

CC10 Reduces Inflammation in Meconium Aspiration Syndrome in Newborn Piglets

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ABSTRACT: Complications from meconium aspiration syndrome (MAS) remain significant despite a variety of therapeutic interventions. Clara cell protein (CC10) is a novel anti-inflammatory agent that can also inhibit phospholipase A₂ (PLA₂) (an important component of meconium). The present study examined whether administration of recombinant human CC10 (rhCC10) would reduce inflammation and improve lung function in a piglet model of MAS. Following meconium instillation, piglets exhibited significant physiologic dysfunction that improved significantly after surfactant administration. Analysis of tracheal aspirates revealed significant increases in both tumor necrosis factor (TNF) α and interleukin (IL)-8 after meconium instillation. rhCC10-treated animals had significantly lower TNF- α levels at 24 h (561 ± 321 versus 1357 ± 675 pg/mL, $p < 0.05$) compared with saline controls. There were no differences between rhCC10-treated and untreated groups with respect to other measured physiologic variables or inflammatory markers, including secretory PLA₂ activity. Histologic analyses revealed marked inflammatory infiltrates and thickened alveolar walls, but no significant differences among rhCC10 and control animals. Newborn piglets with MAS have significant physiologic dysfunction, marked inflammatory changes and histologic abnormalities, which was partially counteracted by a single dose of exogenous surfactant and rhCC10. (*Pediatr Res* 62: 684–688, 2007)

Meconium aspiration syndrome (MAS) can be a devastating illness that primarily affects full-term newborns. It is a multifaceted process that leads to significant mechanical, chemical, and immunologic dysfunction. Despite substantial advances in obstetrical and neonatal care, morbidity and mortality remain unacceptably high (1). The pathophysiology of MAS is often divided into two phases: an early phase characterized by airway obstruction (related to the particulate nature of meconium) and later effects involving severe inflammation (from chemical pneumonitis) and surfactant inactivation (2). Endogenous surfactant function is inhibited by a variety of constituents of meconium, including phospholipases (PLAs) that destroy endogenous surfactant and contribute to the inflammatory response seen in MAS (3). Other effects of meconium include direct lung epithelial cell damage by phospholipase A₂ (PLA₂) with the release of TNF- α (4). All these factors interact synergistically to cause significant lung injury in the newborn.

Interventions designed to mitigate the proinflammatory effects of meconium have met with limited success. Although corticosteroids have been studied in multiple clinical trials, a 2003 Cochrane Review concluded that there was no significant effect on mortality or morbidity (5). This suggests that other anti-inflammatory therapies that might also enhance surfactant function may be beneficial in MAS.

Clara cell protein (CC10) is a novel anti-inflammatory protein that is known to inhibit the activity of secretory PLA₂ (sPLA₂) *in vitro*, notably the pancreatic or type 1b enzyme and the macrophage-derived or type 2a enzyme (6). CC10 deficiency plays a vital role in a variety of disease processes including bronchopulmonary dysplasia, acute respiratory distress syndrome, asthma, cystic fibrosis, renal disease, and cancer (7). While one strain of mice genetically engineered to reduce CC10 activity develops glomerulopathy, pancreatic inflammation, and cancer soon after birth (8), another strain has increased lung injury in response to hyperoxia or infection (9). Recombinant human clara cell protein (rhCC10) has been shown to effectively decrease lung inflammation in a variety of animal models *via* the intravenous (i.v.) or intratracheal (i.t.) route (10–12). In addition, human trials in premature infants at risk of developing acute and chronic lung injury (also characterized by significant inflammatory changes in the lung) demonstrate significant reductions in several important inflammatory mediators in the lung of infants receiving rhCC10 compared with placebo controls (13). These studies suggest that CC10 plays an important role in normal organ function.

Since rhCC10 appears to have significant anti-inflammatory properties and inhibits PLA₂ activity *in vitro*, we hypothesized that i.t. administration in a piglet model of MAS would reduce inflammation and minimize acute lung injury, primarily by inhibiting PLA₂ activity *in vivo*. This hypothesis was tested in a well-established model of MAS in newborn piglets.

