CCN1 secretion and cleavage regulate the lung epithelial cell functions after cigarette smoke

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Cigarette smoking (CS) is the main risk factor for developing chronic obstructive pulmonary disease (COPD), the fourth-leading cause of death in the United States and one of the major causes of mortality and morbidity worldwide (47a). COPD encompasses multiple conditions, including chronic bronchitis, bronchiolitis, and emphysema (47a). Among them, chronic bronchitis and bronchiolitis result from the inflammatory response in the airways (17, 27, 47a). In the alveoli, CS leads to irreversible destruction of the tissues of the lung, known as emphysema (17, 27, 47a). Current literature suggests that the pathogenesis of emphysema involves progressive matrix loss, epithelial cell apoptosis, and alveolar destruction that arise from an imbalance between proteases and antiproteases (17, 27, 47a). Despite the intensive research over a few decades, the underlying mechanisms by which CS induces emphysema remain poorly understood.

The inflammatory mechanisms leading to chronic bronchitis are associated with increased edema, bronchospasm, smooth muscle hypertrophy, increased production of mucus, and squamous cell metaplasia (23, 30, 40). Significantly increased number of neutrophils and macrophages within the airway walls and lumen of both the bronchi and bronchioles are found in the lungs of patients with chronic bronchitis (6, 46). Increased proinflammatory cytokines such as interleukin (IL)-8 have also been demonstrated in the sputum of smokers with chronic bronchitis (31, 32, 49). IL-8 is critical in recruiting neutrophils into the lung tissue and airway that are believed to be important in the pathogenesis of chronic bronchitis (31, 32, 49). In contrast to chronic bronchitis, emphysema presents with increased alveolar wall cell death and loss of lung tissue maintenance (17, 27, 47a). Although chronic inflammation is essential in the development of emphysema, how exactly inflammation contributes to the lung tissue loss remains incompletely understood. Presumably, inflammatory cells are responsible for the secretion of a variety of tissue enzymes that eventually digest the surrounding cells in the interstitial/parenchymal lung tissue (17, 27, 47a). Besides inflammation, reactive oxygen species induce apoptosis, and protease-antiprotease imbalances also contribute significantly to the pathogenesis of emphysema. For instance, matrix metalloproteinases (MMPs) have been well recognized for their functions in the development of emphysema in mice exposed to CS (10, 35, 43). Mice overexpressing MMP1 present with the same emphysematous changes as observed in patients with emphysema (12). More recently, expression of vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) on both epithelial and endothelial cells was reported, and deficiency in VEGFR or VEGF leads to lung cell apoptosis and emphysema (8, 45). Furthermore, both VEGF ligand and VEGFR expression are reduced in human emphysematous lungs (13, 22), supporting...
the critical roles of VEGF in maintaining the integrity of lung tissue.

Our recent studies have revealed a novel protein, CCN1 (former name Cyr61), that potentially plays a crucial role in the development of COPD (33). CCN1, the first member of the CCN protein family, was initially discovered a decade ago (42). It is a cysteine-rich, secreted, extracellular matrix (ECM)-associated protein that regulates many cellular activities, including cell proliferation, adhesion, migration, differentiation, and apoptosis (15, 19, 25). CCN1 has essential roles in vascular system development, and the CCN1-null mice have a lethal phenotype due to vascular defects in the placenta and embryo (19). CCN1 has been shown to bind to the cell surface of at least five different integrins (\(\alpha_V\beta_1, \alpha_V\beta_5, \alpha_\omega\beta_1, \alpha_{IIb}\beta_3, \alpha_{IIIb}\beta_3\)) and heparan sulfate proteoglycans (15, 19, 25). The interaction between different integrins and CCN1 leads to cell type- and function-specific effects. This molecule is known to have five domains that determine different integrin-binding sites (19). CCN1 can be secreted from a variety of cells in the lungs, including the epithelial cells (33). Despite its extensive expression in lung tissue, only a few reports have documented the function of CCN1. Our most recent study uncovered that the epithelial cell-released CCN1 triggers IL-8 secretion and therefore plays an important role in recruiting inflammatory cells in the lung parenchyma (33). Herein, we hypothesized that CCN1 is a potentially crucial factor for the pathogenesis of CS-induced emphysema. We found that full-length CCN1 (fl-CCN1) was cleaved (cCCN1) after prolonged CS exposure and that flCCN1 and cCCN1 have distinct functions. Furthermore, we showed that the pathways of protein secretion and modification also play a crucial role in the pathogenesis of emphysema.

