Clara Cell Protein (CC16), a Marker of Lung Epithelial Injury, Is Decreased in Plasma and Pulmonary Edema Fluid From Patients With Acute Lung Injury*

Jonathan A. Kropski, MD; Richard D. Fremont, MD; Carolyn S. Calfee, MD; and Lorraine B. Ware, MD, FCCP

Background: Acute lung injury (ALI) and ARDS are common clinical syndromes that are underdiagnosed. Clara cell secretory protein (CC16) is an antiinflammatory protein secreted by the Clara cells of the distal respiratory epithelium that has been proposed as a biomarker of lung epithelial injury. We tested the diagnostic and prognostic utility of CC16 in patients with non-trauma-related ALI/ARDS compared to a control group of patients with acute cardiogenic pulmonary edema (CPE). **Methods:** Plasma and pulmonary edema fluid samples were obtained from medical and surgical patients with ALI/ARDS or CPE requiring intubation for mechanical ventilation. The etiology of pulmonary edema was determined using consensus clinical criteria for ALI/ARDS and CPE and the edema fluid-to-plasma protein ratio. Plasma and edema fluid CC16 levels were measured by sandwich enzyme-linked immunosorbent assay. CC16 levels were log transformed for analysis, and comparisons were made by the Student *t* test or χ^2 as appropriate.

Results: Compared to patients with CPE (*n* = 9), patients with ALI/ARDS (*n* = 23) had lower median CC16 levels in plasma (22 ng/mL [interquartile range (IQR), 9 to 44 ng/mL] vs 55 ng/mL [IQR, 18 to 123 ng/mL], respectively; *p* = 0.053) and pulmonary edema fluid (1,950 ng/mL [IQR, 1,780 to 4,024 ng/mL] vs 4,835 ng/mL [IQR, 2,006 to 6,350 ng/mL], respectively; *p* = 0.044). Relative to total pulmonary edema fluid protein concentration, the median CC16 level was significantly lower in patients with ALI/ARDS (45 ng CC16/mg total protein [IQR, 4 to 64 ng CC16/mg total protein] vs 120 ng CC16/mg total protein [IQR, 87 to 257 ng CC16/mg total protein], respectively; *p* = 0.005). Neither plasma nor edema fluid CC16 levels predicted mortality, the number of days of unassisted ventilation, or ICU length of stay.

Conclusion: CC16 is a promising diagnostic biomarker for helping to discriminate ALI from CPE. Larger scale validation is warranted to better characterize the utility of CC16 in the diagnosis of this underrecognized syndrome. (CHEST 2009; 135:1440–1447)

Key words: acute lung injury; ARDS; biomarker pulmonary edema; CC16; Clara cell protein

Abbreviations: ALI = acute lung injury; CC16 = Clara cell secretory protein; CPE = cardiogenic pulmonary edema; *F*_{IO₂} = fraction of inspired oxygen; IQR = interquartile range; LIS = Lung Injury Score; LPS = lipopolysaccharide

Despite extensive research and increasing awareness of acute lung injury (ALI) and the ARDS, the diagnosis remains clinically challenging and may be missed in > 50% of patients.¹ With growing evidence that specific treatments including low-tidal volume ventilation and conservative fluid management improve outcomes in ALI/ARDS,^{2,3} there is a clear need for improved diagnostic approaches.

Clara cell secretory protein, also known as CC16 and CC10, is a secreted product of the respiratory epithelium that within the lung is produced primarily

within the Clara cells of the distal respiratory and terminal bronchioles.^{4,5} The biological function of

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For editorial comment see page 1408

CC16 remains incompletely understood, although CC16 has been demonstrated to interact with multiple components of the inflammatory and coagulation cascades. CC16 inhibits phospholipase A2 activity *in vitro* and *in vivo*,⁶ suggesting it plays a role in attenuating inflammatory responses. CC16 has also

been implicated in feedback inhibition of interferon gamma signaling,⁷⁻⁹ as well as modulation of T helper 2 responses to proinflammatory stimuli.¹⁰ Furthermore, CC16 appears to be activated by tissue transglutaminases including activated Factor XIII,¹¹ and inhibits thrombin-stimulated platelet aggregation,¹² suggesting a possible role in modulating the dysregulated coagulation characteristic of ALI/ARDS.¹³

