Background—Acute lung injury (ALI) after cardiopulmonary bypass (CPB) results from sequential priming and activation of neutrophils. Activated neutrophils release neutral serine, elastase, and matrix metalloproteinases (MMPs) and oxygen radical species, which damage alveolar-capillary basement membranes and the extracellular matrix, resulting in an ALI clinically defined as adult respiratory distress syndrome (ARDS). We hypothesized that treatment with a potent MMP and elastase inhibitor, a chemically modified tetracycline (CMT-3), would prevent ALI in our sequential insult model of ALI after CPB.

Methods and Results—Anesthetized Yorkshire pigs were randomized to 1 of 5 groups: control (n = 3); CPB (n = 5), femoral-femoral hypothermic bypass for 1 hour; LPS (n = 7), sham bypass followed by infusion of low-dose Escherichia coli lipopolysaccharide (LPS; 1 µg/kg); CPB + LPS (n = 6), both insults; and CPB + LPS + CMT-3 (n = 5), both insults plus intravenous CMT-3 dosed to obtain a 25-µmol/L blood concentration. CPB + LPS caused severe lung injury, as demonstrated by a significant fall in PaO\textsubscript{2} and an increase in intrapulmonary shunt compared with all groups (P < 0.05). These changes were associated with significant pulmonary infiltration of neutrophils and an increase in elastase and MMP-9 activity.

Conclusions—All pathological changes typical of ALI after CPB were prevented by CMT-3. Prevention of lung dysfunction followed an attenuation of both elastase and MMP-2 activity. This study suggests that strategies to combat ARDS should target terminal neutrophil effectors. (Circulation. 1999;100:400-406.)

Key Words: lung — metalloproteinases — cardiopulmonary bypass

Each year more than 100 000 people die of complications of the adult respiratory distress syndrome (ARDS).\textsuperscript{1} Despite significant advances in critical care management, mortality from ARDS remains >40%.\textsuperscript{2-4} ARDS after cardiopulmonary bypass (CPB) is often called “postperfusion” or “postpump” syndrome, but it is otherwise indistinguishable from acute lung injury associated with trauma, hemorrhage, or sepsis.

We have previously demonstrated that acute lung injury after CPB can develop after consecutive minor insults, with CPB acting as the initial inflammatory event.\textsuperscript{5} A short period of CPB alone is rather innocuous, but when it is combined with a subsequent, seemingly benign insult (ie, transient hypoxia, ischemia, endotoxemia), the result is an overwhelming inflammatory response leading to endothelial injury, pulmonary edema, and ARDS. It has been well documented that the lung injury in both ARDS\textsuperscript{6,7} and specifically postpump syndrome\textsuperscript{8-9} is neutrophil-mediated. Our previous investigations support this concept, because we have shown an association between pulmonary neutrophil sequestration and physiological lung injury.\textsuperscript{5} Thus, postpump syndrome provides an excellent model of ARDS, since it allows us to investigate the sequence of pathophysiological changes because the timing of the priming and activating stimuli are known.

Activation of sequestered neutrophils leads to the release of proteases and oxygen radical species. Elevated levels of neutrophil elastase and matrix metalloproteinases (MMPs) are present in plasma of patients after CPB\textsuperscript{10,11} and in both plasma and bronchoalveolar lavage (BAL) fluid of patients with ARDS.\textsuperscript{12} MMPs released from activated neutrophils degrade type IV collagen, which provides the framework for the basement membrane of pulmonary capillaries, and interstitial collagen and proteoglycan.\textsuperscript{13,14} Aside from direct collagenolysis, MMPs inactivate endogenous antiproteases, allowing unrestricted protease activity.\textsuperscript{15} Studies by Golub et al...
have confirmed that, by nonantimicrobial mechanisms, chemically modified tetracyclines (CMTs) can directly inhibit MMPs and prevent activation of pro-MMPs to MMPs by scavenging reactive oxygen species.\(^5\) This inhibits direct collagenolysis and protects against inactivation of endogenous antiproteases. We hypothesized that treatment with a CMT-3 will attenuate both MMP collagenolytic activity and MMP-induced inactivation of \(\alpha_1\)-protease inhibitor to prevent basement membrane degradation and protect against the development of acute lung injury in our porcine model.