MATERIALS AND METHODS

Cell culture. Human bronchial epithelial cell line Beas2B cells and primary HBE cells were purchased from the American Type Culture Collection (Manassas, VA). Beas2B cells were cultured in 100-mm dishes containing DMEM with 10% FBS (Life Sciences, Grand Island, NY), 2 mM L-glutamine, 100 \(\mu\)g/ml penicillin, and 100 \(\mu\)g/ml streptomycin, and HBE cells were cultured in an airway epithelial cell basal medium (ATCC) with supplement in a humidified incubator under 5% CO\(_2\) at 37°C, respectively. Cells were exposed to 10% cell basal medium (ATCC) with supplement in a humidified incubator for 5 days/wk for a total of 3 mo using a total body CS exposure chamber as described (33). The smoke machine was adjusted to deliver 10 cigarettes at one time. The chamber temperature was periodically measured for total particulate matter, and concentrations ranged from 100 to 120 mg/m\(^3\).

Preparation of CSE. CSE derived from Kentucky Reference 3R4F research blend cigarettes (University of Kentucky) were prepared as described (33). In brief, CSE was prepared by bubbling smoke from one cigarette in 10 ml serum-free DMEM medium, and CSE was sterilized filtered through a 0.2-\(\mu\)m filter (VWR International, Radnor, PA).

Chemicals and recombinant protein. Tosyllysine chloromethyl ketone (TLCK) hydrochloride was purchased from Santa Cruz Biotechnology, Y-27632 and SB-203580 were from Calbiochem (Darmstadt, Germany), Z-DEVD-FMK was from BioVision (Milpitas, CA), recombinant human plasmin was from Athens Research & Technology (Athens, GA), and recombinant human and antihuman CCN1 proteins were from R&D Systems (Minneapolis, MN).

ELISA. The human IL-8 ELISA kit was purchased from Thermo (Rockford, IL), and human VEGF and MMP-1 duoset was from R&D Systems and followed the manufacturer’s instructions.

Isolation and detection of COOH-terminal and NH\(_2\)-terminal CCN1. Bioactive recombinant CCN1 (10 \(\mu\)g) was incubated with plasmin (1 \(\mu\)g) at 37°C for 1 h. Samples were incubated with anti-CCN1 antibodies (H-2 or N-16; 10 \(\mu\)g/ml) overnight. Antibody-conjugated secondary antibodies were incubated with agarose beads at 4°C for 1 h, and then beads binding positive and negative samples were isolated by centrifugation at 10,000 g for 10 min. Samples were loaded on the H-78 antibody-coated ELISA plate for 3 h at room temperature (RT) and then added N-16 or H-2 antibodies for 2 h at RT. Next, horseradish peroxidase-conjugated secondary antibodies were added for 1 h and protected from direct light exposure. The sample was developed with 3,3′,5,5′-tetramethylbenzidine solution, stopped by 2 N H\(_2\)SO\(_4\), and then read at 450 nm wavelength. To quantify the cCCN1, recombinant CCN1 was used for standard curve.

Small-interfering RNA and CCN1 constructs transfection. Human CCN1 and integrin-\(\alpha_6\), -\(\alpha_5\), -\(\alpha_1\), and -\(\alpha_\omega\) small-interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology, and INTERFERin was from Polyplus-Transfection (Ilikkirchen, France). Transfection procedure was followed by INTERFERin manufacturer’s instructions.

Human CCN1 plasmid constructs were designed by following the pDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen, Grand Island, NY), and CCN1 constructs were transfected by Lipod293 (SignaGen, Rockville, MD) and followed the manufacturer’s instruction. Transfected CCN1 plasmids were detected by anti-V5 antibody.

Statistical analysis. The means of fold change in Figs. 1–7 were calculated by 10.22±0.33 on April 4, 2017 http://ajplung.physiology.org/ Downloaded from

RESULTS

CS augmented CCN1 secretion and induced CCN1 cleavage in lung epithelial cells. In our previous report, we have found that CS immediately induces CCN1 expression and secretion in lungs (33). In our current studies, we first observed that CS augmented the release of exosome from lung epithelial cells.

Western blot analysis. Western blot analysis was according to procedures described (32). CCN1, \(\beta\)-actin, CD9, CD63, and integrin-\(\alpha_6\), -\(\alpha_5\), -\(\alpha_1\), and -\(\beta\) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), and plasmin was from Abcam (Cambridge, MA). Membranes were washed and incubated with appropriate secondary antibodies (Santa Cruz). Detection was performed using the SuperSignal West Pico and Femto system (Pierce, IL) and exposed to Molecular Imager chemiDoc XR+ (Bio-Rad, Hercules, CA). Normalization and relative quantification were performed with Image Lab software (Bio-Rad).