CC16 has been investigated as a potential biomarker of lung epithelial injury in numerous disease states including idiopathic pulmonary fibrosis,¹⁴ sarcoidosis,¹⁴⁻¹⁷ COPD,^{14,15} asthma,^{14,18-21} occupational or environmental lung injury,²²⁻²⁵ bronchiolitis obliterans,^{26,27} chronic tobacco use,^{15,28} and ALI/ARDS.²⁹⁻³³ Several studies have examined whether CC16 levels in BAL fluid or plasma can discriminate patients with ALI/ARDS from those at-risk for ALI/ARDS or healthy control subjects,²⁹⁻³² but results to date have been contradictory. In a 2006 prospective multicenter study of patients with ARDS, plasma levels of CC16 were significantly higher in nonsurvivors compared to survivors.³² However, CC16 levels have not been compared between patients with pulmonary edema due to either a hydrostatic (cardiogenic) mechanism or due to ALI/ARDS.

In this study, we examined the utility of CC16 as a biomarker of lung injury in patients with acute cardiogenic pulmonary edema (CPE) or ALI/ARDS. We hypothesized that injury to the distal lung epithelium would lead to decreased levels of CC16 in both the plasma and pulmonary edema fluid of patients with ALI/ARDS compared to patients with CPE.

MATERIALS AND METHODS

Study Population

Subjects were critically ill adults admitted to the medical, surgical, neurologic, or trauma critical care units at Vanderbilt University Medical Center from January 1, 2002, through De-

cember 31, 2007, with severe CPE or ALI/ARDS requiring endotracheal intubation for mechanical ventilation. Subjects were eligible for inclusion if plasma and pulmonary edema fluid samples could be obtained within 24 h of endotracheal intubation or the onset of acute pulmonary edema in previously ventilated patients. Patients < 18 years of age and patients with a mixed etiology of pulmonary edema (defined later) were excluded from the study. The study protocol was reviewed and approved by the Vanderbilt University Medical Center Institutional Review Board (No. 020260) with a waiver of informed consent because the study posed minimal risk to the participants.

Sample Collection

Pulmonary edema fluid samples were obtained by physicians or trained study nurses immediately following intubation via gentle luminal suction through a soft 14F catheter wedged distally in the bronchial tree, as has been described previously.³⁴ Plasma samples were obtained from leftover samples drawn for routine clinical care of the patient within 24 h of the time of pulmonary edema fluid collection (mean time, 4.1 h). Plasma and edema fluid samples were centrifuged at 3,000g for 10 min and the supernatants were stored at -80°C.

Definition of Pulmonary Edema Type

ALI and ARDS were defined using the North American-European Consensus Committee clinical criteria³⁵ and the edema fluid-to-plasma protein ratio.³⁶⁻³⁸ Briefly, the North American-European Consensus Committee criteria³⁵ include the acute onset of bilateral alveolar infiltrates seen on a chest radiograph, a PaO₂/fraction of inspired oxygen (FIO₂) ratio ≤ 300 (ALI patients) or ≤ 200 (ARDS patients), and the absence of clinical evidence of left atrial hypertension. CPE was defined by clinical evidence of left atrial hypertension due to systolic or diastolic myocardial dysfunction, valvular disease, systemic hypertension, or volume overload. Additionally, a pulmonary edema fluid/plasma protein ratio > 0.65 was required for the diagnosis of ALI/ARDS; a pulmonary edema fluid/plasma protein ratio < 0.65 was required for the diagnosis of hydrostatic pulmonary edema.³⁶⁻³⁸ Patients in whom the clinical scenario and the pulmonary edema fluid/plasma protein ratio were discordant and patients with clinical histories consistent with a mixed cause of pulmonary edema (eg, pneumonia with severe chronic heart failure) were excluded from this study.

Data Collection

Demographic and clinical data including age, gender, race, smoking status, admission diagnosis, as well as hemodynamic and ventilator parameters, were obtained by retrospective chart review. Lung injury severity was assessed by the Lung Injury Score (LIS) of Murray et al.³⁹ Severity of illness was assessed by calculation of the Severe Acute Physiology Score II⁴⁰ on the day of edema fluid sampling.