METHODS

Studies were approved by the Institutional Animal Care and Use Committee (IACUC). Sixteen 1- to 3-d-old piglets were sedated with intraperitoneal

Received March 14, 2007; accepted July 21, 2007.

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This work was funded by National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD (NHLBI R43 HL073572).

Abbreviations: BAL, bronchoalveolar lavage; CC10, Clara cell protein; it, intratracheal; MAS, meconium aspiration syndrome; PLA₂, phospholipase A₂; rhCC10, recombinant human Clara cell protein; sPLA₂, secretory phospholipase A₂; TAF, tracheal aspirate fluid

sodium pentobarbital (30 mg/kg), placed on a heating blanket, and intubated with a 3.5- to 4.0-mm endotracheal tube, and a 3.5-F umbilical arterial line was inserted. Mechanical ventilation was begun using a Bear Cub infant ventilator (VIASYS Healthcare, Palm Springs, CA) with positive end-expiratory pressure (PEEP) of 3 cm H₂O, inspiratory time of 0.5 s., and a flow rate of 8 L/min. Peak inspiratory pressure and ventilatory rate were varied to maintain partial pressure of carbon dioxide (Pco₂) at 40–50 mm Hg. Blood gases were measured every 3–4 h using a Gem Premier 3000 blood gas analyzer (Lexington, MA). Five percent dextrose with 0.33 normal saline was administered at 80 mL/kg/d. Ampicillin (50 mg/kg) and gentamicin (2.5 mg/kg) were given every 12 h. Additional pentobarbital was administered as needed to maintain sedation. Arterial/alveolar oxygen ratios were calculated before and after meconium instillation, 1–2 h after surfactant administration and at 24 h.

Meconium from healthy newborns [3 mL/kg, 20% by weight in phosphate-buffered saline (PBS)] was filtered to remove large particulate matter and instilled *via* the endotracheal tube as previously described (14). Oxygen and mechanical ventilation were then adjusted to maintain arterial blood gases in the physiologic range (with PEEP increased to 4 cm H₂O). After 30 min, animals received 4 mL/kg of surfactant (Survanta, Ross Laboratories, Columbus, OH). Twenty minutes later, half of the piglets received i.t. normal saline (placebo) alone (2 mL/kg), while the other half were given i.t. rhCC10. rhCC10 (5 mg/kg diluted to a total volume of 2 mL/kg with normal saline) was instilled with the piglets in a 30-degree Trendelenberg (head-down) position in two divided doses in the left and right lateral decubitus positions. The rhCC10 was produced in *Escherichia coli* bacteria according to published methods (Claragen, Inc., Germantown, MD) (15). The protein for the study was a >98% pure solution of the human CC10 homodimer. The biologic activity of each batch was compared using a previously published secretory PLA₂ inhibition assay for rhCC10 (15).

At baseline and 24 h, tracheal aspirate fluid (TAF) samples were obtained as previously described (14). At 24 h, piglets were given pentobarbital (30 mg/kg), and static pressure-volume curves were generated as previously described (15). Two piglets in each group were used for histologic analysis. A catheter was secured in the pulmonary artery and an incision made in the left atrium. The vascular system was infused with 180 mL of normal saline followed by 180 mL of 10% formalin. The lungs were then removed *en bloc*. Tissue was paraffin embedded and sections stained with hematoxylin and eosin. Remaining tissues were prepared as above without the formalin fixation. After removal, bronchial alveolar lavage (BAL) was performed with PBS (3 × 25 mL). The BAL underwent a low-speed spin (400 × g) to separate out cells and particulates.