In vivo CS exposure. Mice were exposed to CS (100 cigarettes/day for 5 days/wk) for a total of 3 mo using a total body CS exposure chamber as described (33). The smoke machine was adjusted to deliver 10 cigarettes at one time. The chamber temperature was periodically measured for total particulate matter, and concentrations ranged from 100 to 120 mg/m\(^3\).

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(Fig. 1, A and B). Recent evidence has demonstrated that the exosome secretion exerts key pathophysiological roles in a variety of disease processes (9, 28). This prompted us to explore the molecular contents in the CS-stimulated exosome. The small GTPase Rab27a regulates exosome secretion (4, 9). Interestingly, CS induced Rab27a expression in Beas2B lung epithelial cells in a time-dependent manner, as early as 2 h after exposure to CS (Fig. 1C). We further confirmed our observation using lung tissue homogenates obtained from mice after 6 mo CS exposure (Fig. 1D). To evaluate whether CCN1 was secreted in an exosome-encapsulated manner, we assessed the percentage of CCN1-positive exosome released from Beas2B cells, in the presence and absence of CSE. We found that CSE markedly increased the percentage of CCN1-positive exosome compared with the controls (45 vs. 29%), as shown in Fig. 1E.

Next, we quantified the CCN1 in exosome using ELISA. Robustly augmented CCN1 level was detected in the CS-induced exosome compared with the control when loading the same amount of exosome (Fig. 1F). CCN1 protein is composed of five distinct domains, and the domain-specific function plays essential roles in the pathogenesis of multiple human diseases (15, 39). To determine whether a specific domain of CCN1 protein mediated its exosome-shuttled secretion after CSE, five CCN1 constructs were generated in which one of the specific domains was deleted (Fig. 1G, top). We transfected CCN1 domain-specific plasmids (containing V5 tag) into the Beas2B cells. After 24 h exposure to the 10% CSE, both exosome marker CD9 and CCN1 were determined using Western blot analysis. We found that deletion of domain 1 (SP domain), but not any of the remaining four domains, eliminated the CCN1

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Fig. 1. Cigarette smoke extract (CSE)-induced secretion and cleavage of CCN1 in lung epithelial cells. Beas2B cells were cultured and exposed to 10% CSE as described in MATERIALS AND METHODS. A: left, exosome isolated after CSE were observed using negative-stained transmission electron microscopy (TEM) image. Right, protein concentration in exosome isolated from untreated cells (sham) and CSE-exposed cells. B: Coomassie blue staining. Whole cell lysate (WCL), supernatant, and pellet from ultracentrifuged conditioned media after 10% CSE treatment in Beas2B cells. Each 20 μg protein were loaded and stained by Coomassie blue solution for 20 min and destained. C: expression of exosome marker Rab27a in Beas2B cells in the presence and absence of 10% CSE (time course). D: expression of Rab27a in mouse lung tissue homogenates after room air (RA) or CS (6 mo exposure). Expression level was normalized to β-actin. E: percentage of CCN1-positive exosome that were isolated from control or 10% CSE-treated cells. Anti-CD9 was used as an exosome marker. F: quantification of CCN1 in exosome isolated from control or CSE-treated cells, using ELISA. Equal amount of exosome (1 μg) was loaded for each group. G: each V5-tagged CCN1 construct was transfected into Beas2B cells. After 2 days, these transfected cells were exposed to 10% CSE. After 24 h stimulation, exosome and WCLs were isolated. Exosome was confirmed using anti-CD9 antibodies, and CCN1 was detected using anti-V5 antibodies. H: full-length CCN1 (fCCN1) and cleaved CCN (cCCN1) were detected in soluble or pellet components (exosome) using Western blot analysis. An equal amount (20 μg/each lane) of protein was loaded. *P < 0.05. Each panel represents at least 3 repeats with similar results.