Measurements

CC16 was measured in duplicate by a sandwich enzyme-linked immunosorbent assay (Biovendor; Candler, NC) previously validated in plasma and BAL fluid matrices.

Outcomes

The primary outcome measure was difference in concentration of CC16 in plasma and pulmonary edema fluid between subjects

*From the Division of Allergy, Pulmonary and Critical Care Medicine (Drs. Kropfski, Fremont, and Ware), Department of Medicine, Vanderbilt University Medical Center, Nashville, TN; and the Department of Medicine and Cardiovascular Research Institute (Dr. Calfee), University of California, San Francisco, San Francisco, CA. This study was supported by National Institutes of Health grant No. HL081332.

The authors have reported to the ACCP that no significant conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

Manuscript received October 21, 2008; revision accepted December 16, 2008.

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Correspondence to: Lorraine B. Ware, MD, Division of Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University Medical Center, 1161 Twenty-First Ave South, Suite T-1218 MCN, Nashville, TN 37232-2650; e-mail: Lorraine.Ware@vanderbilt.edu
DOI: 10.1378/chest.08-2465

with CPE or ALI/ARDS. Secondary outcomes included difference in pulmonary edema fluid-to-plasma ratio of CC16, CC16 concentration in pulmonary edema fluid compared to total edema fluid protein, and association of CC16 concentration in ALI/ARDS patient samples with mortality, duration of mechanical ventilation, and ICU length of stay.

Statistical Analysis

Results are expressed as means with SDs or medians with interquartile ranges (IQRs) as appropriate. Continuous data were compared by two-tailed Student's *t* test or Mann-Whitney *U* test based on the normality of distribution. Categorical measures were analyzed using the χ^2 test. Levels of CC16 were not normally distributed and were log-transformed for analysis. The association between baseline demographic and clinical parameters and the levels of CC16 were analyzed by bivariate Spearman correlations. For all analyses, a *p* value < 0.05 was considered statistically significant.

RESULTS

Thirty-two patients met the inclusion criteria for this study. Nine subjects had CPE, and the remaining 23 met criteria for ALI/ARDS. Demographic and baseline clinical and physiologic characteristics of subjects are shown in Tables 1 and 2. At baseline, patients with CPE were significantly older than those with ALI/ARDS. Overall illness severity was similar between the two groups. Subjects with ALI/ARDS

Table 1—Baseline Clinical Characteristics of 23 Patients With ALI/ARDS and 9 Control Patients With CPE*

Characteristics	CPE Patients (n = 9)	ALI/ARDS Patients (n = 23)	p Value
Demographic			
Age	52 ± 14	40 ± 15	0.05
Male gender	44	48	0.86
Smoker	33	32	0.77
Hospital admission type			
Medical emergency	77	91	0.58
Surgical elective	11	4	
Surgical emergency	11	4	
Severe acute physiology score II	53 ± 15	54 ± 20	0.94
Edema fluid/plasma protein ratio	0.44 ± 0.13	0.94 ± 0.20	< 0.001
Etiology of pulmonary edema			
Volume overload/diastolic dysfunction	44		
Congestive heart failure	33		
Hypertensive crisis	22		
Nonpulmonary sepsis		35	
Pneumonia		30	
Aspiration		22	
Transfusion-related lung injury		13	

*Values are given as the mean ± SD or %, unless otherwise indicated.

Table 2—Hemodynamic, Respiratory, and Laboratory Parameters in 23 Patients With ALI/ARDS and 9 Control Patients With CPE*

Parameters	CPE Patients (n = 9)	ALI/ARDS Patients (n = 23)	p Value
Hemodynamic			
Highest MAP, mm Hg	109 ± 27	92 ± 27.1	0.12
Lowest MAP, mm Hg	65 ± 8	56 ± 16	0.04
Heart rate, beats/min	104 ± 23	129 ± 26	0.02
Temperature, °C	37.4 ± 1.2	38.0 ± 1.2	0.23
Urine output, L/24 h	1.0 ± 1.2	1.6 ± 1.3	0.30
Respiratory			
PaO ₂ /FIO ₂ ratio	182 ± 120	77 ± 56	0.02
LIS	2.7 ± 0.7	3.4 ± 0.6	0.02
Laboratory			
Hemoglobin, g/dL	10.4 ± 2.2	9.0 ± 3.8	0.19
WBC, 10 ³ cells/μL	14.0 ± 8.3	14.6 ± 10.0	0.87
Platelets, 10 ³ cells/μL	177 ± 130	105 ± 150	0.19
Creatinine, mg/dL	2.3 ± 1.8	2.6 ± 2.0	0.65

