Acute Uremia but Not Renal Inflammation Attenuates Aseptic Acute Lung Injury: A Critical Role for Uremic Neutrophils

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Acute renal failure (ARF) remains a major clinical challenge, especially in the intensive care setting. Mortality of ARF combined with acute lung injury (ALI) is even higher and may reach 80%. Recent studies have suggested a remote effect of ARF on pulmonary homeostasis. However, it is unknown whether and to what extent ARF clinically affects pulmonary function, in particular oxygenation. For elucidation of the impact of ARF on aseptic ALI, a murine two-hit model that consists of acute uremia (AU) and subsequent ALI was developed. AU was induced by renal ischemia-reperfusion (inflammatory AU) or bilateral nephrectomy (noninflammatory AU). ALI was initiated by intratracheal HCl instillation and characterized by severe, PMN-dependent decrease in arterial partial pressure of O2 (>70%) in nonuremic mice. Uremic mice, by contrast, showed a significant protection from ALI (decrease in arterial partial pressure of O2 <40%); this was independent of the type of AU. Reconstitution experiments, in which uremic neutrophils were injected into nonuremic mice and vice versa, identified uremic neutrophils as the primary mediators. Between normal and uremic neutrophils, there were no differences in apoptosis or superoxide production. Pulmonary recruitment of uremic neutrophils, however, was significantly attenuated compared with that of normal neutrophils. This defect was associated with altered surface expression of L-selectin; sialyl Lewisx, an L-selectin counterreceptor, previously was proved to be critical in aseptic ALI. In conclusion, it is shown that AU but not renal inflammation attenuates aseptic, neutrophil-dependent ALI and exerts an anti-inflammatory effect by attenuating pulmonary neutrophil recruitment.


Acute renal failure (ARF) represents a common problem in critically ill patients (1). Acute tubular necrosis, induced by ischemia or nephrotoxins, is among the leading causes of ARF in the critical care setting (2). ARF itself also is an independent risk factor for death in critically ill patients (3).

Acute lung injury (ALI) delineates another frequent and potentially devastating syndrome in critically ill patients (4–6). The underlying cause can be divided into either extra- or intrapulmonary. Whereas sepsis, trauma, or massive blood transfusion can lead to extrapulmonary ALI, pneumonia and acid aspiration are typical causes of intrapulmonary ALI.

The combination of both ARF and ALI in critically ill patients gives rise to excessively high mortality rates (7) and thereby raises the question of whether renal (dys)function can modulate pulmonary function (or vice versa). Despite obvious and eminent clinical relevance, experimental studies to explore the interactions between ARF and ALI are virtually nonexistent. Currently available studies describe only the impact of ARF on the healthy lung. These findings suggest that the impact of ARF on lung function goes beyond simple fluid overload. In rat models of either renal ischemia-reperfusion (RIR) or bilateral nephrectomy (NET), Rabb et al. (8) found a downregulation of pulmonary epithelial Na\textsuperscript{+}/H\textsubscript{+} channels, Na-K-ATPase, and aquaporin 5. Kramer et al. (9) demonstrated an increase in pulmonary vascular permeability in rats after RIR. In mouse models of ARF, RIR also led to an increase in wet/dry-weight ratios, upregulation of various signaling processes (10), enhanced expression of adhesion molecules, and neutrophil recruitment into the lung (9).

Although these data clearly suggest ARF-induced pulmonary inflammation, they do not provide clinically applicable data. So far, only one experimental study has been able to show a clear impact of ARF on extrarenal organ function (11). In a rat model of ARF, RIR significantly impaired cardiac function as assessed by echocardiography. RIR also increased both systemic and tissue concentrations of TNF-α.