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protein in exosome (Fig. 1G). More interestingly, we further made a novel observation that CS induced CCN1 cleavage in lung epithelial cells (Fig. 1H). Surprisingly, the flCCN1 and cCCN1 were detected in exosome or the soluble component, respectively (Fig. 1H), suggesting a differential route of secretion for flCCN1 or cCCN1 after CSE stimulation despite that CCN1 cleavage has been reported in breast cancer tissue previously (14, 37). CS-induced CCN1 cleavage in lung tissue and its functional roles in lung diseases remain unexplored. These interesting observations prompted us to further characterize the generation of cCCN1 and the underlying mechanisms by which CS induced the cleavage of CCN1 in lung cells and tissue.

CS induced CCN1 cleavage via secreted plasmin. We first evaluated the CCN1 cleavage using both Beas2B cells and primary human lung epithelial cells (HBE). CCN1 was cleaved when plasmin was incubated with the whole cell lysate (WCL) obtained from Beas2B or HBE cells (Fig. 2A). We next measured intracellular or extracellular flCCN1 and cCCN1 after 10% CSE stimulation in Beas2B. Levels of intracellular flCCN1 were diminished while extracellular flCCN1 was enhanced (Fig. 2B). Only extracellular cCCN1 was increased after CSE stimulation (Fig. 2B). This observation suggests that cCCN1 is generated at ECM rather than in cytosol. Next, we measured the level of secreted plasmin after 10% CSE stimulation. Secreted plasmin was increased in a time-dependent manner in both Beas2B and HBE cells (Fig. 2C). We also confirmed that cCCN1 and plasmin levels were both increased...
in lung tissue homogenates obtained from mice after CS exposure compared with the air-exposed group (Fig. 2D). Consistently, elevated flCCN1, cCCN1, and plasmin levels were detected in lung tissue homogenates obtained from COPD patients compared with those obtained from normal controls (Fig. 2E). To further validate our observations, we isolated exosome after 10% CSE stimulation for 24 h and incubated with plasmin for 1 h at 37°C. Levels of exosome-enriched flCCN1 were downregulated, and cCCN1 was augmented by plasmin in a dose-dependent manner (Fig. 2F). We then evaluated the level of flCCN1 and cCCN1 Beas2B cells treated with the serine protease inhibitor (TLCK), which suppresses plasmin activation (5). CSE-induced cCCN1 was significantly reduced after adding TLCK, in a dose-dependent manner (Fig. 2G). We also pretreated the Beas2B cells with plasminogen siRNA to knock down plasmin. The efficacy of plasminogen deletion by siRNA was shown in Fig. 2F, top. Consistently, deletion of plasminogen abolished the CSE-induced cCCN1 production (Fig. 2H).

Differential cellular functions of flCCN1 and cCCN1 in the lung epithelial cells in response to CSE exposure. As above, our data indicated that CS/CSE induced CCN1 cleavage and secretion. These observations suggested distinct cellular functions of flCCN1 and cCCN1 after CS/CSE exposure and promoted us to further dissect their cellular functions. We previously reported that CCN1 promoted IL-8 release by lung epithelial cells after CSE (33). Therefore, we compared the effects of flCCN1 and cCCN1 on IL-8 secretions by Beas2B cells. We first treated the Beas2B cells with the exosome (pellets) or supernatants obtained from the same amount of cells (10 μg/ml). A significantly higher IL-8 secretion was detected in the cells exposed to CS-induced exosome (Fig. 3A). These data suggested that IL-8 secretion was induced by flCCN1 (exosome), but minimally by cCCN1 (supernatant).

Next we treated the Beas2B cells with flCCN1 bioactive recombinant protein (1 μg/ml), plasmin (0.1 μg/ml), or plasmin + flCCN1. After 4 h, IL-8 secretion was the highest in Beas2B cells treated with flCCN1. Adding plasmin alone or plasmin + flCCN1 both eliminated the IL-8 secretion, suggesting that flCCN1 is required for Beas2B cells to release IL-8 (Fig. 3B). To determine which CCN1 domain is critical in mediating IL-8 release, we transfected Beas2B cells with the above-mentioned CCN1 domain-specific plasmids. As shown in Fig. 3C, domain 4 is required for CCN1 to promote IL-8 secretion.

VEGF has been known for its crucial roles in the pathogenesis of COPD. VEGF reduction resulted in more severe forms of emphysema (20, 21, 26). Therefore, we next examined the effects of flCCN1 and cCCN1 on VEGF production and secretion.

