CCN6 as a profibrotic mediator that stimulates the proliferation of lung fibroblasts via the integrin β1/focal adhesion kinase pathway


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Abstract: Idiopathic pulmonary fibrosis is a progressive and lethal disease of the lung that is characterized by the proliferation of fibroblasts and increased deposition of the extracellular matrix. The CCN6/WISP-3 is a member of the CCN family of matricellular proteins, which consists of six members that are involved in many vital biological functions. However, the regulation of lung fibroblasts mediated by CCN6 protein has not been fully elucidated. Here, we demonstrated that CCN6 induced the proliferation of lung fibroblasts by binding to integrin β1, leading to the phosphorylation of FAKY397. Furthermore, CCN6 showed a weak, but significant, ability to stimulate the expression of fibronectin. CCN6 was highly expressed in the lung tissues of mice treated with bleomycin. Our results suggest that CCN6 plays a role in the fibrogenesis of the lungs mainly by stimulating the growth of lung fibroblasts and is a potential target for the treatment of pulmonary fibrosis. J. Med. Invest. 58 : 188-196, August, 2011

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive, lethal disease of the lung that is characterized by distorted lung architecture and loss of respiratory function as a result of proliferation of fibroblasts and increased deposition of the extracellular matrix (1, 2). Although corticosteroids and other immunosuppressants have been used to treat IPF, the 5-year survival rate of patients with the disease is less than 50% (3, 4). For this reason, novel therapeutic modalities are of great interest.

CCN6/Wnt1-inducible signaling protein-3 (CCN6/WISP-3) is a cysteine-rich protein that belongs to the CCN family, which consists of six members, CCN1 (CYR61/cysteine-rich 61), CCN2 (CTGF/connective tissue growth factor), CCN3 (NOV/nephroblastoma overexpressed gene), CCN4 (WISP1/Wnt1-inducible signaling protein-1), CCN5 (WISP2/Wnt1-inducible signaling protein-2), and CCN6 (5-10). Apart from CCN5, all CCN proteins are composed of 4 conserved cysteine-rich modular.
domains; an insulin-like growth factor binding protein-like module (IGFBP); a von Willebrand factor type C repeat module (VWC); a thrombospondin type 1 (TSP1) domain; and a cysteine-knot-containing module (CT), which contains several cysteine-knot motifs (6).

CCN proteins act by binding to specific integrin receptors, heparin sulfate proteoglycans, thereby triggering a wide range of biological functions, such as cell adhesion, proliferation, migration, differentiation, and survival. They can also modulate the activities of other growth factors and cytokines (6, 8, 10).

CCN proteins have been shown to be associated with IPF. In particular, CCN2 frequently acts downstream of TGF-β and cytokines (6, 8, 10). CCN2 protein expression was highly upregulated in a bleomycin-treated pulmonary fibrosis model. In particular, CCN2 neutralizing antibodies have been shown to attenuate of bleomycin-induced lung fibrosis (14). Moreover, CCN2 neutralizing antibodies have been shown to be at blocking pro-fibrogenic CCN2 signaling pathways in vitro and have yielded promising data with respect to the prevention or reversal of fibrosis in several animal models in vivo (11).

A recent study has shown that CCN4 enhances fibrogenesis by inducing EMT in ATII cells and increasing collagen production by fibroblasts. In addition, CCN4 neutralization resulted in marked attenuation of bleomycin-induced lung fibrosis (15). CCN6 has been proposed to take part in cell survival and has been shown to modulate IGF-I-mediated growth and the proliferation of breast epithelial cells (16). In addition, frequent mutations in the CCN6 gene were reported to be associated with autosomal recessive skeletal disorder progressive pseudorheumatoid dysplasia (17). However, no studies have been carried out to investigate the involvement of CCN6 in the proliferation of lung fibroblasts or the pathogenesis of pulmonary fibrosis.

