Injurious Mechanical Ventilation and End-Organ Epithelial Cell Apoptosis and Organ Dysfunction in an Experimental Model of Acute Respiratory Distress Syndrome

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The acute respiratory distress syndrome (ARDS) is a serious form of acute lung injury and has a mortality rate of at least 30%.1,2 Although the most obvious clinical abnormalities are referable to the lung, the most common cause of death is dysfunction of other organs, termed multiple organ dysfunction syndrome (MODS).1,3 Multiple organ dysfunction syndrome is often irreversible, with mortality ranging from 60% to 98%.4-6 To date, there is neither an effective treatment for MODS nor an effective means for preventing its onset.

Mechanical ventilation is essential for patients with ARDS. However, animal and clinical studies have shown that mechanical ventilation can worsen pre-existing lung injury and produce ventilator-induced lung injury (VILI). The spectrum of VILI includes not only air leaks and increases in endothelial and epithelial permeability, but also includes increases in pulmonary and systemic inflammatory mediators.7-9 The importance of VILI has recently been highlighted by clinical trials demonstrating that protective ventilatory strategies were associated with decreased serum cytokine and chemokine levels,10,11 decreased levels of organ dysfunction,11,12 and decreased mortality in patients with ARDS.

Context Recent clinical trials have demonstrated a decrease in multiple organ dysfunction syndrome (MODS) and mortality in patients with acute respiratory distress syndrome (ARDS) treated with a protective ventilatory strategy.

Objective To examine the hypothesis that an injurious ventilatory strategy may lead to end-organ epithelial cell apoptosis and organ dysfunction.

Design and Setting In vivo animals: 24 rabbits with acid-aspiration lung injury were ventilated with injurious or noninjurious ventilatory strategies. In vitro: rabbit epithelial cells were exposed to plasma from in vivo rabbit studies. In vivo human: plasma samples from patients included in a previous randomized controlled trial examining a lung protective strategy were analyzed (lung protection group, n=9 and controls, n=11).


Results The injurious ventilatory strategy led to increased rates of epithelial cell apoptosis in the kidney (mean [SE]: injurious, 10.9% [0.88%]; noninjurious, 1.86% [0.17%]; \( P < .001 \)) and small intestine villi (injurious, 6.7% [0.66%]; noninjurious, 0.97% [0.14%]; \( P < .001 \)), and led to the elevation of biochemical markers indicating renal dysfunction in vivo. Induction of apoptosis was increased in LLC-RK1 cells incubated with plasma from rabbits ventilated with injurious ventilatory strategy at 4 hours (\( P = .03 \)) and 8 hours (\( P = .002 \)). The Fas-Ig, a fusion protein that blocks soluble Fas ligand, attenuated induction of apoptosis in vitro. There was a significant correlation between changes in soluble Fas ligand and changes in creatinine in patients with ARDS (\( R = 0.64, P = .002 \)).

Conclusions Mechanical ventilation can lead to epithelial cell apoptosis in the kidney and small intestine, accompanied by biochemical evidence of organ dysfunction. This may partially explain the high rate of MODS observed in patients with ARDS and the decrease in morbidity and mortality in patients treated with a lung protective strategy.

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ARDS. However, the mechanisms by which mechanical ventilation of patients with ARDS culminates in MODS and how protective ventilatory strategies would reduce mortality are unknown.

Apoptosis is an active gene-regulated mechanism of cell death that maintains cellular homeostasis under physiological conditions; dysregulation of apoptosis may contribute to the pathogenesis of a number of diseases, including acute lung injury and MODS. Inflammatory cytokines and chemokines can modulate apoptosis in various cell types including epithelial cells, and increased epithelial cell apoptosis has been detected in an animal model of sepsis, as well as in patients dying of sepsis and MODS, trauma, and shock.

We proposed that an injurious mechanical ventilation strategy could lead to end-organ epithelial cell apoptosis, and that circulating soluble factors produced by this ventilatory strategy may be involved in this process. To test the first hypothesis, we used an in vivo rabbit acid-aspiration model. The second hypothesis was tested by assessing the effect of plasma from the ventilated animals on development of apoptosis in cultured LLC-RK1 rabbit renal tubular cells. Finally, to ascertain the clinical implications of these findings, we used samples obtained from a previous clinical trial comparing a lung protective strategy with conventional mechanical ventilation, to correlate changes in soluble Fas ligand, a potent pro-apoptotic molecule with changes in renal function.