One can speculate from these findings that the effects of acute (postischemic) renal failure on remote organ (dys)function are due to humoral and/or cellular mediators that circulate in the
blood. The amount of data available regarding cellular and/or humoral components that are involved in clinically relevant remote organ injury during ARF is limited. So far, only a few serum and tissue cytokine profiles after RIR are available; blockade of TNF-α in this setting significantly reduced apoptosis in cardiac myocytes (12). Conversely, the impact of chronic renal failure on certain (inflammatory) diseases, such as atherosclerosis, is well understood. Chronic uremia currently is considered a constant, low-grade, systemic, inflammatory state with altered cytokine homeostasis (13). In addition to an imbalance between proinflammatory and anti-inflammatory cytokines, impaired T cell and neutrophil function have been described. In neutrophils, superoxide production and phagocytosis are severely impaired and thereby compromise antimicrobial killing. Neutrophil apoptosis seems to be enhanced during chronic uremia (14). Chronic uremia also is associated with alterations in B lymphocyte function (e.g., reduced Ig levels, antibody responses) (14). It is unknown whether similar changes also occur during acute uremia (AU) and whether such alterations are actually clinically relevant. We therefore examine the remote effect of inflammatory versus noninflammatory AU in a PMN-dependent murine model of aspiration pneumonitis.

Materials and Methods

Animals

We used adult C57BL/6 wild-type mice (2 to 3 mo of age, 20 to 32 g body wt) from the Jackson Laboratory (Bar Harbor, ME). Mouse colonies were maintained under specific pathogen-free conditions. Before our study, local government authorities had approved all experiments, which were in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reagents

Unless stated otherwise, all reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Surgical Procedures

Mice were anesthetized with intraperitoneal injections of ketamine (125 μg/g body wt; Ketalar; Parke-Davis, Morris Plains, NJ), xylazine (12.5 μg/g body wt; Phoenix Scientific, St. Joseph, MO), and atropine sulfate (0.025 μg/g body wt; Elkins-Sinn, Cherry Hill, NJ). Both renal pedicles were prepared using a median dorsal skin incision and bilateral paramedian opening of the retroperitoneal space. In mice that underwent RIR as described previously (15,16), both pedicles were clamped off for 32 min with hemostatic microclips. Kidneys were inspected for immediate color change, indicating successful clamping. After clamp removal, kidneys were checked for a change in color within 3 min to ensure reperfusion. In mice that underwent NET, both pedicles were ligated and the kidneys were removed. Mice that were subjected to sham operation received identical surgical procedures except that no clamps or ligations were applied. Mice demonstrated comparably severe levels of uremia at 22 h after RIR (creatinine 0.72 ± 0.3 mg/dl; blood urea nitrogen [BUN] 151 ± 3 mg/dl) or at 10 h after the NET (creatinine 0.73 ± 0.3 mg/dl; BUN 151 ± 7 mg/dl), respectively.

At these time points, ALI was induced. Mice were anesthetized as described above. Mice then received 2 ml/kg HCl (pH 1.5) intratracheally, followed by a bolus of air (30 ml/kg) and transtracheal mechanical ventilation for 2 h (tidal volume 10 ml/kg, respiration rate 140/min, fraction of inspired O2 0.21). After that, mice were killed, blood samples were taken, and both kidneys as well as both lungs were harvested. Sham ALI consisted of intratracheal administration of normal saline. A subgroup of mice received an injection of a PMN-depleting antibody (see Isolation and Transfer of Mature Murine Neutrophils from Bone Marrow) or corresponding control antibody 24 h before induction of ALI to study the functional relevance of neutrophils in our model of aseptic ALI. Figure 1A summarizes the sequence of events.

Isolation and Transfer of Mature Murine Neutrophils from Bone Marrow

Mature neutrophils were isolated from murine bone marrow according to previously published protocols (17). Femur and tibia from sham or NET mice were removed and placed in HBSS until further preparation. The bone marrow was flushed from each bone with HBSS, and the plugs were disrupted and filtered through 70-μm nylon meshes. Cells were pelleted, and the entire bone marrow preparation was resuspended in HBSS (5 × 10⁷ cells/ml). Mature neutrophils were separated from the remaining cells by centrifugation over discontinuous Ficoll gradient that consisted of 1.077 and 1.119 Ficoll (2300 rpm for 30 min at room temperature). Mature neutrophils were recovered at the interface of the 1.077 and 1.119 fractions and >90% pure, as determined by flow cytometry (Figure 2).