In the present study, we demonstrate that CCN6 stimulates the proliferation of lung fibroblasts by binding to integrin β1, leading to the autophosphorylation of FAK. CCN6 protein expression was highly upregulated in a bleomycin-treated pulmonary fibrosis model.

MATERIALS AND METHODS

Fibroblast Isolation

The primary culture of murine lung fibroblasts was obtained according to the method reported by Phan et al. (18). The lungs were harvested from untreated 8-week-old C57BL/6 mice. The minced lungs were digested with 0.5% trypsin under stirring, and the cells were collected by centrifugation. The harvested cells were cultured in RPMI 1640 (Nissui Pharmaceutical Co. Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), penicillin (100 U/ml), and streptomycin (50 mg/ml) at 37°C in a humidified atmosphere of 5% CO2 in air for several days. The proliferating cells (doubling time in 10% FBS: 15 to 18 hours) were used as lung fibroblasts at the fifth to tenth passage. These cells were termed C57BL/6 lung fibroblasts (19). MLC lung fibroblasts were obtained from ATCC (Manassas, VA).

Reagents and Antibodies

Recombinant human CCN6/WISP-3 was purchased from Peprotech (Rocky Hill, NJ). PDGF-BB was purchased from Sigma-Aldrich (St Louis, MO). Recombinant TGF-β1 was purchased from Santa Cruz (Santa Cruz, CA). The blocking monoclonal Abs against mouse CD29 (integrin β1) and CD51 (integrin αV) and antibodies for flow cytometry against mouse CD29 (integrin β1), CD18 (integrin β2), CD61 (integrin β3), CD104 (integrin β4), integrin β7, CD49f (integrin α6), CD51 (integrin αV), VLA-5 (CD49e/CD29) (α5β1) were purchased from BD Biosciences, Pharmingen (San Jose, CA). Rabbit anti-phosphoFAK (pY397) was purchased from Invitrogen (Carlsbad, CA). Mouse anti-FAK antibody was purchased from BD Biosciences. The antibody for CCN6 was purchased from Santa Cruz.

Mice and Bleomycin Treatment

Eight-week-old female C57BL/6 mice were purchased from Charles River Japan, Inc. (Yokohama, Japan) and were maintained in the animal facility of the University of Tokushima under specific pathogen-free conditions according to the guidelines of our university. Bleomycin (BLM) was purchased from Nippon Kayaku Co. (Tokyo, Japan). Osmotic minipumps (model 2001; Alza Pharmaceuticals, Palo Alto, CA) containing 200 μl of saline with or without BLM (125 mg/kg) were implanted subcutaneously (20-22) [26-28]. Each experiment involved at least
four mice per group.

Proliferation Assay

The C57BL/6 and MLg lung fibroblasts (8×10^4 cells per well) were cultured in 96-well flat-bottomed tissue plates in RPMI1640 supplemented with 10% FBS, and then serum-starved in medium containing 0.1% FBS overnight (19, 20, 22) [19, 27, 28]. Next, the cells were treated with various doses (1 to 200 ng/ml) of CCN6, and PDGF-BB (10 ng/ml) as a positive control for 72 hours, and labeled with 3H-thymidine deoxyribose (TdR) (6.7 Ci/mmol; Amersham Corp., Arlington Heights, IL) at 1 μCi/well for the final 18 hours. In the blocking experiment, the cells were incubated with CCN6 (200 ng/ml) with or without monoclonal Ab against CD29 (40 μg/ml) or monoclonal Ab against CD51 (50 μg/ml). The cells were harvested in a MASH II cell harvester (Labo Science Co. Tokyo, Japan), and the incorporation of 3H-TdR was determined by liquid scintillation counting. The experiments were performed in triplicate. In addition, proliferation was assessed by cell counting 4 and 7 days after stimulation with CCN6 (200 ng/ml), with counting performed at least 3 times for each condition.

