Regulation and function of CCSP during pulmonary *Pseudomonas aeruginosa* infection in vivo

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Hayashida, Shinya, Kevin S. Harrod, and Jeffrey A. Whitsett. Regulation and function of CCSP during pulmonary *Pseudomonas aeruginosa* infection in vivo. *Am J Physiol Lung Cell Mol Physiol* 279: L452–L459, 2000.—Clara cell secretory protein (CCSP) is a 16-kDa homodimeric polypeptide secreted by respiratory epithelial cells in the conducting airways of the lung. To assess the role of CCSP in bacterial inflammation and to discern whether CCSP expression is influenced by bacterial infection, CCSP-deficient [CCSP(-/-)] gene-targeted mice and wild-type mice were given *Pseudomonas aeruginosa* intratracheally. Infiltration by polymorphonuclear cells was significantly increased in the lungs of CCSP(-/-) mice 6 and 24 h after the administration of the bacteria. The number of viable bacteria isolated from the lungs in CCSP(-/-) mice was decreased compared with that in wild-type mice. Concentrations of the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α were modestly increased after 6 and 24 h, respectively, in CCSP(-/-) mice. The concentration of CCSP protein in lung homogenates decreased for 1–5 days after infection and recovered by 14 days after infection. Likewise, CCSP mRNA and immunostaining for CCSP markedly decreased in respiratory epithelial cells after infection. CCSP deficiency was associated with enhanced pulmonary inflammation and improved killing of bacteria after acute pulmonary infection with *P. aeruginosa*. The finding that *Pseudomonas* infection inhibited CCSP expression provides further support for the concept that CCSP plays a role in the modulation of pulmonary inflammation during infection and recovery.

Clara cell secretory protein; mucosal immunity; bacterial infection

**CLARA CELL SECRETORY PROTEIN (CCSP; also called CC10, CC16, and uteroglobin)** is a 16-kDa homodimeric protein produced by nonciliated bronchiolar cells in the lung (20). CCSP mRNA is also expressed to a lesser extent in the prostate, thyroid, mammary, and pituitary glands and in the uterus during pregnancy (16, 17). In the lung, CCSP is one of the most abundant soluble proteins in the extracellular lining fluid of airways (20). Despite its abundance in the airways of mammals, the physiological function of CCSP in the lung has not been elucidated.

In in vitro studies, CCSP inhibited the secretory phospholipase A2 enzyme (12) and inhibited chemotaxis of macrophages and neutrophils in uterine tissue (24). Likewise, in vitro studies (6, 27) with airway epithelial cells have suggested that CCSP may modulate the activity of various cytokines, including interferon-γ (IFN-γ) and tumor necrosis factor (TNF)-α. In vivo, CCSP-deficient [CCSP(-/-)] mice generated by gene targeting (22) showed increased inflammatory response after lung injury and viral infection. After hypoxic exposure, CCSP(-/-) mice had reduced survival time and increased proinflammatory cytokine production in the lung (10). They also had increased sensitivity to lung injury induced by ozone (14). CCSP also plays a role in immune modulation that follows pulmonary infection. After administration of adenovirus, inflammation, neutrophil migration, and proinflammatory cytokine production were increased in the lungs of CCSP(-/-) mice (9). Collectively, these studies suggest a role for CCSP as an important constitutive protein that modulates inflammatory responses after lung injury.

*Pseudomonas aeruginosa* is a common gram-negative pathogen and causes a variety of infections in compromised hosts and patients experiencing prolonged hospitalization. Ventilator-associated pneumonia caused by *P. aeruginosa* has a mortality rate of 40–68% (4, 23). Mucoid *P. aeruginosa* is a major bacterial pathogen in the airways during cystic fibrosis-related disease and is commonly associated with a decline in lung function in cystic fibrosis patients (8). The respiratory epithelium produces a number of polypeptides that influence host defense and lung inflammation, including collectins (surfactant protein [SP] A and SP-D) (5), defensins (15), lysozyme (11), and other proteins that may influence the pathogenesis of pulmonary *P. aeruginosa* infection. For example, SP-A, an abundant host defense protein produced by the lung epithelium, increases bacterial clearance and decreases the inflammatory response to mucoid *P. aeruginosa* in vivo (13).