**Fig. 3. Differential effects of flCCN1 and cCCN1 on interleukin (IL)-8 and vascular endothelial growth factor (VEGF) secretion in lung epithelial cells.**

A: Beas2B cells were cultured and exposed to control medium or CSE. Cell culture supernatants were collected, and the pellet/soluble portion was separated. The pellet or soluble portions were used to treat naïve Beas2B cells. IL-8 was next measured in these Beas2B cells. B: Beas2B cells were treated with flCCN1 recombinant protein (1 μg/ml), plasmin (0.1 μg/ml), or flCCN1 + plasmin, and IL-8 was measured 4 h later. C: Beas2B cells were transfected with empty vector or CCN1 domain-specific constructs (2 μg). IL-8 in supernatant was measured using ELISA 2 days later. D: Beas2B cells were cultured and exposed to control medium or CSE. Cell culture supernatants were collected, and the pellet/soluble portion was separated. The pellet or soluble portions were used to treat naïve Beas2B cells. VEGF levels in these Beas2B cells were measured by ELISA. E: Beas2B cells were treated with flCCN1 recombinant protein (1 μg/ml), plasmin (0.1 μg/ml), and flCCN1 + plasmin, and VEGF were measured 4 h later. F: Beas2B cells were transfected with empty vector or CCN1 domain-specific constructs (2 μg). After 2 days’ expression, VEGF in supernatant was measured using ELISA. *P < 0.05 compared with sham. **P < 0.05 compared with CCN1 in B and E. Each panel represents at least 2 repeats with similar results.
secretion in lung epithelial cells. Similarly to the situation in IL-8 secretion, a robustly elevated VEGF level was detected in the cells exposed to CS-induced exosome (Fig. 3D). Bioactive flCCN1 recombinant protein (1 µg/ml) augmented VEGF secretion, but not the plasmin + flCCN1, suggesting that the flCCN1, but not the cCCN1, promoted the VEGF secretion in Beas2B cells (Fig. 3E). Again, we determined which CCN1 domain is critical in mediating VEGF release. Using the above-mentioned CCN1 domain-specific plasmids, we confirmed again that domain 4 is critical in regulating the VEGF secretion (Fig. 3F).

Differentially regulated MMP1 secretion by flCCN1 and cCCN1 in lung epithelial cells. CS is well known to induce MMP1 expression (12). Previous studies have demonstrated that MMP1 promotes the emphysematous phenotype in mice (44). Furthermore, mice with lung specific expression of the human MMP1 develop similar emphysematous changes compared with the smokers (11). Therefore, we next investigated whether flCCN1 or cCCN1 modulate the level of MMP1 in lung epithelial cells. As shown in Fig. 4A, we first examined whether deletion of CCN1 affects the MMP1 secretion. CCN1 siRNA significantly reduced the CS-induced MMP1 secretion (Fig. 4A). As shown in Fig. 4A, we first examined whether deletion of CCN1 affects the MMP1 secretion. CCN1 siRNA significantly reduced the CS-induced MMP1 secretion (Fig. 4A). Next, we treated the Beas2B cells with the exosome or soluble factors isolated from the cell supernatants after CSE exposure using an equal amount of protein. Both the exosome and the soluble factors stimulated the MMP1 secretion. However, the soluble factors appeared to have the strongest effects on promoting MMP1 secretion (Fig. 4B). Again, silencing the CCN1 suppressed the MMP1 secretion induced by both the soluble factors and the exosome (Fig. 4B). To differentiate the effects of flCCN1 and cCCN1 on CS-induced MMP1 secretion, we treated the Beas2B cells with the bioactive CCN1 recombinant protein, in the presence and absence of plasmin. Incubation of flCCN1 and plasmin together resulted in cCCN1 and stimulated a significantly higher amount of MMP1 compared with CCN1 or plasmin alone (Fig. 4C). CSE has been known to induce plasmin activation (1, 3, 24). Therefore, we next confirmed the above data using plasmin inhibitor (TLCK). Beas2B cells were pretreated with TLCK followed by CSE exposure. Blocking plasmin using TLCK reduced the MMP1 secretion in a dose-dependent manner (Fig. 4D). To pinpoint the domain that is important in stimulating MMP1 release, we first isolated the NH2-terminal and COOH-terminal fragments of plasmin-cCCN1 as described in Fig. 6B. Both the NH2-terminal and COOH-terminal fragments of CCN1 induced MMP1 secretion from Beas2B cells (Fig. 4E). However, COOH-terminal cCCN1 exerted much stronger effects on induction of MMP1 (Fig. 4E), and also cell death (Fig. 4F).