**METHODS**

**Animal Preparation**

Protocols were approved by the University of Toronto animal research committee. New Zealand white rabbits weighing 3.5 to 4.5 kg (Charles River Labs, St Constant, Quebec) were anesthetized by continuous intravenous anesthesia (ketamine hydrochloride, 10 mg/kg per hour, and xylazine, 2 mg/kg per hour); paralysis was achieved using pancuronium bromide (0.3 mg/kg per hour). An endotracheal tube was inserted through a tracheotomy. Pressure-control ventilation (Servo 300, Siemens, Solna, Sweden; fraction of inspired oxygen [FIO₂, 1.0] was used. Positive end-expiratory pressure (PEEP), peak inspiratory pressure, mean airway pressure, and tidal volume (VT) were measured using ventilator transducers. Lactated Ringers solution (10 mL/kg per hour) was infused intravenously. A carotid arterial line was used to measure arterial blood pressure (Pa 23, Gould Inc, Cleveland, Ohio) and to obtain blood for arterial blood gases (Ciba-Corning Model 248, Bayer, Leverkusen, Germany).

Hydrochloric acid (pH = 1.5, 2.0 mL/kg) was administered intratracheally, followed by a pause at an airway pressure of 30 cm H₂O. Stable mean blood pressure (56-64 mm Hg) and blood gases (PaO₂ 101-149 mm Hg; PaCO₂ 34-44 mm Hg) were required with a PEEP of 3 to 4 cm H₂O and VT of 9 to 10 mL/kg before randomization.

We used 2 ventilatory strategies: noninjurious (n = 12) with low VT (3-7 mL/kg) and high PEEP (9-12 cm H₂O), which is relatively noninjurious, and injurious (n = 12) with high VT (15-17 mL/kg) and low PEEP (0-3 cm H₂O), previously shown to be injurious. Because the pilot studies indicated that animals ventilated with a noninjurious strategy and an FIO₂ of 1.0 would have higher PaO₂, in 6 of the rabbits in the noninjurious group, we used a lower FIO₂ (0.21-0.4) to match the PaO₂ of injurious rabbits. Similarly, 6 rabbits in the injurious group had tubing inserted into the ventilator circuit to increase the dead space to match the PaCO₂ of noninjurious rabbits. Thus, the rabbits were randomly assigned (concealed-allocation method) to noninjurious with high O₂ (FIO₂ = 1.0) (n = 6), noninjurious with low O₂ (FIO₂ = 0.21-0.40) (n = 6), injurious with low CO₂ (n = 6), and injurious with high CO₂ groups (n = 6), and were ventilated for 8 hours. Respiratory rate was 45 to 55/min in all groups. Normal saline (mean [SE], noninjurious, 31.7 [2.2] mL per 8 hours; injurious, 34.2 [1.9] mL per 8 hours; P = .41) was given intravenously to replace fluid losses and to maintain a mean blood pressure of more than 50 mm Hg. Animals were killed at 8 hours with a sodium pentobarbital overdose.

Small fragments of tissue were removed from the left lung, liver, kidney, and small intestine immediately after exsanguination and prepared for electron microscopy (EM). The right lung was fixed by instilling 10% buffered formalin. The liver, kidney, and small intestine were removed and stored in 10% formalin. Control organs were obtained from 2 sham-operated rabbits that were killed after surgical preparation and stabilization.

**Measurement of Chemokines and Biochemical Markers for Organ Dysfunction**

We measured several chemokines, because they have increased in animal models of VILI and in humans with ARDS who underwent ventilation with a conventional ventilatory strategy, and because they play a role in renal apoptosis. Analyses of monocyte chemotactic protein 1 (MCP-1), interleukin 8 (IL-8), and growth-regulated oncogene (GRO) were performed with pulmonary aspirates at 8 hours, and plasma at baseline, 4 hours, and 8 hours in a blinded fashion using enzyme-linked immunosorbent assay with a lower detection limit of 100 pg/mL, with goat polyclonal IgG raised against recombinant rabbit MCP-1, IL-8, or GRO. Aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatinine, and urea nitrogen in plasma were measured at baseline, 4 hours, and 8 hours.

**Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End-Labeling Assay and EM of Lung and Distant Organs**

Apoptosis was quantitated using paraffin sections of lung and kidney (both 4-µm slices) and liver (2-µm slices) by fluorescent terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) and propidium iodide (PI) nuclear staining using the in situ apoptosis detection kit (Oncor, Gaithersburg, Md.). The TUNEL staining can identify an apoptotic positive cell; PI staining helps to identify indi-
ANIMAL MODEL OF ACUTE RESPIRATORY DISTRESS SYNDROME

Figure 1. Gas Exchange, Mean Airway Pressure, and Mean Blood Pressure

![Graphs showing gas exchange, mean airway pressure, and mean blood pressure over time.]

Values are mean (SE). Mean airway pressure and mean blood pressure were not significantly different among the groups during the 8-hour ventilatory period.

vidual cells by highlighting the nucleus of the cells making it easier to quantify cell number. Twelve randomly chosen fields of each section (72 fields for each group) were counted in a blinded fashion. Based on morphological features, we counted well-oriented renal tubular epithelial cells. An apoptotic index was calculated [100% × both TUNEL and PI-positive apoptotic nuclei]/(PI-positive nuclei)].

We used peroxidase TUNEL staining (in situ cell death detection-POD Kit, Roche, Mannheim, Germany) of small intestine, because in a pilot study it provided more distinctive intestinal epithelial morphology than fluorescent TUNEL. Four well-oriented villi and crypts from each small intestinal section were used for quantifying epithelial cell apoptosis. Ultrathin sections were cut from selected Epon blocks, mounted on copper grids, contrasted with uranyl acetate and lead citrate, and examined (Philips 201 electron microscope, NV Philips, Gloeilampenfabrieken, Eindhoven, the Netherlands) by an electron-microscopist (V.E.) and pathologist (E.C.), who were blinded to the protocol.

Studies With LLC-RK1 Cells

The LLC-RK1 rabbit renal proximal tubular cells (ATCC, Rockville, Md) were maintained in Dulbecco’s Modified Eagle Medium with 10% fetal bovine serum (GIBCO, Gaithersburg, Md), incubated in 5% CO2 at 37°C and grown to confluency. Cells were seeded into 6-well plates (Costar, Cambridge, Mass) and cultured until 60% to 80% confluence. Subsequently, the cells were incubated for 12 hours with plasma obtained from the rabbits at baseline and after 8 hours of ventilation with either 2 μg/mL of Fas:lg (Alexis, San Diego, Calif), a control protein of polyclonal IgG1κ (Alexis), or without any reagent. After a 12-hour incubation period, DNA content was measured by flow cytometric analysis.

Human Studies

To examine the clinical implications of our findings, we used plasma samples remaining from a previously described randomized clinical trial10 in which patients with ARDS had received either a conventional strategy (VT = 11.1 mL/kg; PEEP = 6.5 cm H2O) or a protective ventilatory strategy in which VT (7.6 mL/kg) was set to obtain a value of plateau pressure less than the pressure at the upper inflection point, and the PEEP (14.8 cm H2O) was set at 2 to 3 cm H2O higher than the pressure at the lower inflection point. We measured plasma soluble Fas ligand in these samples (n = 11, control; n = 9, lung protection) and examined the correlation of changes from baseline in soluble Fas ligand levels with changes in plasma creatinine levels. Soluble Fas ligand was measured by enzyme-linked immunosorbent assay (Medical and Biological Laboratories, Nagoya, Japan) with a lower detection limit of 50 pg/mL.

Statistical Analyses

Results are presented as mean (SE). Multiple regression with PaO2 and PaCO2 as the regressors was used to analyze the relationship between the following continuous variables: MCP-1, IL-8, and GRO in pulmonary aspirates; plasma MCP-1, IL-8, GRO, aspartate aminotransferase, alanine aminotransferase, lactate dehy-
Blood gases were as per protocol (FIGURE 1). Mean airway pressure and mean blood pressure were not significantly different among the groups during the 8 hours of ventilation.