To explore the role of normal versus uremic PMN in our model, we conducted an adoptive cell transfer. Mice received 1.4 to 1.6 × 10⁶ PMN

Figure 1. Schematic presentation of the intervention sequence in our two-hit model (A) and of all combinations performed in the reconstitution studies (B).
via tail-vein injection 24 h after previous depletion of endogenous neutrophils with a mAb directed against murine PMN (clone RB6–8C5; BD Biosciences, Heidelberg, Germany). In a previous study, this sufficiently depleted circulating PMN but did not affect other leukocyte subsets (18). Leukocytes were counted using Kimura’s stain. Two hours after adoptive cell transfer, ALI was induced by intratracheal HCl application. Figure 1B summarizes the sequence of events.

**Myeloperoxidase Activity**

Myeloperoxidase (MPO), an indicator of global tissue PMN content, was measured according to our previously published protocol (15,16). Samples were homogenized in ice-cold KPO4 buffer. After removal of 17,000 × g supernatants, pellets were resuspended in ice-cold KPO4 buffer, followed by two additional spins. Then 0.5% (wt/vol) hexacyltrimethylammonium bromide-10 mM EDTA in KPO4 was added to the remaining pellet. Suspensions were sonicated, freeze-thawed, and incubated for 20 min at 4°C. Supernatants were used subsequently to determine MPO. Next, assay buffer that contained 0.2 mg/ml o-dianisidine and 158 mM H2O2 in 50 mM KPO4 was added to supernatants. Changes in absorbance were recorded at 460 nm over 3.5 min. Results were expressed as units of MPO/g of protein in the supernatant as determined by bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

**Pulmonary Function: Oxygenation**

After cannulation of the carotic artery, standard arterial blood gas analyses were performed (Radiometer, Willich, Germany).

**Wet/Dry-Weight Ratios**

Lung wet/dry-weight (W/D) ratios were obtained using an established protocol (19). Lungs were cleaned from blood residues and weighed (wet weight). After lung water had been removed by heating (72 h, 90°C), lungs were weighed again (dry weight).

**Pulmonary Chemokine Concentrations**

Using commercially available sandwich ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany), we measured concentrations of the PMN-specific chemokine macrophage inflammatory protein-2 (MIP-2) in lung homogenates. Lungs were homogenized (1:2 wt:vol) in extraction buffer that contained Triton X-100, e-aminocapron acid, heparin, and EDTA. Homogenates were incubated for 18 h by 4°C. After incubation, samples were centrifuged at 10,000 × g for 15 min. Supernatant was collected and used for the actual ELISA, which was carried out according to the manufacturer’s instructions.

**Histology**

Paraffin-embedded lung sections (5 μm) were stained with hematoxylin-eosin to visualize HCl-induced tissue damage.

**Renal Function**

Plasma creatinine and BUN concentrations were measured using commercially available kits (Diazyme, San Diego, CA; and Randox, Krefeld, Germany, respectively).

**Fluorescence Microscopy-Based Tracking of (Uremic) Neutrophils**

We used a PMN-specific cell tracker to analyze pulmonary recruitment of normal neutrophils versus that of uremic neutrophils. PMN were isolated from bone marrow after previous sham surgery or NET and then were incubated with cell tracker CMFDA (Molecular Probes Europe, Leiden, Netherlands; 37°C, 30 min). After incubation, neutrophils (1.4 to 1.6 × 10⁶) were washed, resuspended in PBS, and injected intravenously into a new subgroup of mice. These mice then were subjected to our ALI protocol. After mechanical ventilation for 2 h, the trachea was cannulated and both lungs were inflated by pressure-controlled infusion of formalin (25 cmH2O). Lungs were removed and stored in formalin until further processing. Paraffin-embedded lung sections (5 μm) were assessed for positively stained neutrophils under a fluorescence microscope (blinded investigator, 10 high-power fields per section, ×40).