Methods

Surgical Preparation

Surgical preparation required for our model of acute lung injury after CPB has been described extensively elsewhere.\(^6\) Briefly, healthy Yorkshire hybrid pigs (15 to 20 kg) were anesthetized with sodium pentobarbital (50 mg/mL IV bolus, then 6 mg·kg\(^{-1}\)·h\(^{-1}\)) and bolus infusion of pancuronium bromide. Instrumentation included a transthoracic and intravascular catheters to monitor both systemic and pulmonary arterial and venous pressures. Initial ventilator settings were \(\text{FiO}_2=50\%\), tidal volume = 12 mL/kg, and rate = 10 breaths per minute. Adjustments were made in the respiratory rate to obtain a baseline \(\text{PaCO}_2\) of 45 to 55 mm Hg. Heating pads and warmed IV fluids maintained core temperature between 36°C and 38°C. With the exception of measurements while animals were on CPB, all blood gas measurements were made at 37°C. The potential error is pH ± 0.01, \(\text{PaCO}_2\) ± 2 mm Hg, and \(\text{PaO}_2\) ± 5 mm Hg. All groups received lactated Ringer’s solution (25 mL·kg\(^{-1}\)·h\(^{-1}\)) and bolus infusion of dextan 70 to maintain cardiac output within 10% of baseline.

Cardiopulmonary Bypass

The technique for initiating CPB has been described in detail elsewhere.\(^6\) Briefly, with verification of adequate anticoagulation (activated clotting time >480 seconds), femoral canulas were placed. The pump priming solution consisted of lactated Ringer’s solution (1500 mL), mannitol (5 g), sodium bicarbonate (35 mEq), and porcine lung heparin (300 U/kg). Nonpulsatile, hypothermic CPB was initiated at a flow rate of 120 mL/kg and continued for 1 hour. During weaning, isotropic therapy (4 µg/min) facilitated effective ejection volume to eliminate cardiac distention and prevent ischemia and/or dysrhythmias. Within 30 minutes, animals had returned to baseline status, defined as (1) all blood within the oxygenator transfused back into the animal, (2) heparin reversed with protamine (1.3 mg/100 U heparin), (3) normothermic body temperature, and (4) pulmonary pressures, systemic pressure, and cardiac output all within 10% of baseline without assistance from inotropic agents. Animals not randomized to an arm exposed to CPB received sham CPB (surgical preparation without anticoagulation or bypass).

Endotoxin Infusion

Pigs receiving LPS were infused with 1 µg/kg of *Escherichia coli* lipopolysaccharide (LPS; Sigma 111-B4) mixed in 500 mL of saline and delivered over 1 hour via a volumetric infusion pump (Flo-Guard 8000, Travenol Inc). Pigs randomized to an arm not exposed to LPS received sham LPS (500 mL saline vehicle only).

Chemically Modified Tetracycline

CMT-3 (6-demethyl-6-deoxy-4-dimethylaminotetracycline) is a chemically modified, nonantibiotic tetracycline with a molecular weight of 371.35. CMT-3 (Collagenex Pharmaceuticals) was obtained in powder form, dissolved in dimethyl sulfoxide, and then administered intravenously at a dose to achieve a blood concentration of 25 µmol/L (9.2 µg/mL). We assumed predominantly intravascular distribution for our dosing regimen. This method of delivery only achieved a serum concentration of 1.2±0.367 µg/mL at 1 hour as determined by high-performance liquid chromatography.