Flow Cytometry Assay

To assess the cell surface expression of integrins, C57BL/6 and MLg lung fibroblasts (80% confluent) were trypsinized and washed in cold PBS, and the cells were incubated with 1 μg of primary antibodies for 30 min on ice, washed again with cold PBS, incubated with FITC conjugated secondary antibodies, and resuspended in cold PBS. Flow cytometric analysis was carried out using a FACS Calibur (BD Biosciences), and the results were analyzed with CELLQuest software (BD Biosciences) (23, 24).

Western Blot Analysis

Serum-starved C57BL/6 lung fibroblasts were treated with recombinant CCN6 (200 ng/ml) for 10, 30, or 60 minutes before being harvested (19). Then, the cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing 1 mM PMSF. To detect CCN6 expression in BLM-treated lung tissue, lung tissues were homogenized and lysed in RIPA Buffer (Pierce, Rockford, IL). Their protein concentrations were determined using the Bradford protein assay (Sigma-Aldrich) (19, 20). Immunoprecipitation was performed using 500 μg of total proteins, and immune complexes were recovered with Protein G-Sepharose beads (Zymed Laboratories, South San Francisco, CA) (19, 20). For Western blotting analysis, immunoprecipitates or 30 μg of total protein were resolved by SDS-PAGE (Bio-Rad, Hercules, CA), and the proteins were then transferred to PVDF membranes (Atto, Tokyo, Japan). After being washed 3 times, membranes were incubated with Blocking One (Nacalai Tesque Inc, Japan) for 1 hour at room temperature and then incubated with the primary antibodies against phospho-FAK(pY397), FAK, β-actin, and CCN6 (1 : 1000 dilution) overnight at 4°C. Then, the membranes were incubated for 2 hours at room temperature with species-specific horseradish peroxidase conjugated secondary antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescent substrate (Pierce, Rockford, IL).

Quantitative Real Time-PCR analysis

C57BL/6 lung fibroblasts (1×10^6 cells/well) were seeded on to a 10 cm well plate in RPMI1640 with 10% FBS, before being serum-starved in medium containing 0.1% FBS overnight. The cells were treated with or without CCN6 or TGFβ (200 ng/ml or 5 ng/ml) for 24, 48, or 96 hours. Quantitative real time-PCR analysis was performed according to the previously described method (25). The total RNA of the cells was extracted using the RNAeasy kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). Total RNA were reverse transcribed using an Omniscript RT kit (Qiagen). The expression of fibronectin, type 1 collagen, and β-actin was measured by quantitative RT-PCR analysis on an ABI 7700 Sequence Detection system (Applied Biosystems, Foster City, CA). The quantitative RT-PCR experiments were performed in triplicate, and the relative expression levels were calculated based on the comparative Ct method.

Statistical Analysis

One-way ANOVA with Dunnett’s post-hoc test was performed using GraphPad Prism version 5.01 for Windows, GraphPad Software (San Diego, CA). P<0.05 was considered statistically significant.

RESULTS

Effects of CCN6 Protein on the Proliferation and Expression of Extracellular Matrices of Mouse Lung Fibroblasts

To investigate whether CCN6 stimulates the
proliferation of lung fibroblasts, C57BL/6 and MLg lung fibroblasts were plated in 96 well plates and treated with CCN6, and cell proliferation was measured with the \(^{3}H\)-TdR incorporation assay. As shown in Figure 1A, the proliferation of C57BL/6 lung fibroblasts was induced by recombinant CCN6 at more than 100 ng/ml. Next, we examined the proliferation of MLg lung fibroblasts. CCN6 also stimulated the growth of MLg fibroblasts at more than 100 ng/ml (Figure 1B). To confirm these results, we counted the numbers of C57BL/6 and MLg fibroblasts after treatment with CCN6. Treatment with CCN6 (200 ng/ml) significantly increased the number of C57BL/6 (Figure 1C) and MLg (Figure 1D) fibroblasts on days 4 and 7.