Inflammatory and biochemical assays. Neutrophils were isolated from unventilated control pigs, and neutrophil chemotaxis assays (NCAs) were performed as previously described (15). The concentration of total protein in BAL fluid was quantified using the Pierce bicinchoninic acid (BCA) micro-titer technique (16). IL-8 and TNF- α (two cytokines routinely isolated in lung injury models) concentrations were assayed utilizing a commercially available enzyme-linked immunosorbent assay kit (ELISA) (Quantikine Porcine, R&D Systems, Minneapolis, MN).

Endogenous CC10 and PLA₂ activity was measured in meconium samples from five healthy term infants. Meconium was suspended in PBS, pH 7.4, in a consistent weight: volume (e.g. 50 mg in 200 μ L), mixed thoroughly, and then debris pelleted at 12,000 × g in a microfuge for 2 min. Total protein was determined by micro-BCA assay (Pierce Chemical Co.) and sPLA₂ concentration and activity were measured using an ELISA kit specific for sPLA₂ type 2a and a sPLA₂ activity assay kit (both from Cayman Chemical Co.). CC10 content was determined using a competitive ELISA for CC10 previously described (15).

The biologic activity of rhCC10 was quantified using a novel sPLA₂ inhibition assay. rh-sPLA₂ type 1b was kindly provided by Dr. Wonwha Cho (University of Illinois at Chicago). In this assay, the rh-sPLA₂ type 1b hydrolyzes a specific fluorescent substrate (UNIBIPY; Molecular Probes, Inc.). Reactions were performed in Hanks balanced salt solution (HBSS), pH 7.4, with 1 mM CaCl₂. The ratio of rhCC10 to rh-sPLA₂ type 1b was fixed at 30:1 and the rhCC10 and rh-sPLA₂ type 1b were preincubated for 20 s at 37°C before addition of the substrate (116 μ M UNIBIPY). Reactions were stopped by adding a stopping solution containing an 8:3 mix of 2-propanol:n-hexane. Cleavage products were readily separated using normal phase high-performance liquid chromatography (2-propanol:n-hexane:water 8:3:2 solvent system; Waters Spherisorb Silicon, 5- μ m column) and characterized using fluorescence detection (excitation 480 nm, emission 517 nm). Retention time for UNIBIPY substrate was about 7–8 min, and for product, it was 19–20 min. Inhibition of rh-sPLA₂ type 1b by rhCC10 is highly reproducible, typically >75%. Reaction products were quantitated using HP software to determine the areas of substrate and product peaks.

Statistical methods. Statistical analysis was performed using the SAS System. Analysis of variance (ANOVA) was conducted to examine changes over time within each group. Two-tailed paired or unpaired *t* tests examined differences between groups. A *p* value <0.05 was considered statistically significant. Data are presented as mean \pm SD.

RESULTS

Physiologic measurements. Alveolar-to-arterial (A-a) gradients before meconium instillation were comparable in the control and rhCC10 groups (22 \pm 9 and 47 \pm 59 mm Hg, respectively). After the instillation of meconium and surfactant, the gradients rose significantly in both the control and rhCC10 groups (119 \pm 56 and 113 \pm 78 mm Hg, respectively; *p* < 0.05 compared with baseline). At 24 h, there were no significant differences in the peak inspiratory pressures or inspiratory times between groups. A-a gradients were not statistically different between the control and rhCC10 groups (104 \pm 105 and 130 \pm 181 mm Hg, respectively). Lung weights were also similar in the control and rhCC10 groups (40 \pm 13 g and 35 \pm 10 g, respectively). Pressure-volume curves were generated and revealed no difference between CC10-treated and untreated groups, suggesting that surfactant function was comparable between groups.

Biochemical assays. TAF cell counts could not be performed at 24 h due to significant debris from the meconium present in the cell pellet. IL-8 measurements were 406 \pm 364 and 408 \pm 526 at time 0 and increased to 4837 \pm 1950 pg/mg protein/mL and 5554 \pm 2895 for control and rhCC10-treated groups, respectively. This represents a 12-fold increase from baseline to 24 h, with no statistically significant difference seen between CC10-treated and untreated groups (Table 1). TNF- α measurements were similar between groups at time 0, but by 24 h, control animals had a 17-fold increase while the rhCC10-treated animals had only a 10-fold increase (*p* < 0.05) (Table 1).