*Values are given as the mean ± SD, unless otherwise indicated. MAP = mean arterial pressure.

had higher LIS and lower PaO₂/FIO₂ ratios than those with CPE. There was no difference in serum creatinine or 24-h urine output on the day of sampling between the two groups. As expected, patients with ALI/ARDS had higher mortality, fewer days alive and free of mechanical ventilation, and longer ICU stays than those with CPE, although only ICU length of stay reached statistical significance (Table 3).

At the time of developing pulmonary edema, median CC16 levels in pulmonary edema fluid were significantly lower in subjects with ALI/ARDS (1,950 ng/mL [IQR, 1,780 to 4,024 ng/mL] vs 4,835 ng/mL [IQR, 2,006 to 6,350 ng/mL], respectively; *p* = 0.044) [Fig 1, left, A]. Median plasma CC16 levels were also lower in patients with ALI/ARDS compared to CPE (22 ng/mL [IQR, 9 to 44 ng/mL] vs 55 ng/mL [18 to 123 ng/mL], respectively; *p* = 0.053) [Fig 1, right, B], and this difference nearly reached significance. Patients with pulmonary edema fluid and plasma CC16 levels in the lowest quartile compared to the highest quartile were

Table 3—Clinical Outcomes in CPE Compared to ALI/ARDS*

Outcomes	CPE Patients (n = 9)	ALI/ARDS Patients (n = 23)	p Value
Died, %	33	56	0.28
Days alive free of mechanical ventilation	26 (0–27)	12 (0–20)	0.18
ICU length of stay (days)	3 (2–7)	8 (4–14)	0.02

*Values are given as % or median (IQR), unless otherwise indicated.

FIGURE 1. Box plot summary of CC16 levels in pulmonary edema fluid (*left, A*) and plasma (*right, B*) of patients with CPE or ALI/ARDS. Horizontal line represents median, box includes 25th to 75th percentile, error bars include tenth-ninetieth percentile.

more likely to have ALI/ARDS (edema fluid, 100% vs 43%, respectively [$p = 0.042$]; plasma, 88% vs 44%, respectively [$p = 0.170$]). Patients with ALI also had significantly lower CC16 to edema fluid protein ratios than subjects with CPE (45 ng CC16/mg total protein [IQR, 4 to 64 ng CC16/mg total protein] vs 120 ng CC16/mg total protein [IQR, 87 to 257 ng CC16/mg total protein], respectively; $p = 0.005$) [Fig 2, *left, A*]. The pulmonary edema fluid/plasma ratio of CC16 did not differ between the two groups (Fig 2, *right, B*). Among patients with ALI/ARDS, plasma and pulmonary edema fluid levels of CC16 did not differ

by survivor status or duration of mechanical ventilation or ICU stay (Table 4).

DISCUSSION

In this observational cohort study of patients with ALI/ARDS, levels of the anti-inflammatory CC16 were significantly reduced in both the pulmonary edema fluid and the plasma of patients with ALI/ARDS compared to a control group of patients with CPE. In a prespecified analysis of the group of patients with ALI/ARDS, neither edema fluid nor plasma levels were

FIGURE 2. *Left, A*: box plot summary of edema fluid CC16 levels compared to total pulmonary edema fluid protein (ng CC16/mg total protein) in patients with CPE or ALI/ARDS. Horizontal line represents median, box includes twenty-fifth to seventy-fifth percentile, error bars include tenth-ninetieth percentile. *Right, B*: box plot summary of the pulmonary edema fluid to plasma ratio of CC16 in patients with CPE or ALI/ARDS. Horizontal line represents median, box includes 25th to 75th percentile, error bars include the 10th to 90th percentile.