Calculations

Calculation of venous admixture was performed on an Explorer cardia output computer (Baxter Healthcare Corp) by the following equation:Venous admixture \(Q_{\text{a}}/Q_{\text{t}}\) = [(Hgb\(\times\)1.38) + (0.0031×\(\text{PaO}_2\)-\(C\text{aO}_2\))] / [(Hgb\(\times\)1.38) + (0.0031×\(\text{PaO}_2\)-\(C\text{aO}_2\))], where \(C\text{aO}_2\) and \(C\text{VO}_2\) are arterial and venous blood oxygen content, Q, is venous admixture blood flow, Q, is total blood flow, and \(\text{PaO}_2\) is the partial pressure of alveolar oxygen. \(C\text{aO}_2\), \(C\text{VO}_2\), and \(\text{PaO}_2\) were calculated by use of the following equations: \(C\text{aO}_2\) = (0.0138×Hgb\(\times\)\(\text{SaO}_2\))+0.0031×\(\text{PaO}_2\); \(C\text{VO}_2\) = (0.0138×Hgb×\(\text{SvO}_2\))+0.0031×\(\text{PvO}_2\); and \(\text{PaO}_2\) = (\(\text{FiO}_2\)×\(\text{FiO}_2\))−\(\text{FiO}_2\)×\(\text{FiO}_2\)−(1−\(\text{FiO}_2\))+0.8). Arterial (\(\text{SaO}_2\)) and venous (\(\text{SvO}_2\)) saturations were measured with the OSM3. Ventilatory efficiency index (VEI) has been previously validated\(^7\) and was calculated with the equation VEI (mL·kg\(^{-1}\)·cm\(\text{H}_2\text{O}\)^{-1}) = (5 mL·kg\(^{-1}\)·min\(^{-1}\))/(\(\Delta P\times\text{FiO}_2\times\text{PaCO}_2\)/760), where \(\Delta P\) is the difference between peak- and end-expiratory pressures (mm Hg) and RF is respiratory frequency. The VEI is described in units analogous to compliance and was calculated assuming that the rate of total \(\text{CO}_2\) production was constant at 5 mL·kg\(^{-1}\)·min\(^{-1}\) and \(\text{PaCO}_2=\text{Paco}_2\). The index allowed comparison of respiratory status among animals whose airway pressures, respiratory rates, and \(\text{Paco}_2\) vary throughout the experiment.

Bronchoalveolar Lavage

At necropsy, the bronchus to the left lower lobe was cannulated and secured so that it was isolated from the remaining bronchial tree. Saline (60 mL) was then injected as 3 aliquots of 20 mL each. Each aliquot was injected quickly and then withdrawn slowly 3 times to obtain an optimal BAL specimen. Combined aliquots of BAL fluid were spun at 1000g for 10 minutes to remove cells. Supernatant was frozen at −70°C for subsequent chemical analysis.

Gelatinase Activity

The methods for purification of collagenase have been fully described elsewhere.\(^8\) Briefly, 100 µL of 1× collagenase buffer ([Tris 0.50 mmol/L, NaCl 0.2 mol/L, 5 mmol/L CaCl\(_2\), 0.02% Brij]) was added to 900 µL of BAL fluid. Seventy microliters of this mixture plus 10 µL of 1.0 mmol/L aminophenyl mercuric acetate plus 10 µL of soybean trypsin inhibitor (300 mg/mL) was incubated at room temperature for 1 hour. Next, 10 µL of radiolabeled (tritium) type I rat skin gelatin was added and incubated at 37°C for 4 hours. Then 50 µL of cold gelatin (2 mg/mL) and 100 µL of 45% trichloroacetic acid were added, and the entire mixture was cooled at 4°C for 30 minutes. The reaction mixture was centrifuged at 13 000g for 15 minutes. A 100-µL aliquot was removed to determine the amount of radioactivity released into the supernatant by liquid scintillation counting. Gelatinase activity was determined as % gelatin lyzed=\(\text{DPM in 100 µL supernatant−DPM of the blank}\times2.5\)/\(\text{DPM in 10 µL substrate}\). Ten microliters of the substrate contained 10 µg of the gelatin. By multiplying lys by the substrate concentration and dividing by the time of incubation, we were able to calculate the quantity of substrate degraded per milligram of protein per hour.

Elastase Activity

Elastase activity was determined by incubating 100 µL of the BAL fluid and 400 µL of the 1.25 mmol/L specific synthetic elastase substrate methoxysuccinyl-Ala-Ala-Pro-Val-nitroanilide in a 96-well ELISA plate at 37°C for 18 hours. After incubation, the optical density was read at 405 nm. Data were expressed as nanomoles elastase substrate degraded per milligram of protein per hour. These methods are described in full detail elsewhere.\(^8\)

BAL Protein

BAL protein analysis was based on the Bradford protein assay (BioRad) with albumin as the standard. Standards ranged from 70 µg/mL to 1.40 mg/mL. Twenty milliliters of Coomassie blue dye solution was diluted to 100 mL with saline. Either 100 µL of standard solution or 100 µL of BAL fluid was added to 5 mL of Coomassie blue solution, and the optical density was read at 575 nm.
in a spectrophotometer. The results were reported as micrograms of protein per 100 μL of BAL fluids.