cCCN1 functions via integrin-α7 in lung epithelial cells after CS exposure. We next explored the underlying mechanisms by which cCCN1 exerted its cellular functions in epithelial cells after CSE. Integrins are known cell surface receptors for CCN1 (19). We first evaluated the integrin mRNA expressions in the presence of flCCN1, plasmin, and plasmin + flCCN1 (cCCN1). As shown in Fig. 5A, nine α-integrins and six...
β-integrins were evaluated using previously published primers (7). Compared with control, flCCN1-alone, or plasmin alone-treated cells, the cCCN1 (flCCN1 + plasmin) treatment induced mRNA transcription of integrin-α6, -α7, -αv, and -α11 (Fig. 5A). To determine the effects of these integrins on CS-induced MMP1, we transfected Beas2B cells with siRNAs of integrin-α6, -α7, -αv, and -α11 and -β1 (Fig. 5B). Our data showed that silencing of these integrins all reduced the CS-induced MMP1 secretion (Fig. 5C). Among them, siRNAs of integrin-α7 and -α11 exerted the most significant effects (Fig. 5C). To evaluate the effects of these integrins on cCCN1-mediated MMP1 secretion, we transfected the Beas2B cells using the siRNAs of integrin-α6, -α7, -αv, and -α11. After stimulating the cells with flCCN1, plasmin, or flCCN1 + plasmin, we measured the total CCN1 and cCCN1 in mouse lung tissue homogenates and bronchoalveolar lavage fluid (BALF). C57/BL6 mice were exposed to filtered air or CS. After 6 mo, mouse lung tissue homogenates were obtained and subjected to ELISA. A: 6-mo CS exposure induced emphysematous changes in mouse lung. B: Level of cCCN1 (NH2-terminal) or COOH-terminal fragments. Bioactive recombinant CCN1 was incubated with plasmin at 37°C for 1 h. Samples were incubated with anti-CCN1 antibodies (H-2 or N-16) at 4°C overnight. Antibody-conjugated samples were incubated with agarose beads at 4°C for 1 h, and then beads binding positive and negative samples were isolated by centrifugation at 10,000 g for 10 min. Samples were loaded on the H-78 antibody-coated ELISA plate for 3 h at room temperature (RT) and then added N-16 or H-2 antibodies for 2 h at RT. Next, horseradish peroxidase (HRP)-conjugated secondary antibodies were added for 1 h and protected from direct light exposure. The sample was developed with 3,3′,5,5′-tetramethylbenzidine solution, stopped by 2 N H2SO4, and then read at 450 nm wavelength. To quantify the cCCN1, recombinant CCN1 was used for standard curve. Total CCN1 level (C), cCCN1 level (D), and the ratio between cCCN1/total CCN1 (E); C57/BL6 mice were exposed to filtered air or CS. After 1 or 6 mo, BALF was obtained and subjected to ELISA. Total CCN1 level (F), cCCN1 level (G), and the ratio of cCCN1/total CCN1 (H) in 1 mo (n = 5) and 6 mo (n = 7) BALF.
plasmin (cCCN1), we compared the MMP1 level. Interestingly, only cells transfected with integrin-α7 siRNA abolished the effects of cCCN1 on induction of MMP1 (Fig. 5D). Silencing of integrin-α7 did not affect the expression of integrin-α6, -α11, and -αV (Fig. 5E). Also integrin-α7 interacts with the β1-subunit (29, 48). To determine whether integrin-α7 or -β1 is crucial for MMP-1 secretion after CS exposure, we first silenced integrin-α7, -β1, or both together in Beas2B cells.
MMP-1 secretion induced by cCCN1 was abolished after integrin-α7 silencing but enhanced after integrin-β1 silencing (Fig. 5F). Furthermore, CS induced integrin-α7 expression significantly after CS exposure in mouse lung tissue (Fig. 5G). To investigate the signaling pathways involved in the cCCN1-induced MMP-1 secretion, we pretreated the Beas2B cells with a variety of signaling pathway inhibitors (1 h) followed by cCCN1 protein for another 8 h. MMP1 secretion was then measured. We found that MMP-1 secretion was diminished after Rho signaling inhibitor but not the rest of the inhibitors, including ERK, MAPK, and caspase-3 pathway inhibitors (Fig. 5H).

Level of cCCN1 correlated with the development of emphysema.

