. Pharmacological studies of FUT-175, nafamostat mesilate. I. Inhibition of protease activity in in vitro and in vivo experiments. *Jpn J Pharmacol* 35: 203-227, 1984
- 4) Ikari N, Sakai Y, Hitomi Y, Fujii S. New synthetic inhibitor to the alternative complement pathway. *Immunology* 49: 685-691, 1983
- 5) Fujii S, Hitomi Y. New synthetic inhibitors of C1r, C1 esterase, thrombin, plasmin, kallikrein and trypsin. *Biochim Biophys Acta* 66: 342-345, 1981
- 6) Kreil E, Montalescot G, Greene E, et al. Nafamostat mesilate attenuates pulmonary hypertension in heparin-protamine reactions. *J Appl Physiol* 67: 1463-1471, 1989
- 7) Issekutz AC, Roland DM, Patrick RA. The effect of FUT-175 (Nafamostat Mesilate) on C3a, C4a and C5a generation in vitro and inflammatory reactions in vivo. *Int J Immunopharmacol* 12: 1-9, 1990
- 8) Gridelli B, Gatti S, Andreani P, et al. Xenogeneic orthotopic liver transplantation from sheep to pig: delay of rejection by FUT 175 Infusion. *Transplant Proc* 28: 784-787, 1996
- 9) Miyagawa S, Shirakura R, Matsumiya G, et al. Prolonging discordant xenograft survival with anticomplement reagents K76COOH and FUT175. *Transplantation* 55: 709-713, 1993
- 10) Kabat EA, Mayer MM. Complement and Complement Fixation. *In Experimental Immunochemistry* (2nd ed, Springfield, IL) pp. 133-149, 1961
- 11) Tavakoli R, Devaux JY, Nonnenmacher L, Louvel A, Weill B, Houssin D. Discordant lung xenograft rejection in the rat. *Transplantation* 53: 235-237, 1992
- 12) Luh SP, Tsai CC, Shau WY, et al. Protective effects of inhaled nitric oxide and gabexate mesilate in lung reperfusion injury after transplantation from non-heart-beat donors. *J Heart Lung Transplant* 21: 251-259, 2002
- 13) Ishikawa N, Oda M, Kawaguchi M, Tsunozuka Y, Watanabe G. The effects of a specific neutrophil elastase inhibitor (ONO-5046) in pulmonary ischemia-reperfusion injury. *Transpl Int* 16: 341-346 Epub, 2003
- 14) Kirk AD, Heinle JS, Mault JR, Sanfilippo F. Ex vivo characterization of human anti-porcine hyperacute cardiac rejection. *Transplantation* 56: 785-793, 1993

## Abbreviations:

FUT-175 (nafamostat mesilate)

CH50 units (complement activity causing 50% hemolysis)

PAP index (pulmonary arterial pressure index)