**Ventilatory Strategy and Chemokines and Biochemical Markers for Organ Dysfunction**

Levels of MCP-1, IL-8, and GRO in pulmonary aspirates and plasma were significantly higher in the injurious group compared with the noninjurious group at 8 hours (TABLE). The pulmonary aspirates have no comparisons at baseline and 4 hours because we did not want to interfere with the ventilatory strategy at these times to perform aspiration.

**Ventilatory Strategy and Epithelial Cell Apoptosis in Distant Organs**

No TUNEL-positive cells were observed in lung, liver, or kidney in the control group (2 sham-operated rabbits without injection of hydrochloric acid) and very few positive cells were observed in small intestine (apoptotic index, approximately 2%). In the lung, TUNEL-positive nuclei were increased in the noninjurious group (FIGURE 2A). The apoptotic index in the lung was higher in the noninjurious group (FIGURE 3). The TUNEL signal was not increased in the liver (data not shown). In the kidney, TUNEL-positive tubular epithelial cells were numerous in the injurious group, whereas few such cells were observed in the noninjurious group (Figure 2B). Similarly, the apoptotic index of tubular epithelial cells in the kidney was higher in the injurious group (mean [SE], injurious, 10.9% [0.88%]; noninjurious, 1.86% [0.17%]; P < .001) (Figure 3). In small intestinal villi, TUNEL-positive apoptotic epithelial cells were greater in the injurious group and no differences in apoptosis were observed in the crypts of the small intestine (FIGURE 4). The apoptotic index of epithelial cells in small intestinal villi was significantly higher in the injured group in the noninjurious group [injury, 6.7% [0.66%]; noninjurious, 0.97% [0.14%]; P < .001] (Figure 3).

We performed an EM examination as an independent method of assessing apoptosis and for the identification of specific cell types involved in apopto-
sis and necrosis. Criteria used to assess apoptosis on EM samples were shrunken nuclei with chromatin condensation and fragmentation, and presence of apoptotic bodies. The EM findings were qualitatively in agreement with results obtained by the TUNEL assay. In the control group, there were no significant changes in EM findings observed in any organs. In the lung, EM demonstrated apoptotic changes in type II alveolar epithelial cells and early membrane damage in the noninjurious group, although the injurious group showed decreased apoptosis and significant injury with necrosis of type II alveolar epithelial cells (FIGURE 5A).

In the kidney, EM demonstrated mild cytoplasmic blebbing of tubular epithelial cells in the noninjurious group and more marked bleb formation and apoptosis in tubular epithelial cells in the injurious group. No necrotic changes were found in the kidney in any groups (Figure 5B). In the small intestine, EM demonstrated increased intraepithelial lymphocytes and phagolysosomes in the surface epithelium in the noninjurious group. Epithelial cells appeared generally well preserved in the noninjurious group. In contrast, in the injurious group, intraepithelial lymphocytes and phagolysosomes were increased in surface epithelium, and the epithelial cells and lymphocytes showed characteristic changes of apoptosis. No necrotic changes were found in the small intestine in any of the groups (Figure 5C). There were no significant changes in EM findings observed in the liver in both injurious and noninjurious groups (data not shown).

**Injurious Ventilatory Strategy and Apoptosis in LLC-RK1 Cells**

Incubation of LLC-RK1 cells for 12 hours with plasma obtained from the rabbits in the injurious group resulted in a larger sub-G0/G1 peak on the DNA histogram by flow cytometric analysis, which represents a population of cells with reduced DNA stainability suggesting apoptosis (data not shown).33,34
Compared with the noninjurious group, induction of apoptosis (percentage change from baseline) was increased in cells incubated with plasma obtained from rabbits in the injurious group at 4 hours ($P = .03$) and 8 hours ($P = .002$) (Figure 6). Viability of LLC-RK1 cells incubated for 12 hours with plasma obtained from ventilated rabbits in either the noninjurious or the injurious group was not impaired (viability in all experiments $>97\%$).