In TAF collected at baseline (before meconium administration), a low level of CC10 background could be detected (mean, 19.1 \pm 15.9 ng/mL; range, 6.8–68.5). Twenty-four hours following meconium instillation, CC10-treated piglets had a mean of 2600 \pm 5800 ng/mL, while the placebo animals had a mean of 38.9 \pm 59.9 ng/mL. The background level of CC10 (measured by the ELISA), doubled in the first 24 h following meconium instillation, presumably due to cross-reactivity with endogenous piglet CC10. Mean sPLA₂ activity

Table 1. Inflammatory markers in TAF, BAL, and serum

	Time 0 h		Time 24 h	
	Control	rhCC10	Control	rhCC10
Total protein	0.34 \pm 0.012	0.52 \pm 0.24	1.54 \pm 0.90	1.41 \pm 0.70
TA IL-8	406 \pm 364	408 \pm 526	4837 \pm 1951	5554 \pm 2895
TA TNF- α	80 \pm 84	57 \pm 59	1357 \pm 676*	561 \pm 321*
Serum TNF- α	27 \pm 10	28 \pm 5	72 \pm 81	36 \pm 26
BAL NCA			42 \pm 25	29 \pm 18

There were no differences seen in total protein levels, IL-8 levels, or neutrophil chemotaxis activity between groups. There is a significant decrease in TAF TNF- α levels (*p* < 0.05) and a trend toward decreased serum TNF- α levels (*p* > 0.05) in piglets following rhCC10 administration. TA, tracheal aspirate.

* *p* < 0.05 in control vs rhCC10.

in the baseline pooled TA sample (equal mixture of all 16 piglets) was 0.64 nmol/min. Mean sPLA₂ activity in 24 h TAF showed no significant difference between controls (0.97 ± 0.29 nmol/min) and CC10-treated animals (0.99 ± 0.43 nmol/min). Therefore, sPLA₂ activity in TAF increased 44% between baseline and 24 h, irrespective of treatment group.

Serum. IL-8 levels were undetectable in serum. Although TNF-α levels were similar at time 0 (27 ± 10 and 28 ± 5 pg/mL), by 24 h, serum levels in the control animals had increased almost threefold while rhCC10-treated animals had remained the same (Table 1). These values were not statistically significant due to biologic variability. CC10 was detectable in serum at 24 h in both control (3–6 ng/mL “background”) and CC10-treated piglets (220–230 ng/mL).

BAL. IL-8 was undetectable in BAL, and there were no differences between groups in TNF-α levels at 24 h. NCA measurements on concentrated BAL were 42 ± 25% in controls and 29 ± 18% in the rhCC10-treated animals. Although this 50% difference favored the rhCC10 treatment group, these values did not reach statistical significance, again due to biologic variability.

Meconium. The mean CC10 concentration in normal newborn meconium was 18.8 ± 5.2 ng/mg meconium (soluble fraction only, *n* = 5). The mean sPLA₂ activity was 5.7 ± 3.5 nmol/min in meconium (*n* = 5). Mean concentration of sPLA₂ type 2a was 20.5 ± 7.6 pg/mg of meconium (*n* = 7). Total protein was consistent between meconium extract samples, and the mean was 18.3 ± 8.0 μg/mg meconium (*n* = 5). We found no correlation between the amount of endogenous CC10 and the level of PLA₂ activity in meconium.

Histopathology. Histopathology revealed significant pneumonitis in all untreated and rhCC10-treated piglets (Fig. 1). Polymorphonuclear cells and edema were seen in the alveolar spaces in addition to thickened cellular walls. The lungs contained significant cellular debris and mucus. There were patchy areas of pneumonitis alternating with areas of severe

atelectasis and normal lung. Areas without evidence of pneumonitis still showed thickening of the alveolar walls. There were no qualitative differences between control and rhCC10-treated groups.

