Antithrombin III Prevents Early Pulmonary Dysfunction After Lung Transplantation in the Dog

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Background—Ischemia-reperfusion injury with the resulting inflammatory response is a devastating complication of lung transplantation; much of the tissue damage could be diminished by control of the inflammatory response. Recent studies have shown that antithrombin III (AT III) has an anti-inflammatory effect in addition to its established role in the regulation of blood coagulation. Thus, we hypothesized that the administration of AT III might help to prevent ischemia-reperfusion injury after lung transplantation.

Methods and Results—The study was performed in a dog model of orthotopic lung transplantation. Dogs were randomly assigned to receive either vehicle (controls) or AT III. We observed that in control dogs, during the 180-minute period after lung transplantation, the arterial O2 partial pressure decreased and both the alveolar-arterial O2 difference and the pulmonary vascular resistance increased. By contrast, these parameters remained unchanged in the group of dogs receiving AT III. Dogs with transplants receiving AT III did not show an increase in cell adhesion molecules, and histological examination revealed almost an absence of inflammatory response. The administration of AT III produced a marked increase in serum prostacyclin (PGI2) levels, whereas in control dogs, the PGI2 levels did not change. The beneficial effect of AT III was not observed when dogs received indomethacin to prevent the stimulation of PGI2 release by AT III.

Conclusions—Our results demonstrate that AT III prevents ischemia-reperfusion injury in a dog model of lung transplantation and that this effect is conditioned by an increase in PGI2 production. (Circulation. 2001;104:2975-2980.)

Key Words: ischemia ▪ reperfusion ▪ thrombin ▪ lung ▪ transplantation ▪ coagulation

Primary graft failure resulting from ischemia-reperfusion injury is a devastating complication of lung transplantation. It accounts for almost 33% of perioperative deaths.1 Primary graft failure also contributes to early and late postoperative complications, precluding and jeopardizing a successful recovery after transplantation.2

The pathophysiology of the ischemia-reperfusion injury in the lung is not fully understood. It is known, however, that endothelial damage, together with leukocyte activation and the increased production of mediators of inflammation, plays a critical role in the development of ischemia-reperfusion injury.3 An effective therapeutic strategy should be directed against key pathogenic factors involved in ischemia-reperfusion injury.

Antithrombin III (AT III) is the main inhibitor of serine proteases generated during blood coagulation. AT III inhibits activated coagulation factors of the intrinsic and common pathways, including thrombin; factors IXa, Xa, Xla, and XIIa; and kallikrein.4 In addition to its anticoagulant activity, AT III has been shown to have an anti-inflammatory effect; AT III induces endothelial cell release of prostacyclin (PGI2), which inhibits cytokine production5–8 and suppresses leukocyte and T-cell activation.9,10 Taking into consideration all the actions of AT III, we hypothesized that the administration of AT III may prevent or modify ischemia-reperfusion injury after lung transplantation.

The present study was designed to evaluate the effect of AT III administration on short-term lung function in a dog model of orthotopic lung transplantation. Our results show that the administration of AT III prevents ischemia-reperfusion injury associated with lung transplantation. This beneficial effect of AT III is explained, at least in part, by the increase in PGI2 levels induced by AT III administration.

Methods

Animal Model

Twelve left lung allotransplants were performed in adult mongrel dogs matched for size and weight (18 to 31 kg). The first experiment was performed to evaluate the effect of AT III administration on graft lung function shortly after transplantation. Pairs of dogs were

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randomly assigned in a blinded fashion to experimental (n=6) or control (n=6) groups. The animals were treated identically except that either vehicle or AT III concentrate (Laboratory Grifols; 50 IU/kg to the donors and 200 IU/kg to the recipients) was administered systemically to control or experimental (AT III) dogs, respectively. Vehicle or AT III concentrate was given 30 minutes before occlusion of cardiac inflow in the donors and 30 minutes before initiation of lung reperfusion in the recipients. Because the administration of AT III resulted in a marked improvement in lung function, a second set of experiments was performed to analyze whether the beneficial effect of AT III was due to an increase in PGII levels. Thus, 2 pair of dogs received indomethacin (5 mg/kg IV) 30 minutes before the administration of AT III.

Donor Lung Harvest and Recipient Procedure

The left lung was harvested from the donor dog according to a procedure previously published. The lung was stored in a 10°C normal saline bath for a period of 28 hours until transplantation. In the recipient dog, a left single-lung transplantation was performed as previously described. Ten minutes after the completion of implantation, hemodynamic and gasometric parameters were obtained, then the right PA was ligated and the right lung ventilation arrested.