**Soluble Fas Ligand Blocking in LLC-RK1 Cells Incubated With Plasma From the Injurious Group**

Because our results indicated that LLC-RK1 cell apoptosis was induced by plasma from the injurious group, we examined whether soluble Fas ligand was involved in this process by using Fas: Ig. The Fas: Ig reduced the induction of apoptosis, calculated as a percentage of control apoptotic cells (ie, cells incubated with no plasma from ventilated rabbits), in response to treatment with the 8-hour plasma obtained from the injurious group (mean [SE], 8-hour plasma only, 180% [12.0%]; 8-hour plasma + control protein, 182% [13.3%]; 8-hour plasma + Fas: Ig, 141% [9.6%]; $P = .88$ [8-hour plasma only vs 8-hour plasma + control protein]; $P = .03$ [8-hour plasma only vs 8-hour plasma + Fas: Ig]; $P = .02$ [8-hour plasma + control protein vs 8-hour plasma + Fas: Ig]). The Fas: Ig did not have a significant effect on the apoptosis induced by baseline plasma. The control protein did not have any effect on the apoptosis induced by the injurious group plasma, either at baseline or at 8 hours.

**Soluble Fas Ligand Levels and Creatinine Levels in Patients With ARDS**

Mean (SE) plasma soluble Fas ligand levels (pg/mL) at time 1 (24-30 hours after study entry) and time 2 (36-40 hours after study entry) were significantly higher in the control group (time 1, 250 [21.3] pg/mL; time 2, 343 [42.1] pg/mL) compared with the protective ventilatory strategy group (time 1, 195 [13.6] pg/mL, $P = .047$; time 2, 239 [12.3] pg/mL, $P = .03$). There was a significant correlation between changes in soluble Fas ligand (values at time 2 – values at entry) with changes in creatinine (values at 72 hours after admission – values at entry) ($R = 0.64$, $P = .002$) (Figure 7).

**COMMENT**

We present evidence suggesting a novel mechanism of remote organ injury resulting from VILI. Our in vivo data demonstrate that an injurious ventilatory strategy administered to the lung can lead to epithelial cell apoptosis in organs distal to the lung, such as the kidney, and our in vitro data suggest that 1 of the mechanisms mediating this effect could be related to circulating soluble factors, such as soluble Fas ligand. Examination of samples obtained from a previous clinical trial demonstrated a correlation between changes in soluble Fas ligand and changes in plasma creatinine. This...
mechanism of ventilator-induced end-organ dysfunction may also partially explain the high rate of MODS observed in patients with ARDS who have undergone mechanical ventilation, and explain the decrease in mortality observed in a recent ARDS Network multicenter clinical trial using a protective ventilatory strategy.11

The elevation of biochemical markers indicating renal dysfunction is compatible with studies demonstrating that kidney dysfunction is common in MODS.37 In a recent study, we found a higher incidence of renal failure in patients who have undergone ventilation with conventional strategy compared with a lung protective strategy.12

and a recent randomized controlled trial found increased renal failure–free days in the group receiving the protective ventilatory strategy.13 Renal tubular cell apoptosis has emerged as a final common pathway to renal injury and dysfunction in response to a wide variety of cellular insults.38 Plasma obtained from rabbits that underwent the injurious ventilation strategy induced greater apoptosis in cultured LLC-RK1 cells in vitro, suggesting that circulating soluble factors associated with the injurious mechanical ventilation might be involved in this process. However, in our in vivo studies, we cannot be certain that apoptosis was the cause or the consequence of cell injury, and both mechanisms can coexist. Indeed, renal tubular epithelial cells have been shown to undergo apoptosis following ischemia reperfusion injury,39 toxic injury,40 sepsis,41 and disseminated intravascular coagulation,42 although this finding is not universal.23