The present study was designed to discern the potential role of CCSP in the host immune response and to determine whether acute *Pseudomonas* pneumonia influences the expression of CCSP by respiratory epithelial cells. CCSP(-/-) gene-targeted and wild-type
mice were infected with a mucoid strain of *P. aeruginosa*. CCSP deficiency was associated with increased influx of polymorphonuclear cells and improved killing of *P. aeruginosa* in vivo. Pulmonary infection with *P. aeruginosa* markedly decreased CCSP mRNA and protein in vivo.

**MATERIALS AND METHODS**

**Mice.** CCSP(−/−) (129J Ola/129J hybrid) and wild-type (129J; Taconic Farms, Germantown, NY) mice were housed under pathogen-free conditions in the Children’s Hospital Research Foundation vivarium following American Association for Accreditation of Laboratory Animal Care guidelines.

**Preparation of bacteria.** A stock culture of a mucoid strain of *P. aeruginosa* from a clinical isolate was kindly provided by Dr. J. R. Wright (Duke University, Durham, NC). Bacteria were suspended in 2× yeast tryptone (2×YT) agar with 20% glycerol and were frozen at −80°C. Before each experiment, an aliquot was thawed and plated on 2×YT agar. It was then inoculated into 5 ml of 2×YT broth and grown for 15 h at 37°C with continuous shaking. The broth was centrifuged and resuspended in 1 or 2 ml of PBS. The concentration of the suspension was determined by quantitative culture on 2×YT agar; concentration was then adjusted by dilution with sterile PBS to 1×10³ colony-forming units (CFU)/ml.

**Intratracheal administration of *P. aeruginosa*.** Eight- to twelve-week-old CCSP(−/−) and wild-type control mice (n = 6–12 mice/group) were used. Mice were anesthetized with isofluorane vapor, and a ventral midline incision was made to expose the trachea. Intratracheal inoculation of 1×10⁶ CFU of *P. aeruginosa* in 100 µl of PBS was performed with the use of a bent, 27-gauge tuberculin syringe. The incision was closed with one drop of Nexaband. At a predetermined time for each analysis, mice were killed by a lethal injection of pentobarbital sodium. A midline incision was made in the abdomen. Mice were exsanguinated by transection of the inferior vena cava to reduce pulmonary hemorrhage.

**Bronchoalveolar lavage.** Mice were killed 6 and 24 h after bacterial administration as described in Intratracheal administration of *P. aeruginosa*. The lungs were lavaged three times with 1 ml of sterile PBS. Bronchoalveolar lavage fluid (BALF) was centrifuged at 2,000 rpm for 10 min and was then resuspended in 0.5–2 ml of PBS. Cell suspension (100 µl) was mixed with 100 µl of 0.4% trypan blue (GIBCO BRL, Life Technologies, Grand Island, NY), and total cell counts were determined with a hemocytometer and trypan blue exclusion. Differential cell counts were made on cytospin preparations stained with Diff-Quik (Baxter Healthcare, Miami, FL). Photographs of randomly selected fields in light microscopy were taken for each sample. In the photographs, each of which contains 500–1,000 cells, the percentage of neutrophils and macrophages was determined. The number of each cell type was calculated from this percentage and the total cell count.

**Bacterial killing.** Mice were killed 6 and 24 h after infection. Whole lungs were removed and weighed, then homogenized in 1 ml of sterile PBS. Serial dilutions of lung homogenates were plated on 2×YT agar to determine the number of CFU of *P. aeruginosa*.

**Cytokine and chemokine production.** Lung homogenates were centrifuged at 2,000 rpm for 20 min, and the supernatants were stored at −20°C. Interleukin (IL)-1β, TNF-α, and IL-6 were quantitated in supernatants with ELISA kits (Endogen, Woburn, MA) according to the manufacturer’s directions. The neutrophilic chemokines macrophage inflammatory protein (MIP)-1α and MIP-2 were also quantitated with ELISA kits (R&D systems, Minneapolis, MN). All plates were read on a microplate reader (Dynatech, Chantilly, VA), and data were analyzed for significance with Excel 98 (Microsoft, Seattle, WA).