We further verified our observations using mouse emphysema models. The mice were exposed to CS for 6 mo and confirmed for emphysematous changes (Fig. 6A). Mouse lung tissue homogenates and bronchoalveolar lavage (BALF) were obtained and analyzed for total CCN1 level, cCCN1 level, and the cCCN1-to-total CCN1 ratio following procedures as described in Fig. 6B. We found that, in the lung tissue homogenates, total CCN1 levels had no significant changes between the lung homogenates from control mice and CS-exposed mice (Fig. 6C). Interestingly, markedly lower cCCN1 level and cCCN1-to-total CCN1 ratio were found in lung tissue homogenates from CS-exposed mice (Fig. 6, D and E). Given that CCN1 secretion was highly augmented after CS exposure, we hypothesized that the lower cCCN1 level in lung tissue reflects higher secretion of cCCN1. Therefore, we analyzed the total and cCCN1 level in BALF obtained from control or CS-exposed mice. As predicted, total CCN1 levels in BALF did not have any differences (Fig. 6F). The cCCN1 level in BALF was highly elevated in BALF obtained from mice after 6 mo CS exposure but not from those after 1 mo exposure (Fig. 6G). The ratio between cCCN1 and total CCN1 was also significantly increased in the BALF obtained from mice after 6 mo CS exposure (Fig. 6H). Only the mice after 6 mo, but not 1 mo, CS exposure expressed emphysematous changes; thus, the elevated cCCN1 was only shown in those mice with significant emphysema.

**DISCUSSION**

Our studies revealed that the matricellular CCN1 potentially is an important switch and may mediate the cross talk between inflammation in the airways and tissue loss in the lung parenchyma. /flCCN1 was upregulated and secreted by lung epithelial cells after CS exposure. As illustrated in our previously published study (33), this increased secretion of /flCCN1 facilitated the IL-8 release by epithelial cells, subsequently recruited more immunomodulatory cells in the lung tissue, and promoted lung inflammation (33). In our current work, we further identified that /flCCN1 was secreted in a tightly regulated manner, i.e., via epithelial-derived exosome. We found that CS induced a large amount of exosome release from lung epithelial cells (Fig. 1). /flCCN1 was secreted in an exosome-shuttled manner. The exosome-mediated secretion of /flCCN1 potentially allows “inflammatory” signals to be transmitted to distant portions of the lungs. More importantly, prolonged CS exposure cleaved the cCCN1 into truncated forms (Figs. 1 and 2). These cCCN1 fragments existed mainly in the soluble portion of the ECM fluid (Fig. 1G). In this study, we targeted plasmin as a generator of cCCN1. However, other proteases, such as MMPs, elastase, and proprotein convertases, also mediate CS-induced emphysema (32). Whether these proteases participate in the cleavage of CCN1 after CS exposure requires further investigation and may be included in our near-future directions.

The different compartments of /flCCN1 and cCCN1 potentially allow them to carry distinct cellular functions via the domain-specific interactions with integrins simultaneously at the same location. This observation prompted us to further investigate the cellular functions of cCCN1s. The significance of this study falls into the connected but distinct biological functions that /flCCN1 or cCCN1 carries after CS exposure. /flCCN1 triggers the inflammatory responses via mediating IL-8 release and subsequent neutrophil recruitment. On the other hand, /flCCN1 may also play a role in maintaining the integrity of lung tissue structure by facilitating the VEGF secretion.

CCN1 is probably a critical factor involved in the lung homeostasis. We identified that the CS-upregulated plasmin was responsible for the CS-induced CCN1 cleavage (Fig. 2). Previous studies have reported that plasmin cleaves proteins between lysine (K)-arginine (R) and lysine (K)-valine (V) (41). We analyzed the published protein sequence of CCN1 as shown in Fig. 7A (16). The major potential site (K/R) for plasmin-mediated cleavage falls into the linked region that locates between domains 3 and 4 (Fig. 7A). Therefore, the cellular functions of cCCN1 potentially reflect two fragments...
consisting of domains 1–3 (NH2-terminal fragment) and/or domains 4–5 (COOH-terminal fragment). If the plasmin-mediated cleavage occurs at the K/V sites, as shown in Fig. 7A, the SP domain likely is deleted from the main fragments. This potentially explains the fact that cCCN1 was not secreted via an exosome-shuttled manner given that SP domain was required for exosome encapsulation (Fig. 1G).