DISCUSSION

MAS was induced in our piglet model and treated with a combination of surfactant and rhCC10 or placebo. rhCC10-treated animals had significantly less TNF-α in their TAF compared with placebo controls at 48 h. TNF-α is a cytokine that has multiple proinflammatory effects, acting locally as a paracrine and autocrine regulator of leukocytes and endothelial cells (17). The principal actions of TNF-α include increased expression of adhesion molecules, activation of lymphocytes, and increased cytokine production by mononuclear cells. TNF-α enhances the synthesis of CC10 in cell culture models by stabilizing CC10 mRNA, suggesting that a physiologic role for CC10 is down-regulation of TNF-α-induced inflammation (4). Meconium has been shown to induce TNF-α secretion in bronchial epithelial cells, suggesting that TNF-α is an important mediator of chemical pneumonitis and the inflammatory response in MAS (18).

This is the first report of CC10 activity in normal newborn meconium. Its presence is not surprising since CC10 rises to 1 μg/mL in amniotic fluid after 32 wk of gestation as a result of fetal lung maturation (19). CC10 was shown previously to inhibit sPLA₂ protein *in vitro* (6,20), yet the CC10 in meconium was not sufficient to completely inhibit PLA₂ activity. Moreover, soluble PLA₂ activity increased an average of 44% in TAF samples from both treatment groups 24 h after meconium instillation. It is not clear whether the increase in PLA₂ activity was due to the instillation of the meconium or whether overall activity level was augmented by host sPLA₂ released as part of the inflammatory response. Nevertheless, there were no differences in levels of PLA₂ activity between the control and rhCC10-treated groups, despite the fact that rhCC10 demonstrated potent inhibition of sPLA₂ activity *in vitro*. This suggests that the decrease in TNF-α concentrations in TAF from the treated group was not due to the ability of rhCC10 to inhibit sPLA₂ activity *in vivo*.

Meconium reportedly contains significant amounts of IL-8, sufficient to mediate neutrophil chemotaxis (21). While we did not find detectable levels of IL-8 in serum from meconium-treated piglets, elevated IL-8 in was found in TAF from meconium-treated piglets. In contrast, inflammatory responses that illicit IL-8 release in the lungs typically result in significant systemic levels as well. This result is consistent with a previous report of the highly localized nature of the inflammatory response to meconium in piglets (22).

The present study illustrates that meconium aspiration elicits a potent inflammatory response in which sPLA₂ activity, IL-8, and TNF-α in TAF were elevated 24 h post-instillation. It was somewhat surprising that only the local and systemic TNF-α response, and not IL-8 or sPLA₂ activity levels, was suppressed by rhCC10. It is possible that timing of peak responses were missed in this “snapshot” or the dose was insufficient to completely inhibit the inflammatory response

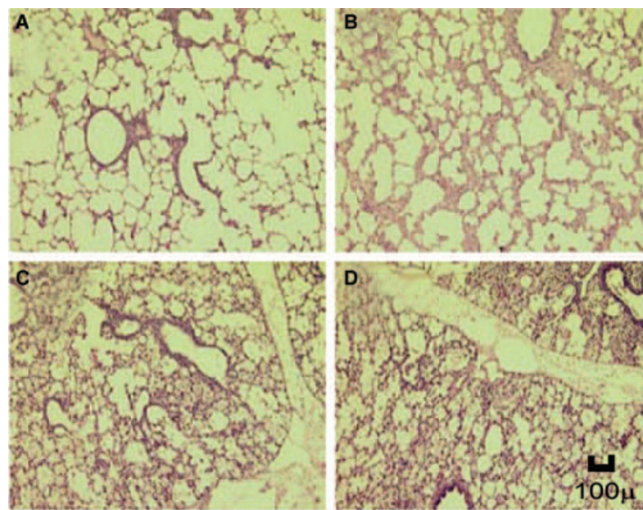


Figure 1. Lung histology after meconium aspiration. (A) Normal piglet lung (2–4 d old). (B) Diffuse septal thickening without evidence of meconium. (C, D) Significant neutrophil infiltration, mucus production, and squamous cell accumulation in both control and rhCC10-treated piglets. Scale bar = 100 μm. Magnification is ×100.