**Western blot analysis.** Wild-type mice were killed 6, 24, and 48 h and 5 and 14 days after bacterial infection. Right lobes were clamped with a hemostat. Right middle lobes and half of the right lower lobes were removed and homogenized in 1 ml of PBS. Lung homogenates were centrifuged at 2,000 rpm for 20 min, and total protein concentration of the supernatants was measured by the Bradford assay (Bio-Rad, Richmond, CA). Ten micrograms of protein from each sample were electrophoresed on 16% SDS-polyacrylamide gels (Novex, San Diego, CA). Western transfer to a nitrocellulose filter was performed at 60 V for 1 h. The membrane was incubated with rabbit anti-rat CCSP polyclonal antibody for 15 h at room temperature. Anti-rat CCSP antibody was a generous gift from Gurmukh Singh and Sikandar Katyal (Veterans Affairs Medical Center, Pittsburgh, PA). After being washed with Tris-buffered saline, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG antibody for 3 h at room temperature. Detection of CCSP was performed with Western blotting detection reagents (Amer sham Pharmacia Biotech, Piscataway, NJ).

**Immunohistochemical analysis.** At each predetermined time point after *Pseudomonas* administration, left lobes of wild-type mice were inflated with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) at 20 cmH₂O pressure for 1 min and were used for histological examination and immunohistochemical analysis. Inflation-
fixed lungs were washed in PBS and divided in half for paraffin embedding. Paraffin-embedded lungs were sectioned at 5 μm. The sections were deparaffinized, blocked with 2% normal goat serum, and incubated with rabbit anti-rat CCSP polyclonal antibody diluted 1:100,000 for 15 h at 4°C. The sections were washed and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 for 30 min at room temperature. CCSP was detected with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), then counterstained with 0.1% nuclear fast red. Immunohistochemical staining was analyzed by light microscopy with a Nikon microscope.

**S1 nuclease protection assay.** S1 nuclease protection assay was performed as described previously (1, 18). At each set time point after *Pseudomonas* administration, right upper lobes and the other half of the right lower lobes of wild-type mice were homogenized in 1 ml of TRIzol reagent (Life Technologies, Grand Island, NY) and used for RNA isolation. Total RNA was isolated from lung homogenates according to the manufacturer’s directions. The RNA was quantified by absorbance at 260 nm. Radiolabeled cDNA probes for CCSP, SP-A, SP-B, SP-C, and L32 were generated by end labeling with T4 kinase according to manufacturer’s instructions (GIBCO BRL, Life Technologies, Gaithersburg, MD). Labeled cDNA was purified by centrifugation in Sephadex G-50 spin columns (Boehringer Mannheim, Indianapolis, IN). Briefly, 2 μg of lung total RNA were incubated overnight at 55°C with radiolabeled cDNA probes. S1 nuclease digestion of unhybridized probe was performed at room temperature for 1 h. Labeled transcripts were separated by 6% PAGE for 1.5 h at 30 W, and the gel was dried at 80°C under vacuum. After 3–15 h, transcript abundance was detected by autoradiography at −70°C with Kodak film (Rochester, NY).

**Statistical analysis.** Significance was determined by Student’s t-test with the use of Microsoft Excel software.

**RESULTS**

**Cell counts and cell differential analysis in BALF in CCSP(−/−) mice after bacterial infection.** To assess the role of CCSP in modulating inflammatory cell migration into the air space, CCSP(−/−) gene-targeted mice and wild-type mice (8–12 wk of age) were injected with 1 × 10^8 CFU of *P. aeruginosa*. Mice recovered within 1 h after administration, with no lethality observed in either group. Total cell counts in BALF were assessed 6 and 24 h after infection. Total cell counts in BALF were increased after 6 h in lungs from both CCSP(−/−) mice and wild-type mice com-
pared with those in uninfected mice. In both groups of animals, total cell counts were further increased 24 h after administration of bacteria. At both 6 and 24 h after bacterial administration, total cell counts were significantly greater in the BALF from the lungs of CCSP(−/−) mice compared with those in wild-type mice (Fig. 1).