Neutrophil Infiltration

At necropsy, the right middle lobe was excised and its bronchus cannulated. Glutaraldehyde fixative (2.5%, phosphate-buffered) was slowly instilled until air was no longer displaced from the bronchus. The lung was immersed in glutaraldehyde, and additional fixative was infused with a syringe until airway pressure of the fixative stabilized at 25 mm Hg. The cannula was clamped, and the lobe was stored in glutaraldehyde at room temperature for 24 hours. One tissue block from the fixed lobe of each animal was randomly chosen and processed for routine paraffin sectioning. Ten serial sections 7 μm thick were made, individually mounted, and numbered consecutively. A random selection of either odd or even sections was stained with hematoxylin and eosin for histological assessment. On each of the 5 sections per animal, a randomly placed sampling probe, consisting of 10 equidistant sampling points each, was established along the vertical axis. This method avoided overlap of sampling probes between sections from the same animal. Each area was located with the vernier scales of the microscope stage and then viewed with ×100 oil immersion through a high-resolution video camera. Areas featuring bronchi, connective-tissue septa, or blood vessels other than capillaries were discarded by advancing the stage 0.5 mm along the vertical axis of the section. This process limited quantification of neutrophils to the alveoli and interstitium only. Total neutrophil count was obtained in all focal planes from a sampling area of 6400 μm².

Lung Water

Representative tissue samples from both lungs were sharply dissected free of nonparenchymal tissue, with care taken to avoid contact with the tissue. Samples were placed in a dish and weighed. The specimen was then oven-dried at 65°C for 24 hours and reweighed. Lung water was expressed as a ratio of wet to dry weight.

Protocol

See Figure 1 for a schematic. The control (n=3) group consisted of animals subjected to 1 hour of sham CPB (surgical preparation without bypass) followed by sham LPS (infusion of saline vehicle only) over 60 minutes. Animals were euthanized at 270 minutes (3 hours after onset of LPS/sham LPS).

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Protocol

See Figure 1 for a schematic. The control (n=3) group consisted of animals subjected to 1 hour of sham CPB (surgical preparation without bypass) followed by sham LPS (infusion of saline vehicle without LPS) and then monitored for 2 hours. Neither heparin nor protamine was given.

The CPB (n=5) group consisted of animals subjected to 1 hour of CPB followed by LPS infusion and monitored for 2 hours. Neither heparin nor protamine was given.

The CPB+LPS (n=6) group consisted of animals subjected to 1 hour of CPB followed by LPS infusion and monitored for 2 hours. The CPB+LPS+CMT (n=5) group consisted of animals subjected to 1 hour of CPB followed by LPS infusion and monitored for 2 hours.

Statistics

Differences between physiological parameters, neutrophil count, and protease activity were assessed by 1-way ANOVA with Newman-Keuls post hoc analysis for between-group comparisons and a repeat ANOVA for within-group comparisons. Mortality rate between groups was analyzed by Fisher’s exact test. All evaluations used a 95% CI.

Animals

Animals were euthanized with an overdose of pentobarbital (90 mg/kg IV). Experiments described in this study were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals in research. The protocol was approved by the Committee for the Humane Use of Animals at our institution.

Results

Physiological Changes

CPB+LPS was the only group that developed severe physiological lung injury typical of ARDS. Survival in this group was 60% at 270 minutes, compared with 100% in all other groups. Lung injury manifested as a significant fall in arterial oxygenation (Figure 2), a fall in the VEI (Figure 3), and an increase in venous admixture (Figure 4). The VEI fell in all groups subjected to CPB. However, VEI improved in the group treated with CMT, to remain significantly better than the CPB+LPS group at 270 minutes (Figure 3). There was a significant, progressive decrease in cardiac output over time in all groups. Cardiac output was significantly lower at 60 minutes in groups undergoing CPB because venous return limited pump flow to 2 L/min. LPS infusion without CMT treatment caused a significant increase in pulmonary artery pressure by 150 minutes (Table 1). No significant change was noted in the pulmonary artery occlusion pressure for all groups.
Biochemical Changes