The accumulation of ECM proteins, including collagens, fibronectin, proteoglycans, and elastin, has long been considered a hallmark of fibrosis. The ECM plays a vital early role in the repair process, orchestrating the modulation and transition of cell phenotype and function (26). It has been shown that CCN6 stimulates collagen type II, aggrecan, and superoxide dismutase expression and activity in chondrocytes (27, 28). To investigate whether CCN6 affects on ECM production, we treated C57BL/6 cells with CCN6 and examined the mRNA expression of

![Figure 1](image_url)

**Figure 1** Effects of CCN6 protein on the proliferation and expression of the extracellular matrixes of mouse lung fibroblasts C57BL/6 (A) and MLg (B) mouse lung fibroblasts (8 \(\times\) 10\(^3\) cells/well) were cultured in 96-well flat-bottomed tissue culture plates in RPMI1640 containing 10% FBS for 24 hours and then serum starved in medium containing 0.1% FBS overnight before treatment with various doses of CCN6, or PDGF-BB (10 ng/ml) as a positive control. The cells were labeled with \(^{3}H\)-TdR at 1 \(\mu\)Ci/well for the final 18 hours, and the incorporation of \(^{3}H\)-TdR was measured with a liquid scintillation counter. C57BL/6 (C) or MLg (D) fibroblasts (4 \(\times\) 10\(^4\) cells/well) were cultured in 6-well flat-bottomed tissue culture plates and then treated with CCN6 (200 ng/ml) for 7 days. The number of cells was counted on Days 4 and 7. Data are presented as the mean±SD of triplicate cultures. C57BL/6 fibroblasts (1 \(\times\) 10\(^6\) cells/well) were serum starved for 24 hours before being incubated with CCN6 (200 ng/ml) or TGF-\(\beta\)-1 (5 ng/ml) for the indicated time periods. Then, the total RNA of the cells was extracted, and the mRNA expression levels of type I collagen and fibronectin were determined by quantitative real-time RT-PCR. The mRNA expression levels of fibronectin (E) and type I collagen (F) presented have been normalized to \(\beta\)-actin using the \(C\) method. Similar results were obtained in three separate experiments. (*) \(p<0.05\) vs untreated control (one-way ANOVA with Dunnett’s post-hoc test). The data is representative of three independent experiments.
fibronectin and type I collagen by real-time RT-PCR analysis. Treatment with TGF-β1 significantly stimulated the expression of both fibronectin (Figure 1E) and type I collagen mRNA (Figure 1F). On the other hand, CCN6 slightly but significantly stimulated the expression of fibronectin mRNA (Figure 1E), but not type I collagen mRNA (Figure 1F).

**Integrin Expression on the Surface of Mouse Lung Fibroblasts**

Integrins mediate cellular adhesion to extra-cellular matrices (ECM), which are important for many cellular processes including growth, transformation, migration, survival, and differentiation (29). Most of the activities of CCN proteins in isolated cell systems can be attributed to their direct interaction with integrin receptors, which function as co-receptors with HSPG in all cases in some contexts (10). Therefore, we examined the expression of integrins by C57BL/6 and MLg lung fibroblasts. As shown in Figure 2, C57BL/6 and MLg fibroblasts expressed integrins β1, αV, α6 and α5β1. However, the expression of integrins α1, α2, α3, α4, β2, β3, β4 and β7 was not detected in both fibroblasts (data not shown).

**Blocking Monoclonal Antibodies against Integrins Inhibit the Proliferative Effect of CCN6 on C57BL/6 Lung Fibroblasts**

Since most activities of CCN proteins are known to be mediated through integrins (6, 10, 30, 31), we next examined which integrin is involved in the signaling pathway mediated by CCN6 in lung fibroblasts. We performed blocking experiments to block the growth of lung fibroblasts using two antibodies against integrin β1 (CD29) and αV (CD51). C57BL/6 lung fibroblast cells were treated with CCN6 protein and antibodies against integrin β1 (CD29) or αV (CD51). As depicted in Figure 3A, CCN6-induced proliferation of lung fibroblasts was partially inhibited by anti-β1(CD29) antibody, but not by anti-αV (CD51) antibody. These findings suggest that CCN6 exerts a proliferative effect on lung fibroblasts at least in part by binding to integrin β1.