The inflammatory cell population in the lungs of CCSP(−/−) mice 6 and 24 h after Pseudomonas administration was determined by differential staining of the cells in BALF. Cells in the BALF of uninfected mice in both groups consisted mainly of alveolar macrophages. At both time points, BALF from infected CCSP(−/−) and wild-type mice contained predominantly neutrophils. The ratio of neutrophils to total cell count in BALF was significantly increased in CCSP(−/−) mice compared with that in wild-type mice at 6 and 24 h (Fig. 2). Although the ratio of macrophages to total cell count in BALF was decreased in CCSP(−/−) mice, there was no difference in the number of macrophages in BALF between the two groups at either time point (Fig. 2).

Bacterial killing in CCSP(−/−) mice. To discern the role of CCSP in lung bacteria killing after Pseudomonas infection, lung homogenates from CCSP(−/−) mice and wild-type mice were assessed for bacterial CFU 6 and 24 h after P. aeruginosa infection. Bacterial CFU in lung homogenates from CCSP(−/−) mice were significantly decreased compared with those in wild-type mice 24 h (Fig. 3). Although bacterial CFU in the lungs of CCSP(−/−) mice at 6 h were also decreased compared with those in wild-type mice, this difference did not reach significance.

Cytokine and chemokine concentrations in lungs from CCSP(−/−) mice after bacterial infection. To assess the possible mechanism by which CCSP deficiency causes increased neutrophilic recruitment after infection, cytokines (IL-1β, TNF-α, and IL-6) and chemokines (MIP-1α and MIP-2) were measured in lung homogenates from CCSP(−/−) mice and wild-type mice. After administration of P. aeruginosa, IL-1β, TNF-α, IL-6, MIP-1α, and MIP-2 concentrations were markedly elevated in both CCSP(−/−) mice and wild-type mice compared with those in uninfected mice (Fig. 4). Compared with wild-type mice, IL-1β and TNF-α concentrations were modestly but significantly increased in the lungs of CCSP(−/−) mice 6 and 24 h, respectively, after infection. MIP-1α, MIP-2, and IL-6 concentrations were similar in CCSP(−/−) mice and wild-type mice 6 or 24 h after infection.

Decreased CCSP expression after acute Pseudomonas infection. To determine whether CCSP expression was influenced by bacterial infection of the lung, CCSP protein was estimated in lung homogenates from wild-type mice by Western blot analysis 6, 24, and 48 h and 5 and 14 days after administration of P. aeruginosa. CCSP was readily detected in lung homogenates from uninfected wild-type mice. CCSP protein was decreased 6, 24, and 48 h after infection (Fig. 5), returning to the levels seen in uninfected mice 14 days after infection.

Immunohistochemical staining for CCSP was performed on lung sections from wild-type mice. Histologically, lung inflammation consisted of cellular consolidation and exudation in the lung parenchyma 6 h after infection. The severity of the inflammation increased 24 and 48 h after infection. Before infection, CCSP staining was readily detectable in the nonciliated bronchiolar cells (Clara cells) of large airways and bronchioles (Fig. 6). Six hours after infection, the intensity and distribution of CCSP staining were not altered. In contrast, CCSP staining was markedly decreased 24 h after infection and was undetectable 48 h after infection, coinciding with histological evidence of increased lung inflammation. CCSP staining in Clara cells was detected 5 days after infection, coinciding with some resolution of lung inflammation. Fourteen days after infection, both lung histology and the intensity and distribution of CCSP staining had returned to normal.

Decreased CCSP mRNA after acute Pseudomonas infection. To determine whether the decrease in CCSP protein seen after bacterial infection was regulated by changes in CCSP mRNA, an S1 nuclease protection assay of total RNA from lung homogenates was performed at various time points after bacterial infection of wild-type mice. In addition, SP-A, SP-B, and SP-C mRNAs were determined as markers of lung epithelium-specific gene expression. Whereas the abundance of mRNA for the housekeeping gene L32 was unchanged after bacterial infection, CCSP mRNA was decreased 24 and 48 h after bacterial infection (Fig. 7). Likewise, SP-B and SP-C mRNAs were decreased 24 and 48 h after infection. In contrast, P. aeruginosa infection did not alter pulmonary SP-A mRNA at any
time. Thus CCSP, SP-B, and SP-C mRNAs were reduced 24 and 48 h after *P. aeruginosa* infection of the lung.