BAL analysis demonstrated a significant increase in elastase and gelatinase (Figure 6) activity in the CPB+LPS group compared with all other groups. Both elastase and gelatinase activities were reduced to levels observed in controls with CMT-3 treatment. Furthermore, total protein in BAL fluid increased significantly in animals exposed to LPS and increased still further with CPB+LPS (Table 2). However, treatment with CMT-3 ablated the increase in BAL protein seen in the CPB+LPS group (Table 2). Lung water, as determined by the ratio of wet to dry weight, was highest in the CPB+LPS group but was not statistically different from all other groups (Table 2). Lung water in the CPB+LPS group remained insignificantly different because there was a single animal (1 of 6) that did not demonstrate a measurable increase in lung water, although gross parenchymal edema was noted, as with all animals randomized to this group. Repeat analysis without this animal achieved significance (P<0.05). Nonetheless, because all other physiological, histological, and biochemical parameters for this single animal were as anticipated for the CPB+LPS group and because no obvious technical failure was identified, we did not have
legitimate reason to exclude the single poor responder in the results for lung water.

**Discussion**

This study confirms our previous investigations, which have demonstrated that acute lung injury after bypass can be caused by sequential, seemingly innocuous insults. These findings support the theory that accepts the mechanism of postpump syndrome as a neutrophil-mediated destruction of the alveolar-capillary interface. In our model, CPB primes neutrophils, causing them to sequester in the lung, and subsequent exposure to low-dose LPS activates these primed neutrophils, causing degranulation and the release of oxygen radical species, serine proteases, and MMPs. The CPB+LPS group demonstrated a high-protein pulmonary edema with an insignificant change in pulmonary artery occlusion pressure and a pulmonary artery pressure that increased significantly over time, findings consistent with noncardiogenic pulmonary edema. The process was associated with damage to the pulmonary endothelium, high-permeability edema, and acute lung injury. CMT-3 treatment reduced both elastase and MMP activity, decreased neutrophil infiltration into the pulmonary interstitium, decreased extracapillary extravasation of protein, and prevented the physiological lung dysfunction typical of clinical ARDS.

The CMT-induced reduction of BAL elastase and MMPs could reflect direct inhibition of enzyme, diminution of neutrophil infiltration, inhibition of neutrophil degranulation, or a combination of these possibilities. Treatment with CMT-3 decreased pulmonary neutrophil infiltration compared with CPB+LPS without treatment. Importantly, neutrophil infiltration was reduced only to the level observed in animals that received a single insult (CPB or LPS), a level significantly greater than control. However, CMT treatment completely ablated elastase and MMP activity, with enzyme levels similar to controls at 6 hours. This suggests that
prevention of lung injury by CMT was due, in part, to direct enzyme inhibition and not solely to a reduction in neutrophil infiltration.

It is well established that the initial response to a systemic inflammatory stimulus includes sequestration of neutrophils in the pulmonary capillaries and their elaboration of cytokines and enzymes.\textsuperscript{5–9} Multiple investigators have demonstrated large numbers of neutrophils sequestered in the lung during both ARDS\textsuperscript{6} and postpump syndrome.\textsuperscript{5,7–9} The local cytokine activity serves to activate sequestered neutrophils, which then secrete numerous mediators. These neutrophil-derived bioactive lipids, cytokines, oxygen metabolites, and granular enzymes all have the capability of injuring basement membranes and the extracellular matrix. Those of greatest importance are toxic oxygen radicals, neutral serine proteases, and MMPs.\textsuperscript{19,20}

Although the progression of neutrophil-mediated lung injury is very complex, it is evident that the destruction of the endothelial basement membrane has a central role.\textsuperscript{13,14} The basement membrane of the capillary endothelium and the alveolar epithelium is a complex of type IV collagen. MMPs and serine proteases are the primary neutrophil products that can target the basement membrane. Elastase and MMP-9 (92-kDa type IV collagenase) are present in neutrophils and released on activation. It has been shown that the number of neutrophils in the BAL fluid correlates with an increase in MMP-9 levels and that quantities of both MMP-9 and MMP-2 are elevated in the BAL fluid of patients with ARDS.\textsuperscript{12} In addition, the level of MMPs correlates directly with an increase in the concentration of degradation products from type IV collagen within the basement membrane.\textsuperscript{12} Our data again support these findings, demonstrating significantly increased activity for both elastase and gelatinase in the BAL fluid of animals exposed to sequential insults.