Hemodynamic Measurements

The following parameters were measured at baseline, immediately after graft reperfusion (before right pulmonary artery [PA] occlusion), and at 60, 120, and 180 minutes after the right PA occlusion: systemic arterial pressure, heart rate, PA pressure, central venous pressure, pulmonary capillary wedge pressure (PCWP), cardiac output, partial pressure of arterial oxygen (PaO₂), partial pressure of arterial carbon dioxide (PaCO₂), partial pressure of mixed venous blood oxygen (PvO₂), arterial oxygen saturation (SaO₂), mixed venous blood oxygen saturation (SvO₂), and hemoglobin.

The following hemodynamic and pulmonary function parameters were calculated on the basis of previously published formulas: mean arterial pressure, mean PA pressure (MPAP), cardiac index (CI), systemic vascular resistance index (SVRI), pulmonary vascular resistance index (PVRI), arteriovenous oxygen difference (a-vDcO₂), and pulmonary shunt (Q s /Q t%)

Laboratory Measurements

PaO₂, PaCO₂, pH, hemoglobin level, SaO₂, and SvO₂ were measured with a blood gas analyzer (ABL-2, Radiometer). Mixed venous blood samples were collected from the distal portion of the Swan-Ganz catheter.

Leukocyte counts were determined by Coulter counter, and the differential cell count was determined manually. AT III activity was determined in platelet-poor plasma on the basis of a chromogenic substrate method using the Berichrom AT III test kit (Behringwerke AG).

The production of PGII was assessed by measuring the plasma levels of 6-keto-PGF₁α, a stable metabolite produced by nonenzymatic hydration of PGII (Pharmacia LKB). The 6-keto-PGF₁α levels were measured with a commercial immunoassay (R&D Systems) in plasma samples supplemented with the PG synthetase inhibitor indomethacin (2 mmol/L).

Cytometric study was performed in peripheral blood white cells isolated by use of Ficoll-Hypaque discontinuous gradient or cells from airway edema fluid. Cells were incubated in a fluorescence-activated cell sorter (FACS) medium containing 10% canine serum, 1% BSA, and 0.1% NaN₃ in PBS. Thereafter, indirect immunofluorescence was performed by use of the standard protocols for flow cytometry using as first monoclonal antibodies YTH 81.5 (CD11), YFC 118.3 (CD18), YKIX 337.8 (CD44), and 84H10 (CD54) from Serotec (Laboratory Tebrib SA) and a FITC-conjugated rat antismouse antibody (Serotec) as secondary antibody. Cells were analyzed with a Becton Dickinson FACScan.

Airway edema fluid from the left lung was collected by aspiration at the end of each experiment; the cell content was analyzed by flow cytometry according to the same protocol as used for peripheral blood white cells.

At the end of the experiment, dogs were euthanized by sodium pentobarbital overdose. The left lung was excised, dripped, weighed, and fixed in 10% formalin. Five representative samples were taken from the left middle lobe, and these samples were stained with hematoxylin and eosin for histological analysis by light microscopy.

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 Academy of Science and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Statistical Methods

The results are expressed as mean±SD. Comparisons among means were made by ANOVA followed by Scheffe’s test for multiple comparison. Statistical significance was defined as a value of \( P<0.05 \).

Results

Experiment 1: Effect of AT III on Pulmonary Function After Lung Transplantation

Alveolar-Capillary Gas Exchange

In control dogs, the PaO₂ decreased immediately after lung reperfusion, and the decrease was progressive throughout the 180 minutes of observation. By contrast, the PaO₂ remained unchanged in dogs with transplants receiving AT III (Figure 1A). The decrease in Po₂ observed in control dogs was associated with a parallel increase in Qs/Qt%. In dogs receiving AT III, the Qs/Qt% did not change (Figure 1B). Thus, at 180 minutes, the AT III group had greater Po₂ and lower Qs/Qt% than controls.
Hemodynamic and Gasometric Parameters, Control vs AT-III Groups