**DISCUSSION**

Neutrophil recruitment into the air spaces of CCSP(−/−) mice was increased compared with that in wild-type mice 6 and 24 h after administration of *P. aeruginosa*. Surprisingly, bacteria were killed faster in CCSP(−/−) mice 24 h after infection. This, perhaps, was mediated by the increased inflammatory response.

The current studies in CCSP(−/−) mice suggest that CCSP modulates lung inflammation during pulmonary bacterial infections. Likewise, the marked decrease in CCSP expression in wild-type mice after bacterial infection suggests that changes in CCSP concentration may play a role in initiation or resolution of lung inflammation.

In the present study, CCSP(−/−) mice killed *P. aeruginosa* similarly or slightly faster than wild-type mice.
mice, suggesting that CCSP does not play a direct role in host defense against *P. aeruginosa*. Findings in the CCSP(−/−) mice are distinct from recent studies in SP-A(−/−) mice. Increased lung inflammation and decreased *P. aeruginosa* killing were observed in the SP-A(−/−) mice in vivo (13). As seen in SP-A(−/−) mice, the current study demonstrated that CCSP(−/−) mice also have increased inflammation in response to *P. aeruginosa*, yet in contrast to the findings in SP-A(−/−) mice, bacterial killing was enhanced in CCSP(−/−) mice. The increase in bacterial killing in the lungs of CCSP(−/−) mice may be explained in part by the increased recruitment of inflammatory cells in the lung. Indeed, both alveolar macrophages and neutrophils have been shown to influence the clearance of *P. aeruginosa* from the lungs (7, 21, 25). However, the

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**Fig. 5.** Western blot analysis of lung homogenates from WT mice after infection. Monomeric CCSP under reducing conditions was detectable migrating with relative molecular weight = 5,000 in the lung homogenates from uninfected WT mice. After intratracheal inoculation with $1 \times 10^8$ CFU of *P. aeruginosa*, CCSP was estimated by Western blot in 2–3 samples/time point. Data are representative of 6–8 determinations/time point. Sample loading was normalized to total protein as determined by Lowry assay. d, Day. Nos. on left, molecular mass markers in kDa.

**Fig. 6.** Immunohistochemical staining of CCSP in the lungs of wild-type mice after intratracheal *P. aeruginosa* administration. Bronchi (b), bronchioles (br), vessels (v), and inflammatory cells (arrowheads) are shown. CCSP was readily detected in bronchiolar epithelial cells of uninfected mice. Inflammatory cell infiltration was observed 6 h after infection with $1 \times 10^8$ CFU of *P. aeruginosa*. Staining for CCSP was decreased 24 h after infection and was further decreased at 48 h. Thereafter, CCSP staining increased and was similar to that in uninfected mice by 14 days after infection. Numbers of inflammatory cells were increased at 24 h and were further increased 48 h after infection. Lung inflammation was no longer apparent 14 days after infection. Figures represent 5 determinations/time point. Original magnification, ×220.
current work supports the concept that CCSP limits inflammation in the lung during bacterial infection through a mechanism that is independent of bacterial killing. The observed increase in lung inflammation in CCSP(–/–) mice after bacterial infection is consistent with a previous study (9) performed by this laboratory in which adenovirus-mediated lung inflammation was increased in CCSP(–/–) mice. Intratracheal administration of adenovirus to CCSP(–/–) mice caused increased inflammation, production of proinflammatory cytokines, and enhanced clearance of the virus (9).