**Apoptosis and Superoxide Production**

Apoptosis in neutrophils was determined using a commercially available apoptosis detection kit (R&D Systems). Isolated neutrophils (1 × 10⁶ to 1 × 10⁷) were incubated with annexin V and propidium iodide (according to the manufacturer’s instruction). Next, a so-called binding buffer was added, and samples were centrifuged (500 × g, 5 min). After the cells were washed, the PMN-containing pellets were
resuspended in PBS and analyzed by flow cytometry for annexin V–FITC and propidium iodide.

For quantification of intracellular H$_2$O$_2$ production, we used a previously published protocol (20). Dihydrorhodamine-123 (DHR; Invitrogen, Karlsruhe, Germany) and phorbol myristate acetate (PMA) were prepared according to the manufacturer’s instructions (DHR 10 mM; PMA 10 and 100 pg/ml). DHR was added to isolated neutrophils ($1 \times 10^6$ neutrophils). After a brief incubation (5 min, 37°C), PMA was added to each sample, which then was incubated again (10 min, 37°C). Cell activation was stopped by addition of ice-cold PBS, and samples subsequently were analyzed by flow cytometry. Cells that were incubated with DHR only served as controls.

Surface Staining and Flow Cytometry

We analyzed surface expression of leukocyte adhesion molecules on peripheral blood leukocytes by incubating whole-blood samples with FITC-conjugated anti-murine PMN antibody (Ly-6G) in combination with L-selectin (MEL-14).

L-Selectin Reverse Transcriptase–PCR

Total RNA was extracted from isolated neutrophils using a TRIzol/chloroform protocol (Invitrogen). Total RNA was eluted in diethylpyrocarbonate-treated water. Reverse transcription was accomplished using an L-selectin–specific primer (5’-TATAGATCTCCACACAGTCC-3’; Invitrogen SuperScript II-Protocol). The detection PCR was carried out with gene-specific primers (upstream primer 5’-ACTGCTCTGTTGGACTCTCC-3’ and downstream primer 5’-TATAGATCTCCACAAGATTGAC-3’), gaining a specific product of 324 bp. PCR was performed using the Platinum TaqDNA polymerase kit (Invitrogen). RNA extraction and L-selectin reverse transcriptase–PCR (RT-PCR) were normalized via glyceraldehyde-3-phosphate dehydrogenase–specific RT-PCR (upstream primer 5’-CCGAGTCAAGTTGCTCCTGAT-3’ and downstream primer 5’-AGCCTTCTCCTCATGGTGTTGAC-3’, revealing a distinct product of 307 bp).

Statistical Analyses

Statistical analysis included one-way ANOVA, Student-Newman-Keuls test, and t test where appropriate. All data are presented as mean ± SEM.

Results

Intratracheal HCl Instillation Causes Severe, PMN-Dependent ALI

Intratracheal HCl administration led to a significant decrease in arterial partial pressure of O$_2$ (Pao$_2$) when compared with control (Figure 3A). Neutrophil-depleted mice displayed full protection from HCl-induced ALI (Figure 3A). A strong rise in pulmonary MPO 2 h after induction of ALI indicated a massive influx of PMN into the lungs. Neutrophil-depleted mice exhibited no change in lung MPO after HCl application (Figure 3B). When compared with sham mice (Figure 3C), lung histology of mice with ALI showed interstitial edema, massive influx of inflammatory cells, hyperemia, and fluid accumulation in the alveoli (Figure 3D).