Oxygen metabolites released during neutrophil activation include superoxide (O\textsubscript{2}\textsuperscript{-}), along with the hydroxyl ion (\textit{OH}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). In the presence of H\textsubscript{2}O\textsubscript{2} and free chloride, myeloperoxidase from the neutrophil forms hypochlorous acid (HOCI). HOCI was initially felt to be directly cytotoxic; however, recent evidence has demonstrated that HOCI is far less toxic than commonly assumed. The more important role of HOCI in lung injury may be its capacity to oxidize \textit{α}-protease inhibitor, an important endogenous antiprotease. This results in unopposed serine protease activity and consequent degradation of the basement membrane and the interstitial matrix.\textsuperscript{19,20}

When devising a plan to prevent or treat acute lung injury after CPB, 4 distinct strategies could be used to protect against neutrophil-mediated injury. First, because neutrophil activation is central to the pathogenesis of ARDS, an obvious approach would be to simply deplete the number of circulating neutrophils. As a prophylactic measure, this strategy is plausible during CPB but is impractical in treatment of all causes of ARDS. As a treatment strategy, this would not be functional, because a large percentage of neutrophils are already sequestered by the time a diagnosis of acute lung injury is made, rendering them impervious to a leukoreduction filter. In addition, pure leukodepletion will inhibit the activity of neutrophils toward humoral immunity and the phagocytic clearance of pathogens.

Another strategy would be to modulate neutrophil surface-receptor binding of mediators or to scavenge the very cytokines and mediators that influence primed neutrophils. A third treatment strategy would be to modulate the signal transduction pathways or the synthesis of mediators within the neutrophil itself. The problem with the latter 2 strategies is that the control of bioactive lipids, cytokines, and cell signal pathways would influence multiple cell lines, not neutrophils alone. In addition, clinical trials using these strategies have had limited success.

A final approach for the treatment of ARDS, investigated in this study, targets the neutrophil-specific terminal effectors. This strategy provides a substance (CMT) that antagonizes or neutralizes active neutrophil-derived mediators, specifically the oxygen metabolites, serine proteases, and MMPs. In their comprehensive review, Gadek and Pacht\textsuperscript{1} determined that ARDS develops from a protease-mediated destruction of the alveolar-capillary basement membrane that results from an imbalance in the antioxidant-antiprotease ratio within the pulmonary parenchyma. Tetracycline-based drugs have been
used as antibacterial agents for decades. Numerous studies have demonstrated that tetracyclines and their chemically modified, nonantimicrobial analogues (CMTs) inhibit MMP activity and prevent the activation of MMP precursors (pro-MMPs) by HOCl.16–25 This inhibition of MMPs not only blocks collagenolysis but also prevents α1-protease inhibitors from becoming inactivated either directly by MMPs or indirectly by oxidation from HOCl.26 This results in preserved antiprotease (antielastase) function, which attenuates neutrophil elastase activity and further protects the basement membrane.21

The inflammatory signals that provoke the cascade of events leading to acute lung injury after CPB cannot always be anticipated or prevented. A therapeutic alternative is to treat the downstream, neutrophil-derived effectors. We have isolated the role of enhanced activity of neutrophil-derived serine proteases and MMPs and provided a clear association with the physiological and histological aberrations typical of clinical ARDS. This study strongly suggests that strategies to combat acute lung injury should target the terminal effectors of neutrophil activation. Furthermore, our data confirm that CMT tempers the activity of these neutrophil-derived MMPs and serine proteases. CMTs may provide a useful new therapy for the prevention and treatment of acute lung injury after CPB.

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Matrix Metalloproteinase Inhibitor Prevents Acute Lung Injury After Cardiopulmonary Bypass

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