Although IL-1β and TNF-α concentrations in lung homogenates were modestly increased in CCSP(–/–) mice after infection, these differences were small, and it is unclear whether differences in cytokine production mediated the increase in neutrophils observed after infection. Although the neutrophilic chemokines MIP-1α and MIP-2 were not increased in CCSP(–/–) mice after bacterial infection, pulmonary infiltration with polymorphonuclear cells was consistently more severe in CCSP(–/–) mice after infection. Vasanthakumar et al. (24) demonstrated that uteroglobin (CCSP) inhibited chemotaxis of macrophages and neutrophils in vitro. Dierynck et al. (6) demonstrated that CCSP inhibited IFN-γ production from mononuclear cells and that the biological activity of IFN-γ was diminished by CCSP. On the other hand, Yao and colleagues (26, 27) found that IFN-γ and TNF-α stimulated CCSP production from human airway epithelial cells in vitro. Taken together, this evidence shows that CCSP plays an anti-inflammatory role in the lung; however, the mechanisms by which CCSP influences neutrophil recruitment into the lung remain unclear.

In a previous study (22) performed by this laboratory, the ultrastructure of Clara cells was found to be altered in CCSP(–/–) mice. The importance of this observation to the present findings regarding bacterial killing and inflammation is unclear at present. The concentrations of SP-A and SP-B mRNA were unchanged in CCSP(–/–) mice, suggesting no direct effect of CCSP deficiency on these SPs expressed with CCSP in Clara cells. Nevertheless, the current study does not exclude the possibility that a general alteration in Clara cell function secondary to CCSP deficiency may influence the host response after Pseudomonas infection. The current studies, however, strongly support a role for CCSP, whether directly or indirectly, in modulating lung inflammation after infection.

The concentrations of CCSP in lung homogenates and CCSP mRNA were decreased in wild-type mice after intratracheal administration of P. aeruginosa. Whereas CCSP was present at high concentrations in the lung homogenates from wild-type mice, it was nearly undetectable in the lungs of mice after infection. Decreased CCSP was noted as early as 6 h after infection, coincident with the early influx of inflammatory cells into peribronchiolar and alveolar regions of the lung. It is unclear whether the loss of CCSP that occurred in the lungs of wild-type mice early in the course of infection enhanced pulmonary inflammation, perhaps in a manner similar to that seen in the CCSP(–/–) mice.

A number of mechanisms may be involved in the observed decrease in CCSP during lung inflammation, including changes in gene transcription or mRNA and protein stability. CCSP mRNA was also decreased after P. aeruginosa infection; however, the decrease in CCSP preceded the changes in CCSP mRNA, suggesting that CCSP clearance was also influenced by the infection. The Clara cell is thought to be a progenitor cell for the epithelium in injured bronchi and bronchioles in vivo (20). Cell injury may also be involved in the decrease in CCSP protein and mRNA. However, histological evaluation of the tissue did not demonstrate cell necrosis, and L32 and SP-A mRNA were not decreased, suggesting that the effects of infection on CCSP expression were not caused by cell loss.

P. aeruginosa is a common pathogen in immunocompromised hosts and a frequent cause of nosocomial infection of the lung (2). Colonization and infection of the respiratory tract by P. aeruginosa is a major cause of morbidity and mortality in patients with cystic fibrosis (8). Ventilator-associated pneumonia caused by P. aeruginosa has a mortality rate of 40–68% (4, 23). Despite advances in therapeutic practices and the advent of useful antibiotics, P. aeruginosa pneumonia is still associated with high mortality. The present find-
ings support the concept that CCSP serves a role in the regulation of pulmonary inflammation but is not critical for clearance of the bacteria. The finding that TNF-α production and associated lung inflammation are required for Pseudomonas clearance (3) supports the concept that recruitment of polymorphonuclear neutrophils to the lung is critical for bacterial clearance. However, administration of the anti-inflammatory cytokine IL-10 improved survival rates and decreased lung inflammation in P. aeruginosa pneumonia in mice (19). The present study demonstrated that deficiency of CCSP increased the clearance of P. aeruginosa but was also associated with increased neutrophil recruitment after infection. CCSP inhibited neutrophilic recruitment after P. aeruginosa infection in the lung. The timing of the changes in CCSP expression after P. aeruginosa infection support its role in modulating inflammation after acute pneumonia and during recovery.

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