The Fas-Fas ligand system is composed of the membrane receptor Fas (CD95), a 45-kDa membrane receptor that belongs to the tumor necrosis factor-α family of proteins, and its natural ligand, Fas ligand. Fas ligand exists as a membrane-bound form and a soluble
form (soluble Fas ligand), both of which can activate Fas. The Fas-Fas ligand system is involved in renal tubular epithelial cell apoptosis in renal failure, endotoxemia, and disseminated intravascular coagulation. In the present study, Fas:lg, a fusion protein that binds and blocks soluble Fas ligand, attenuated the rate of apoptosis in LLC-RKI cells induced by coincubation with plasma of the injured group. A previous study also showed that Fas:lg blocked alveolar epithelial cell apoptosis and lung injury in rabbits. Soluble Fas ligand has been shown to induce alveolar epithelial cell apoptosis and lung injury in rabbits and human distal lung epithelial cells in vitro. Soluble Fas ligand is increased in bronchoalveolar lavage of patients with ARDS and in serum of patients with cardiopulmonary arrest and chronic renal failure. We found that plasma soluble Fas ligand was increased in patients with ARDS who underwent ventilation with a conventional strategy compared with the lung protective strategy, and changes in plasma creatinine were correlated with changes in soluble Fas ligand, suggesting but not proving a role for soluble Fas ligand in the renal dysfunction.

We also found apoptosis in epithelial cells of the villi of the small intestine of the rabbits that underwent ventilation with the injurious ventilatory strategy. Although apoptosis is a normal physiological process regulating cell turnover of the intestine, the degree of apoptosis in the injurious group was far in excess of baseline apoptosis present in the control group. The extent of small intestinal epithelial cell apoptosis in the injurious group is consistent with observations in an animal model of cecal ligation and puncture and Pseudomonas aeruginosa pneumonia–induced sepsis, as well as in patients dying of sepsis and multiple organ dysfunction and trauma. It has been proposed that translocation of bacrovin from the intestine is the driving force of the MODS. Our data suggest a mechanism by which this may occur in the context of VILI with a cascade of mediators leading to increased intestinal apoptosis, which could lead to bacterial translocation and subsequent organ dysfunction.

The decreased apoptosis and the increased necrosis of type 2 cells observed in the EM findings in the lungs of rabbits that underwent ventilation with the injurious strategy was opposite to those observed in the kidney and the small intestine villi. These data are in agreement with other models of lung injury, such as rat ischemia-reperfusion injury after lung transplantation in which low levels of injury caused high levels of apoptosis, whereas increased lung injury was associated with decreased apoptosis and increased necrosis. In the present study, the lungs were exposed to 2 hits, acid aspiration and VILI, producing relatively severe injury. It is possible that with milder injury, a greater degree of apoptosis would have been obtained in the lungs.

Numerous studies have shown the role of inflammatory mediators including chemokines and cytopathic hypoxia in systemic inflammatory response syndrome and MODS. We found increases in plasma chemokines (MCP-1, IL-8, and GRO) in the injurious group. The MCP-1 has been shown to promote renal tubular cell apoptosis and renal dysfunction in vivo. Apoptosis has been suggested as a final common pathway accounting for the progression of the exaggerated inflammatory response leading to MODS. Our data support this concept and suggest a mechanism to explain the increased organ failure free days of the ARDS Network trial in which patients with ARDS who underwent ventilation with the protective ventilatory strategy, although from the present study, we cannot definitively conclude whether the end-organ apoptosis is the cause or the consequence of cell injury.

In conclusion, an injurious ventilatory strategy can lead to increased epithelial cell apoptosis in the kidney and small intestine, accompanied by abnormal elevations of biochemical markers for organ dysfunction compared with a noninjurious ventilatory strategy in vivo. Circulating pro-apoptotic soluble factors, such as soluble Fas ligand, may be involved in this process. Together, these data suggest that mediators enhancing end-organ epithelial cell apoptosis may be reasonable therapeutic targets for MODS related to mechanical ventilation in patients with ARDS.

**Figure 7.** Correlation Between Plasma Soluble Fas Ligand and Plasma Creatinine in Patients With Acute Respiratory Distress Syndrome

There was a significant correlation between changes in plasma soluble Fas ligand (36-40 hours after entry minus values at entry) with changes in plasma creatinine (72 hours after entry minus values at entry) from patients with acute respiratory distress syndrome (R=0.64, P<.002).

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