<table>
<thead>
<tr>
<th>Time</th>
<th>MAP, kPa</th>
<th>CI, L·min⁻¹·m⁻²</th>
<th>PCWP, kPa</th>
<th>SVRI, kPa·L⁻¹·min⁻¹·m²</th>
<th>a-vDcO₂, mL/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>AT-III</td>
<td>13.8±1.98</td>
<td>2.9±0.25</td>
<td>1.15±0.37</td>
<td>262.3±33.6</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14.5±1.92</td>
<td>3.1±0.62</td>
<td>1.48±0.39</td>
<td>252.1±34.9</td>
</tr>
<tr>
<td>Tx, 0 min</td>
<td>AT-III</td>
<td>12.7±1.4</td>
<td>2.5±0.15 ‡</td>
<td>1.72±0.39*</td>
<td>266.0±21.3</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14.2±4.1</td>
<td>2.7±0.63 ‡</td>
<td>1.88±0.21*</td>
<td>262.7±56.0</td>
</tr>
<tr>
<td>60 min</td>
<td>AT-III</td>
<td>11.7±0.9</td>
<td>2.6±0.25‡</td>
<td>1.61±0.38</td>
<td>236.4±36.2</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14.3±4.5</td>
<td>1.8±0.41‡</td>
<td>2.42±0.34‡</td>
<td>419.7±84.7‡</td>
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<tr>
<td>120 min</td>
<td>AT-III</td>
<td>10.9±0.8*</td>
<td>2.5±0.23‡</td>
<td>1.54±0.36</td>
<td>219.9±22.9</td>
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<tr>
<td></td>
<td>Control</td>
<td>12.2±3.2</td>
<td>1.7±0.43‡</td>
<td>2.34±0.41‡</td>
<td>380.8±96.5‡</td>
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<tr>
<td>180 min</td>
<td>AT-III</td>
<td>11.4±1.2*</td>
<td>2.4±0.25‡</td>
<td>1.62±0.28</td>
<td>243.5±38.1</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>10.1±3.7†</td>
<td>1.5±0.31‖</td>
<td>2.42±0.49‖</td>
<td>335.0±105.7‖</td>
</tr>
</tbody>
</table>

Baseline indicates values obtained before transplantations; Tx, 0 min, values obtained immediately after reperfusion of the transplanted lung and contralateral native lung exclusion. Data are given as mean±SD.

*P<0.05, †P<0.01 vs control. ‡P<0.05, §P<0.01 vs baseline.

**Hemodynamic Parameters**

Hemodynamic parameters are shown in Figure 1. Immediately after lung reperfusion, there was a moderate increase in MPAP in both the control and AT III groups. However, although in control dogs the MPAP increased progressively until 180 minutes, the AT III group did not show a further increase in MPAP. Thus, at 180 minutes, the values of MPAP in controls were 2-fold increased compared with the AT III group (Figure 1C). The increase in MPAP in controls was associated with an increase in PVRI; by contrast, in AT III dogs, the PVRI did not increase after lung transplantation (Figure 1D). The CI declined shortly after reperfusion in both groups; thereafter, the CI decreased only in control dogs, and at 180 minutes, the CI in AT III dogs was greater than in controls (Table). After reperfusion, PCWP values were similar in the 2 groups; in controls, however, the PCWP increased significantly throughout the 180 minutes of observation, and in AT III dogs, the PCWP did not change (Table). In both groups, changes in central venous pressure were parallel to those in PCWP, and heart rate did not change in either group (data not shown).

The mean arterial pressure decreased in both AT III and control dogs; however, the decrease in mean arterial pressure was more marked in controls than in the AT III group (Table). In controls, the SVRI increased during the first 60 minutes after reperfusion and decreased from 60 to 180 minutes. Nonetheless, values of SVRI in control dogs were maintained above baseline, whereas they were unchanged in the AT III group. The decrease in CI observed in control dogs was accompanied by a significant increase in a-vDcO₂. The a-vDcO₂ also increased in dogs receiving AT III, but the values were lower than in controls at 120 and 180 minutes (Table).

At the end of the experiment, lungs from control dogs were heavier than those from AT III dogs (269±63 versus 149±29 g; P<0.01).

**Plasma Levels of AT III and 6-Keto-PGF₁α**

The administration of AT III produced an expected increase in circulating levels of AT III that remained elevated until the end of the experiment. By contrast, in control dogs, the serum levels of AT III progressively decreased after lung graft reperfusion (Figure 2A). At 180 minutes, the mean plasma levels of AT III relative to baseline were 133.6±12.9% and 29.5±7.7% in the AT III and control groups, respectively (P<0.001). In dogs receiving AT III, the increase in AT III plasma levels was accompanied by an elevation in 6-keto-PGF₁α with a peak at 60 minutes; then the values decreased at 120 and 180 minutes but were still almost 3-fold increased compared with baseline. In control dogs, the concentration of 6-keto-PGF₁α remained unchanged (Figure 2B).