and/or sPLA₂ activity. Previous studies have suggested a strong linkage between sPLA₂ enzyme activity and TNF- α levels, TNF- α signaling and IL-8 transcription, and sPLA₂ activity and IL-8 activation during inflammatory responses to a variety of stimuli (23). Similarly, sPLA₂ protein can act as a ligand for the M-type sPLA₂ receptor in bronchial epithelial cells and human lung macrophages, stimulating cPLA₂ activation and initiation of the arachidonic acid cascade, resulting in synthesis of eicosanoid mediators and the release of IL-8 and TNF- α (23,24).

Our NCA did reveal a decreased chemoattractant capability of the BAL in rhCC10-treated animals compared with placebo controls, although the values failed to reach statistical significance. Given the incomplete suppression of the overall inflammatory response by rhCC10 in this model, it is not surprising that the chemoattractant capability of BAL was blunted, but not significantly reduced, in CC10-treated animals.

Surfactant, like meconium, is a very complex mixture of components and the anti-inflammatory properties of exogenous lung surfactant must also be considered (25). All animals received exogenous surfactant after meconium instillation, since this treatment is now considered routine. A standard dose of surfactant contains at least fourfold more surfactant than a full-term lung (26) and may largely overcome the phospholipase activity of the meconium and ameliorate inactivation of endogenous surfactant. The anti-inflammatory properties of the surfactant itself may have masked the full inflammatory response, explaining the differences between the IL-8, sPLA₂, and TNF- α responses.

It is of interest that histopathologic analysis revealed areas of alveolar wall thickening in areas where there was no evidence of pneumonitis. Other sections from the same lungs did have pneumonitis, suggesting that the inflammatory response from the areas with active disease caused cytokine-induced lung injury, resulting in hyperplasia (27). IL-8 is not only a potent neutrophil chemoattractant, but also stimulates infiltration by fibroblasts (28), which could account for the generalized thickening observed in both treatment groups. The physiologic advantage of this type of hyperplasia might be to provide a greater capacity to absorb and remove the particulate debris.

Our experimental period of 24 h was also relatively short considering that MAS develops over a variable period of time, often being initiated *in utero* and increasing in severity in the days following birth. At 24 h, CC10 levels were doubled in untreated animals and were increased by 137-fold after CC10 treatment. This suggests that the CC10 remains present and may have more biologic and physiologic effects if the exposure is longer. The maximal anti-inflammatory effects of rhCC10 (*e.g.* decreased total cell counts in TAF, particularly neutrophils) in premature human infants were observed 48–72 h after rhCC10 administration (13). Likewise, the maximal anti-inflammatory effects of rhCC10 in ventilated newborn piglets were observed at 48 h, but not 24 h post-administration (15). The lack of *in utero* effects and short experimental period represent major limitations of the piglet model.

In conclusion, *i.t.* administration of rhCC10 to piglets with MAS significantly reduced the amount of TNF- α in TAF, without affecting IL-8 or sPLA₂ activity. The nature of the inflammatory response in MAS, the relatively short experimental period (24 h), the lack of *in utero* hypoxia, and the use of exogenous surfactant contribute to this complicated picture and represent limitations of the model. Both meconium and surfactant contain components that trigger numerous pro- and anti-inflammatory cellular pathways simultaneously. More detailed studies will be required to further delineate the anti-inflammatory pathways of rhCC10 action in this model.

Acknowledgments. The authors thank Ellen Gurzenda, Hshi-Chi Koo, Jeffrey Kazzaz, and Lynda Adrouche-Amrani, M.D., for their expert technical assistance. They also gratefully acknowledge the contribution of Simcha Pollack, Ph.D., for his assistance with statistical analysis.

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