Improvement of Oxygenation through Previous Induction of AU

In all subgroups with a two-hit approach (i.e., AU plus ALI), HCl application significantly impaired oxygenation as shown by lower Pao$_2$ values (Figure 4A). However, in mice that presented with AU at the time of ALI induction, the decline in Pao$_2$ was significantly attenuated (Figure 4A). This protection was independent of ongoing renal inflammation. The induction of AU itself had no impact on oxygenation in mice without ALI. All groups with aseptic ALI (A), Pulmonary myeloperoxidase (MPO) increased after HCl administration, indicating a strong PMN influx into the lungs (B). Compared with sham mice (C), lungs from mice with aseptic ALI (D) showed interstitial edema, influx of inflammatory cells, hyperemia, and some fluid accumulation within the alveolar compartment (hematoxylin-eosin stain).

Uremic Neutrophils Are the Primary Mediators in Attenuating ALI and Show Impaired Pulmonary Recruitment

To unravel the mechanisms underlying these observations, we conducted so-called reconstitution experiments (Figure 1B). After depletion of endogenous neutrophils, uremic mice received an injection of nonuremic neutrophils before induction
of ALI or vice versa. Only mice that had received an injection of uremic neutrophils displayed an improved oxygenation similar to that seen in mice with AU at the time of ALI induction (Figure 5). Circulating humoral factors alone (e.g., uremic plasma) did not provide any detectable protection.

Isolated uremic and nonuremic neutrophils were labeled with cell tracker CMFDA before re-injection in an additional set of experiments. As opposed to MPO as a marker of global tissue PMN content, this strategy allowed for specific tracking of uremic neutrophils versus nonuremic neutrophils during ALI (Figure 6, A and B). Morphometric analysis of various lung sections revealed impaired pulmonary recruitment of uremic neutrophils when compared with nonuremic neutrophils (Figure 6C).

ALI also caused an increase in lung tissue concentrations of the neutrophil-specific chemokine MIP-2. However, MIP-2 concentrations were not different between normal and uremic mice (data not shown).

Superoxide Production and Apoptosis Are Not Different in Uremic and Nonuremic Neutrophils

There were no changes in neutrophil apoptosis or superoxide production during AU (Figure 7).

AU Modulates Surface Expression of L-Selectin on Neutrophils

When compared with normal neutrophils, uremic neutrophils exhibited downregulation of surface L-selectin expression before and after induction of ALI (Figure 8A). L-selectin downregulation seemed to be caused by increased L-selectin shedding, because AU did not affect L-selectin mRNA expression (Figure 8B).

Discussion

Although both ARF and ALI carry extremely high mortality rates, especially when they are combined, only limited information is available regarding kidney–lung interactions under such circumstances. Here, we show that preexisting AU attenuates pulmonary neutrophil recruitment and greatly improves oxygenation during aseptic ALI; concurrent renal inflammation had no clinically relevant effect in this model.

Mimicking aspiration pneumonitis, intratracheal HCl instillation in our model led to a strong influx of neutrophils into the
lung and severe, PMN-dependent impairment of oxygenation. This is in excellent agreement with previous studies that also have demonstrated a pivotal role for neutrophils and their recruitment into the lung during HCl-induced ALI (21–23). Members of the selectin class of adhesion molecules leukocyte, as well as sialyl Lewisx, a “pan-selectin” counterreceptor, are known to be critical in experimental aspiration pneumonitis, because they control pulmonary PMN recruitment (24,25).

Figure 6. Reduced pulmonary recruitment of uremic PMN during aseptic ALI. Immunofluorescence lung microscopy after reconstitution with stained (CMFDA) nonuremic PMN (A) or stained uremic PMN (B) and subsequent induction of ALI demonstrated a decreased pulmonary recruitment of uremic neutrophils (C; blinded investigator, 10 high-power fields).

The positive effects of AU on oxygenation during aseptic ALI seem to defy obvious hypotheses that one might have derived from previous studies that have examined ARF and pulmonary homeostasis (8–10). Moreover, these findings seem to contradict current clinical wisdom and data that clearly demonstrate higher morbidity and mortality when ARF and ALI are combined (7). Despite these controversies, the following careful evaluation and interpretation of our results allow for a better understanding of the systemic effects of AU and might provide directions for future studies.