**Expression of Adhesion Molecules in Mononuclear Cells**

In control dogs, the percentage of peripheral blood mononuclear cells expressing adhesion molecules increased during the 180-minute study period: CD11b 42±7% versus 83±14% (P<0.001), CD18 37±8% versus 52±17% (P<0.001), CD44 28±3% versus 45±18% (P<0.05), and CD54 62±5% versus 82±19% (P<0.03). Contrary to control dogs, the expression of adhesion molecules did not change in AT III dogs throughout the 180-minute experiment. A repre-
sentative histogram of cell adhesion molecules obtained with the FACScan is shown in Figure 3. The expression of adhesion molecules was also evident in cells from airway edema fluid in control dogs. The absence of airway fluid in AT III dogs did not allow cell analysis.

Histological Examination
Tissue samples from control dogs demonstrated intra-alveolar edema with interstitial neutrophil migration and capillary beds filled with neutrophil aggregates (Figure 4A). AT III dogs had only small areas of edema, with nearly absent intra-alveolar and interstitial neutrophil migration (Figure 4B).

Experiment 2: Effect of the Combination of AT III and Indomethacin on Pulmonary Function Shortly After Lung Transplantation
To evaluate whether the beneficial effect of AT III administration was caused by an increase in PG\textsubscript{I\textsubscript{2}}, additional experiments were conducted in 2 dogs that received AT III and indomethacin, an inhibitor of PG\textsubscript{I\textsubscript{2}} production. In these dogs, the plasma levels of AT III were similar to those of the dogs receiving AT III alone (data not shown). At 180 minutes, however, the plasma levels of 6-keto-PGF\textsubscript{1\textalpha} (a measure of PG\textsubscript{I\textsubscript{2}} production) were lower than the values observed in any of the dogs receiving the same dose of AT III alone (Figure 5A). Furthermore, the plasma levels of 6-keto-PGF\textsubscript{1\textalpha} in dogs receiving AT III plus indomethacin were indistinguishable from those of controls throughout the 180 minutes of observation (Figure 5A).

The inhibition of PG\textsubscript{I\textsubscript{2}} production in dogs receiving AT III plus indomethacin resulted in a decrease in P\textsubscript{O\textsubscript{2}} to values that were similar to those observed in controls (Figure 5B) and markedly reduced compared with any of the AT III dogs.

Systemic and pulmonary hemodynamic parameters in dogs receiving AT III plus indomethacin were similar to those of controls (data not shown).

Discussion
The present study evaluates the effect of AT III administration on short-term lung function in a dog model of orthotopic lung transplantation. Our results show that the administration of AT III concentrates prevented both hypoxemia and the increase in pulmonary vascular resistance, both critical events that develop as a result of ischemia-reperfusion injury after lung graft implantation.

Although in controls, there was a marked impairment in pulmonary function, in dogs receiving AT III, pulmonary function was preserved during the 180 minutes after lung graft implantation. It is important to emphasize that in this model, pulmonary function is fully supported by the transplanted lung. Furthermore, the beneficial effect of AT III administration is even more relevant when one considers that the organ was preserved for 28 hours before implantation. This is in contrast to the 6 to 8 hours of ischemic time recommended for human lung transplantation.

After lung transplantation, the P\textsubscript{O\textsubscript{2}} decreased markedly in controls and remained unchanged in dogs receiving AT III.
The hypoxemia observed in controls can be explained by the increase in Q/Qs%, a ventilation/perfusion mismatch resulting from the ischemia-reperfusion injury that normally follows lung graft transplantation.11 AT III administration prevented the development of ventilation/perfusion mismatch, and the Po2 did not decrease. In control dogs, there was an increase in pulmonary vascular resistance that had systemic hemodynamic repercussions. These hemodynamic changes were also caused by ischemia-reperfusion injury. None of these hemodynamic changes were observed in dogs receiving AT III. These results strongly suggest that AT III is able to prevent the development of ischemia-reperfusion injury observed after lung graft implantation.