Table 4—CC16 Levels and Clinical Outcomes in 23 Patients With ALI or ARDS

Outcomes	CC16 Levels, ng/mL	
	Edema Fluid	Plasma
Survivors (n = 10)	1,810 (176–4,861)	20 (10–38)
Nonsurvivors (n = 13)	2,119 (360–3,759)	22 (7–50)
p Value	0.44	0.99
≥ 7 d unassisted ventilation (n = 12)	1,762 (276–2,966)	22 (10–42)
< 7 d unassisted ventilation (n = 11)	2,772 (164–4,711)	22 (7–49)
p Value	0.76	0.83
< 7 ICU d (n = 10)	2,118 (164–3,249)	28 (16–51)
≥ 7 ICU d (n = 13)	1,902 (702–4,757)	20 (8–23)
p Value	0.34	0.28

*Values are given as the median (IQR), unless otherwise indicated.

significant predictors of mortality, duration of mechanical ventilation, or ICU length of stay, although the total number of patients studied was small.

CC16 concentrations detected in plasma were similar to those reported in other studies, although concentrations in pulmonary edema fluid were five to tenfold higher than those reported for BAL fluid, as would be expected due to dilution of BAL fluid during collection.^{29–32} These findings suggest that differences in patient populations or sample collection, preparation, or storage are unlikely to explain the differences between our findings and those reported by other groups.

Decreased CC16 concentration in BAL fluid or pulmonary edema fluid has not previously been described in ALI/ARDS but has been reported in chronic tobacco use^{15,28} and bronchiolitis obliterans syndrome,^{26,27} as well as in animal models of ALI.^{41,42} In one study of rats exposed to intratracheal lipopolysaccharide (LPS), a dose-dependent reduction in BAL fluid CC16 concentration was reported 24 h after LPS administration.⁴¹ Nearly tenfold reductions in lung homogenate CC16 were demonstrated, along with markedly decreased CC16 staining within terminal bronchioles by immunohistochemical analysis and reduced total lung homogenate CC16 messenger RNA. Reduced CC16 messenger RNA expression has also been reported in an acid aspiration rat model of ALI/ARDS.⁴³ However, in an observational study of post-cardiopulmonary bypass patients at risk for ALI/ARDS, mean CC16 levels in BAL fluid were not different between patients with ARDS and those at risk.³¹ In contrast to our findings, in that study CC16 levels were significantly lower in nonsurvivors than survivors, although this may be due to differences in the patient populations.

Interestingly, although we found plasma CC16 concentration to be lower in patients with ALI/

ARDS than those with CPE, other studies in animal models^{41,42} and humans³³ have shown acute increases in plasma CC16 concentration following inspired or intratracheal LPS administration. Additionally, a study of critically ill mechanically ventilated patients found higher plasma CC16 levels in patients with ALI/ARDS compared to those at risk, and patients with ALI/ARDS who died had higher CC16 levels than survivors.³² However, a recent study of chemical-induced lung injury in rats reported decreased serum CC16 levels after an initial transient increase compared to saline solution-treated control subjects.⁴⁴ We are not aware of any prior reports directly comparing patients with ALI/ARDS to control patients with severe acute respiratory failure due to CPE.

There are several potential explanations as to why CC16 levels may be lower in plasma of patients with ALI/ARDS compared to those with CPE. Patients with ALI/ARDS were significantly younger than those with CPE; however, age has not been shown to affect CC16 levels significantly.^{29,45} Renal function is a significant predictor of plasma CC16 levels.^{29,45} Both subject groups in the current study had moderate renal impairment, although this did not significantly differ between patients with ALI/ARDS and those with CPE. Plasma creatinine was not significantly correlated with plasma CC16 concentration, and after controlling for creatinine, plasma CC16 remained significantly lower in patients with ALI/ARDS (data not shown). Nonetheless, it remains possible that baseline differences in renal function may have contributed to relative differences in CC16 clearance between the two groups. The effect of CPE on plasma CC16 levels has not been reported, and it is possible that severe CPE that requires mechanical ventilation, as was the case in patients in this study, is accompanied by changes in alveolar-epithelial permeability that permit small alveolar proteins such as CC16 (molecular weight, 16 kD) to cross the alveolar-epithelial border but do not permit transit of larger molecules including albumin (molecular weight, 66 kD). Compared to healthy control subjects, patients with CPE do have increased total protein in BAL fluid,⁴⁶ suggesting that some modest alteration in alveolar epithelial permeability occurs permitting bulk-flow transit of interstitial fluid into the airspace during the alveolar flooding phase of CPE⁴⁷ without permitting transit of alveolar proteins into the interstitial and vascular spaces. We found that the ratio of CC16 to total protein, comprised largely of albumin and other larger proteins^{46,48} in pulmonary edema fluid was significantly lower in patients with ALI/ARDS than those with CPE, suggesting that alternative mechanisms of alveolar flooding in ALI/ARDS and CPE may be responsible

for different relative concentrations of CC16 and total edema fluid protein between the two groups.