Both NET and RIR had similar clinical effects with respect to oxygenation and lung W/D ratios. AU, irrespective of the under-
Figure 8. AU leads to decreased L-selectin surface expression in neutrophils without affecting L-selectin mRNA expression. Gated on the Ly-6G+ cells, flow cytometry showed a significantly reduced L-selectin surface expression in these cells during AU (A). These data are representative of three similar experiments performed with neutrophils that were derived from different mice. Reverse transcriptase–PCR analysis of L-selectin mRNA expression (B) revealed no differences between uremic or nonuremic neutrophils that were derived from pooled bone marrow: Nonuremic neutrophils (lanes 1 and 2), uremic neutrophils (lanes 3 and 4), and water (lane 5).

As opposed to chronically uremic neutrophils (14), acutely uremic neutrophils did not display changes in either superoxide production or rate of apoptosis, two key parameters of neutrophil function during aseptic inflammation. Acutely uremic neutrophils, however, exhibited a substantially impaired recruitment into the lung during aseptic ALI, when compared with normal neutrophils. Because AU did not alter the expression of the PMN-specific chemokine MIP-2 within the lung, attenuation of neutrophil-specific inflammatory responses within the lung as a result of AU seems less likely. This is supported further by the loss of protection from aseptic ALI when mice received an injection of normal neutrophils beforehand. Because members of the selectin class of adhesion molecules and their counterreceptors have been implicated in the pathogenesis of experimental aspiration pneumonitis (24,25), we evaluated L-selectin expression on normal and uremic neutrophils. L-selectin is the only selectin molecule that is expressed exclusively on leukocytes, including neutrophils (26). Flow cytometry revealed a significant decrease in L-selectin surface expression in uremic neutrophils when compared with nonuremic neutrophils. This difference, although present throughout the entire experiment, was especially pronounced before induction of aseptic ALI. Downregulation of L-selectin surface expression can give rise to significantly impaired recruitment of leukocytes to the site of inflammation (26). Like other adhesion molecules, L-selectin surface expression can be regulated by gene transcription or cleavage from the surface (26). RT-PCR analysis of L-selectin mRNA expression did not reveal any differences between normal and uremic neutrophils, suggesting increased L-selectin shedding rather than decreased transcription rates as the underlying mechanism.

On the basis of these findings, we conclude that preexisting AU impairs neutrophil recruitment during inflammation and thereby induces an anti-inflammatory state. Although this effect translates into significant and clinically relevant protection from severe aseptic pneumonitis, great caution is warranted when extrapolating these findings to routine clinical practice. Whereas impaired neutrophil recruitment seems to be beneficial in our model of aseptic pneumonitis, the opposite could be true if infectious organisms were involved, because neutrophils also are key components of innate immunity. These concerns are substantiated further by previous studies that proved L-selectin to be crucial for neutrophil recruitment (27) as well as for survival during bacterial pneumonia (28). A recent clinical study also identified ARF as an independent variable associated with death in the course of community-acquired pneumonia (29).

Conclusion
We have shown that preexisting AU greatly improves oxygenation and attenuates pulmonary PMN recruitment in a neutrophil-dependent murine model of aspiration pneumonitis. Underlying renal inflammation had no clinically relevant effect in this setting. Uremic neutrophils emerged as the primarily protective mediators under these circumstances. As opposed to chronic uremia, AU did not change superoxide production or rate of apoptosis in neutrophils. However, AU led to a downregulation of L-
selectin surface expression on PMN. Although AU has been protective in this model, our findings warrant careful interpretation. Recent experimental and clinical studies provide evidence that both L-selectin–dependent PMN recruitment and ARF have a critical impact on outcome in bacterial pneumonia.

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References