**FAK Signaling Pathway is involved in the CCN6-induced Proliferation of C57BL/6 Lung Fibroblasts**

Focal adhesion kinase (FAK), a potential candidate-signaling molecule, has been shown to be
capable of regulating integrin-mediated survival signaling (32). Integrin engagement with extracellular matrix proteins activates the phosphorylation of the Tyr397 of FAK, which plays a critical role in integrin-mediated cell functions, such as cell proliferation and migration (33, 34). Therefore, we investigated whether CCN6 can stimulate the FAK signaling pathway using Western blotting for pFAKY397 (Figure 3B). As previously reported (35), TGF-β1 stimulated the phosphorylation of FAKY397 30 minutes after its addition. In this experimental condition, CCN6 also induced the phosphorylation of FAKY397.

These findings suggest that CCN6 stimulates the proliferation of lung fibroblasts by binding to integrin β1 and leads to the autophosphorylation of FAKY397.

Increased Expression of CCN6 in the Lungs of BLM-treated Mice

Finally, to investigate the expression of CCN6 in lung fibrosis, we analyzed the expression of CCN6 in a BLM-treated pulmonary fibrosis model. The expression of CCN6 in BLM-treated lungs was examined by Western blotting. As shown in Figure 4, CCN6 exhibited increased expression on Day 3 after BLM administration, and the high level of CCN6 expression continued until Day 14, before decreasing on Day 28.

Figure 3 Integrin β1 and focal adhesion kinase are involved in the signaling pathway mediated by CCN6

A : The blocking antibodies against integrins inhibit the proliferation of C57BL/6 lung fibroblasts induced by CCN6. C57BL/6 lung fibroblasts (8 × 103 cells/well) were cultured in 96-well flat-bottomed tissue culture plates and treated with CCN6 (200 ng/ml) in the presence or absence of anti-β1(CD29) antibody (40 μg/ml) and anti-αV (CD51) antibody (50 μg/ml). PDGF-BB (10 ng/ml) was used as a positive control. The cells were labeled with 3H-TdR at 1 μCi/well for the final 18 hours, and the incorporation of 3H-TdR was measured. Data are presented as the mean ± SD of triplicate cultures. (* p < 0.05 CCN6 vs CCN6+anti-β1 (CD29). B : CCN6 induces the autophosphorylation of FAKY397. C57BL/6 fibroblasts (80% confluent) were serum-starved overnight, and incubated with CCN6 (200 ng/ml) or TGF-β1 (5 ng/ml) for the indicated time periods before being harvested. The autophosphorylation of FAKY397 was determined by Western blot analysis. For semiquantitation, the intensities of the bands for FAK, pFAK, and β-actin were calculated using a densitometer. The bars show the relative pFAK/FAK ratio. Equal protein loading was confirmed by β-actin. Similar results were obtained in three separate experiments.

Figure 4 The level of CCN6 in lung homogenate was elevated in the lungs of bleomycin-treated mice

Eight-week-old C57BL/6 mice were implanted with osmotic minipumps containing saline or bleomycin (125 mg/kg). On the days indicated, the mice were killed, and lung tissues were homogenized. The expression level of CCN6 was determined by Western blotting assay using CCN6 antibody (1 : 1000 dilution range). For semiquantitation, the intensities of the CCN6 and β-actin bands were calculated using a densitometer. The bars show the relative CCN6/β-actin ratio. Equal protein loading was confirmed by β-actin.
DISCUSSION

Numerous studies have shown that CCN1, CCN2, and CCN3 have no intrinsic ability to stimulate cell proliferation on their own, but can enhance DNA synthesis induced by other mitogenic growth factors (6, 18, 30, 36, 37). A recent study by Königshoff et al. has shown that CCN4 acts as a proliferative mediator of ATII cells, but has no effect on the proliferation of lung fibroblasts. Moreover, they demonstrated that CCN4 treatment resulted in a marked increase in fibronectin and type 1 collagen production by fibroblasts (15). In the current study, we showed that, CCN6 has the ability to induce the proliferation of lung fibroblasts and the production of fibronectin by fibroblasts. These results suggest that CCN6 is involved in the proliferation of fibroblasts and ECM production by lung fibroblasts.