In mice, lung injury is characterized by dynamic changes in the composition of the bronchial epithelium, including sloughing of Clara Cells in the acute phase and a dedifferentiation and spreading of the ciliated epithelial cells, along with altered transcriptional activity within ciliated and nonciliated epithelial cells.⁴⁹ Furthermore, high levels of inspired oxygen, commonly required and employed in patients with ALI/ARDS, decrease CC16 transcription through a hyperoxia-sensitive promoter element.⁵⁰ Thus we believe the following three potential mechanisms explain why CC16 levels decrease in the setting of ALI/ARDS: (1) alterations in alveolar epithelial permeability; (2) Clara cell death; and (3) changes in transcriptional activity within remaining Clara cells.

A major strength of this study is the comparison of patients with ALI/ARDS to a control group of patients who are often difficult to distinguish using clinical and radiographic criteria only. Furthermore, use of undiluted pulmonary edema fluid offers the most direct measure of alveolar fluid composition, and it avoids the generation of iatrogenic inflammation or epithelial injury as can occur during BAL.⁵¹ This study also has some limitations. First, the overall number of subjects was modest, limiting the statistical power of the study as well as our ability to control statistically for potential confounding variables in multivariate analysis. Furthermore, the study subjects represented only a small percentage of all patients who presented with severe acute pulmonary edema during the study period and included only subjects in whom pulmonary edema fluid samples could be obtained. Second, clinical parameters and diagnoses were obtained and adjudicated by retrospective chart review, and classification of the type of pulmonary edema was performed retrospectively by the authors. Third, patients with mixed edema, a group in whom a biomarker may be most useful in determining the cause of pulmonary edema, were *a priori* excluded from the study due to the lack of a gold standard for determining the etiology of pulmonary edema. Additionally, blood samples were not collected specifically for this study, and the analyzed plasma samples were collected up to 23 h after edema fluid sampling, at which time a patient's physiology may have significantly changed. Furthermore, there has been suggestion of circadian variation in CC16 levels in plasma,⁵² although the magnitude of this is relatively small. After controlling for time of sampling, both plasma and pulmonary edema fluid CC16 levels remained significant predictors of edema type (data not shown). Finally, although both plasma and pulmonary edema fluid CC16 levels differed significantly between patients

with CPE and ALI/ARDS, in this small exploratory study there was moderate overlap between the two groups. Larger scale validation is necessary to evaluate the diagnostic value of CC16 levels.

In summary, we have described CC16 as a potential diagnostic biomarker that is decreased in the setting of ALI/ARDS. This stands in contrast to most other proposed biomarkers, including the receptor for advanced glycosylation end products,⁵³ surfactant protein-D,⁵⁴ von Willebrand factor antigen,⁵⁵ plasminogen activator inhibitor-1,^{56,57} and Fas-Fas ligand,⁵⁸ which are elevated in the setting of ALI/ARDS. We believe that a marker whose levels are reduced may offer particular benefit as a specific biomarker and a component of multimarker panels for the diagnosis of ALI/ARDS.

CONCLUSIONS

ALI and ARDS constitute a spectrum of severe hypoxic respiratory failure leading to significant morbidity and mortality in hundreds of thousands of patients each year. In this study of patients with CPE or ALI/ARDS, the levels of CC16 in both plasma and pulmonary edema fluid were lower in patients with lung injury than control subjects. Larger scale validation of these findings is warranted to better characterize the diagnostic, prognostic, and pathogenic role of CC16 in ALI/ARDS.

ACKNOWLEDGMENT: We thank Nancy Wickersham and Fred Bossert for their technical assistance.

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