The mechanisms involved in the development of ischemia-reperfusion injury in the lung are not fully understood; a period of tissue ischemia followed by reperfusion results in endothelial damage, with leukocyte activation and increased production of inflammatory mediators.3 An anti-inflammatory effect of AT III has been shown in patients with sepsis and trauma4,5; in these patients, AT III administration inhibits the production of elastase, soluble cell adhesion molecules, and proinflammatory cytokines, all of which are mediators of an acute inflammatory response.5 Furthermore, in a rat model of sepsis, intravenous administration of AT III not only attenuated acute inflammation and pulmonary vascular injury but also prevented the accumulation of leukocytes in the lung.7 In our study, dogs with lung transplants receiving AT III did not show an increase in cell adhesion molecules, and the histological examination revealed almost an absence of inflammatory response; these results are in accordance with previous findings and add evidence in favor of an anti-inflammatory action of AT III. Nevertheless, the beneficial effect of AT III in sepsis is probably due to the combined anticoagulation and anti-inflammatory effects of AT III.5,17,18

The anti-inflammatory action of AT III may be mediated by its ability to stimulate the release of PGI2 by endothelial cells. The effects of PGI2 include vasodilatation, inhibition of platelet aggregation, and inhibition of leukocyte activation.19 In our dog model of lung transplantation, the administration of AT III induced an increase in PGI2 production, which confirms results by others7 and also identifies a mechanism by which AT III may prevent ischemia-reperfusion injury.

As already mentioned, in addition to its primary anti-inflammatory effect, the beneficial effect of AT III in lung graft function may also be explained by the modification of the inflammatory response due to inhibition of the coagulation cascade. Some observations may help to explain how natural anticoagulants might modulate the inflammatory response. Recent reports have demonstrated that factor VIIa, factor Xa, and thrombin act on cells directly, probably through the cleavage of cell-surface protease-activated receptors.20 Thrombin acts on protease-activated receptor 1, inducing changes in intracellular calcium20 with the subsequent activation of intracellular signals involved in cell activation.21 In the endothelium, thrombin promotes leukocyte adhesion through the elaboration of adhesion molecules and the stimulation of platelet-activating factor production, a potent agonist for neutrophils.22 Thrombin also increases IL-6 and IL-8 production from endothelial cells and monocytes.23 The fact that AT III decreases the circulating levels of thrombin may explain, at least in part, the beneficial effect observed in our dogs with lung transplants.

To determine whether the beneficial effect of AT III on lung function was mediated by the stimulation of PGI2 production, we performed an additional experiment in dogs that received both indomethacin and AT III; the inhibition of PGI2 production by indomethacin was shown by the lack of increase in the serum levels of 6-keto-PGF1α after administration of AT III. In these dogs, PO2 and hemodynamic parameters were indistinguishable from those of control dogs. After 2 experimental procedures, the results obtained were so markedly different from the group receiving AT III alone that we questioned whether the use of more animals (a pair of dogs in each experiment) was justified to demonstrate that the beneficial effect of AT III was mediated, to a large extent, by the stimulation of PGI2 production.

Taken together, our results show that AT III may prevent the inflammation and severe tissue injury observed in transplanted lungs, and the stimulation of PGI2 production by AT III seems to play an important role.

In a recent study, using a similar experimental model of lung transplantation, we showed that the administration of C1 inhibitor, another serine protease inhibitor, reduces the pulmonary dysfunction induced by ischemia-reperfusion injury.11 These findings confirm the beneficial effect of serine protease inhibitor. The long-term beneficial effect of AT III, however, which combines anti-inflammatory and anticoagulant activity, may be superior to that of C1 inhibitor. Nevertheless, more work is necessary to identify other mechanisms by which AT III exerts this beneficial effect.

Lung transplantation has become an effective therapeutic approach for a variety of patients with end-stage lung disease.
Donor lungs, however, are particularly vulnerable to ischemia-reperfusion injury. Therefore, pulmonary graft failure is still a major clinical problem. A mortality rate as high as 60\% has been reported among patients with pulmonary graft failure, and in those who survive, the clinical recovery is often protracted.\(^{24}\) Thus, even a modest reduction in the rate of pulmonary graft failure would have a significant impact on the overall long-term survival. In the present study, the observational period was limited to 180 minutes, and there was no follow-up; the beneficial effect of AT III during the 3 hours after lung transplantation, however, suggests that AT III may provide an innovative therapeutic strategy to prevent primary graft dysfunction.

In conclusion, AT III prevents ischemia-reperfusion injury in a dog model of lung transplantation. This effect is conditioned by an increase in PGI\(_2\) production. Thus, AT III may provide an innovative therapeutic strategy to prevent primary graft dysfunction.

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