CCN proteins utilize distinct integrins depending on the target cell type and the activity being mediated. In fibroblasts, CCN1 promotes growth factor-induced cell proliferation, adhesion, and migration through αVβ3, α6β1, and αVβ5, respectively. In contrast, it stimulates cell migration in endothelial cells and vascular smooth muscle cells by binding to αVβ3 and α6β1, respectively (6, 38). It has been reported that CCN6 stimulates the migration of undifferentiated mesenchymal stroma cells through integrin αVβ5 (31). In the current study, we showed that fibroblasts expressed integrins β1, αV, α6, and α5β1 and that CCN6-induced proliferation of lung fibroblasts was partially inhibited by anti-β1 (CD29) antibody, but not by anti-αV (CD51) antibody. These findings suggest that α5β1 or α6β1 play a critical role in the signaling pathway of CCN6 in lung fibroblasts. In addition, we demonstrated the autophosphorylation of FAKγ397 in response to CCN6. Although western blotting analysis showed the similar time course of the phosphorylation of FAKγ397 by CCN6 as compared to TGF-β stimulation (figure 3B), the further analysis is required to clarify the signaling pathway involved in the phosphorylation of FAKγ397.

A recent study by Königshoff et al. (15) showed that CCN6 mRNA was detectable in primary fibroblasts and ATII cells isolated from BLM-treated mouse lungs, which supports our findings. However, they reported that CCN6 was not elevated in the lung homogenates of BLM-treated mice, which is not consistent with the elevated CCN6 in the lungs of BLM-treated mice in the current study. The reason for the discrepancy between the two studies is unclear. The differences in BLM-models used in the two studies might have affected the expression of CCN family proteins in the lungs. In addition, we examined the expression of CCN6 by Western blotting at the protein level, whereas they performed RT-PCR and microarray analysis at the mRNA level. Further analyses of the expression of CCN6 protein might be required.

When considering the roles of CCN6 for lung fibroblasts in vitro experiments, which has been showed in the current study, it is reasonable to expect that the early elevation of CCN6 expression in the lungs may be associated with the growth of fibroblasts and ECM production by fibroblasts in the fibrotic areas of the lungs and resulting in progression of lung fibrosis in BLM-treated mice. In addition, Kleer and colleagues reported that CCN6 was essential to induce the process of epithelial-mesenchymal transition (EMT) with repression of E-cadherin gene expression and induction of a protein expression program characteristic of EMT in human mammary epithelial cells (39). Considered together, CCN6 may also play an important role in development of lung fibrosis via inducing EMT, which is one of critical cellular modulators of fibrosis, although further studies including in vitro and in vivo experiments are necessary to test whether CCN6 is involved in induction of EMT in the lungs, resulting in progression of lung fibrosis.

CONCLUSIONS

CCN6, a member of the CCN family of secreted cysteine-rich matricellular proteins, induces the proliferation of lung fibroblasts by binding to integrin β1 and leads to the autophosphorylation of FAKγ397. Moreover, CCN6 was highly expressed in the lungs of BLM-treated mice and induced fibronectin production in lung fibroblasts. Since these data strongly suggest that CCN6 plays a role in the pathogenesis of pulmonary fibrosis in mice, further studies in humans are necessary to understand the molecular mechanisms of CCN6 signaling in pulmonary fibrosis.

CONFLICTS OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.
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