Our continued studies uncovered a few important aspects of the cellular functions mediated by cCCN1s. First, the truncated CCN1, cCCN1, failed to induce as much of the proinflammatory cytokine IL-8 as the fCCN1 (Fig. 3, A–C). The domain 4 was required for the CCN1-mediated IL-8 and VEGF production (Fig. 2). Cleavage of CCN1 abolished the effects of fCCN1 protein on VEGF and IL-8 production, and, additionally, cCCN1 fragments promoted the production of MMP1 (Fig. 3). The COOH-terminal fragment of cCCN1 (domain 4–5) stimulated the MMP1 production stronger than the NH2-terminal one. Furthermore, COOH-terminal cCCN1 induced cell death (Fig. 4). The upregulated MMP1 and downregulated VEGF both have been well documented promoting emphysematous change in lungs (11, 12, 26). Therefore, we postulated that the accumulation of CSE-induced cCCN1 mainly via secreted plasmin. This secretion mechanism determined the “local” feature of cCCN1. Therefore, localized accumulation of cCCN1 may be responsible for the “patchy” appearance of the emphysematous changes in lung tissue. Consistent with our hypothesis, we found that cCCN1 level was highly elevated in BALF obtained from the mice with emphysematous changes after 6 mo exposure of CS (Fig. 6). CCN1 binds with the cell surface integrins and functions in a domain-integrin specific way (19). Our data suggested that integrin-α2 was required for the cCCN1 (domain 1–3) to promote the MMP1 secretion, and this integrin-α2-mediated effect was probably via the Rho pathway (Fig. 5F). However, thus far, to our best knowledge, previous reports have not revealed a binding site of integrin-α2 in CCN1. It is not clear whether the CCN1 cleavage led to a novel binding site for integrin-α2 or represents on that have not yet been reported. Although we did not observe any off-target effects using integrin-α2 siRNAs (Fig. 5), it remains a possibility that some other integrins are also reduced after deletion of integrin-α2. This is certainly one of the limitations in our reports. Human Beas2B and HBE cells are convenient cellular models to illustrate the signaling pathways of CCN1 regulation and function. However, the results and conclusions obtained from these cultured cells should not be readily extrapolated to human alveolar cells in physiological conditions.

In our studies, although both NH2-terminal and COOH-terminal fragments carried proemphysema functions, the COOH-terminal fragment (domain 4–5) seemed to play the more critical role, by not only inducing MMP1, but also promoting cell death. Integrin-α6β1 binding site localizes in the thrombospondin (domain 4) domain and is known to induce apoptosis (19), thus potentially exacerbating the lung tissue loss. Furthermore, integrin-β1 activation has been shown to correlate with severe emphysema (2). Besides the integrin-α3β1, integrin-α6β3, -α5β1, -α1β1, and -αMβ2 are also known as CCN1-binding receptors (19). The role of integrins in the pathogenesis of emphysema has not been extensively explored. That said, integrin-β6 seems to function oppositely compared with β1, and deletion of β6 resulted in MMP12 induction, which potentially aggravates emphysema (34). Apparently, the roles of integrins in the development of emphysema require further research.

Although our studies showed that cCCN1 was markedly elevated in BALF obtained from emphysematous mice (Fig. 6), future studies should include the determination of local concentrations of cCCN1 in the areas with emphysematous changes or normal lung tissue. One additional limitation of our current studies is that the function of the inhibition or depletion of CCN1 should be investigated in the development of CS-induced emphysema using in vivo models. cCCN1 level, but not the full-length form, was positively correlated with emphysematous changes, suggesting that cCCN1 probably was involved in the pathogenesis of CS-induced emphysema. However, direct confirmation requires generation of lung epithelial Cre-cCCN1 null mice. We are in the process of generating CC10 Cre-fCICN1 null mice. Moreover, generation of a specific deletion of the cleaved form is required to directly answer this question. This is also included in our near future plans. The next step in our work will be to obtain exact sequences of the fragmented CCN1.

Related to genetic predisposition of COPD, such as the α1-antitrypsin deficiency, elevated plasmin levels have also been reported (47). Because elevated plasmin leads to increased cCCN1, it will also be interesting to determine the level of cCCN1 in these patients. CCN1 has been reported to be expressed in an alternative spliced form (38). The CCN1 mRNA alternative splicing also induces a truncated CCN1 protein with domains 1–3 only, although CS did not induce the CCN1 mRNA alternative splicing in epithelial cells in our studies. Further research is required to explore the CCN1 mRNA alternative splicing in all other lung cells in response to CS exposure.

In summary, CCN1 was secreted in an exosome-shuttled manner or fragmented form (cCCN1). If confirmed using transgenic/knock out mouse models, cCCN1 level potentially can serve as a biomarker to predict the development of emphysema. Targeting on decreasing the cCCN1 generation may help on preventing the rapid progression of emphysema.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS


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