Cardiac Fibrosis Is Associated With Decreased Circulating Levels of Full-Length CILP in Heart Failure

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VISUAL ABSTRACT

HIGHLIGHTS

- After in vitro stimulation or in vivo pressure overload injury, activated cardiac fibroblasts express Ltbp2, Comp, and Cilp.
- In ischemic heart disease, LTBP2, COMP, and CILP localize to the fibrotic regions of the injured heart.
- Circulating levels of full-length CILP are decreased in patients with heart failure, suggestive of the potential to use this protein as a biomarker for the presence of cardiac fibrosis.

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Cardiac fibrosis is a pathological process associated with various forms of cardiac disease that contributes to impaired cardiac function, development of arrhythmias, and ultimately heart failure (1,2). The formation of fibrosis can be initiated by either an acute ischemic event to the heart, such as myocardial infarction, or through a chronic progression driven by increased cardiac load. Cardiac fibroblasts (CFBs) are the main participating cells in the development of myocardial fibrosis (3). Under homeostatic conditions, resident CFBs are responsible for maintaining the structural integrity of the heart by regulating extracellular matrix (ECM) production (4). However, under pathological conditions, CFBs become activated, proliferate, and secrete an excess amount of ECM proteins, contributing to scar tissue (3,4). This scar replaces healthy myocardium, renders the substrate arrhythmogenic, induces stiffening of the heart, and leads to adverse remodeling. Collectively, the sequelae of fibrosis can have deleterious effects on the ability of the heart to pump blood effectively and hinders the recovery of cardiac function. There are currently limited treatment options for the reversal of cardiac fibrosis, and available therapies for heart failure are ineffective at preventing the formation of scar tissue (5). It has been suggested that identifying diagnostic markers for fibrosis may provide prognostic value for clinicians (6). Considering the critical role of CFBs in myocardial fibrosis, we hypothesized that CFBs may release factors that could serve as promising biomarkers for cardiac fibrosis (7). Identification of circulating biomarkers would serve as a noninvasive clinical tool to determine the presence, extent, and progression of fibrosis in cardiac disease patients.

In the present study, we isolated CFBs from C57BL/6J mice that underwent transverse aortic constriction (TAC), a pressure overload injury model, or sham operation and performed ribonucleic acid (RNA) sequencing to identify key upregulated genes in response to injury (8). From this data, we identified 3 genes encoding secreted proteins that could be potential biomarkers for myocardial fibrosis: latent transforming growth factor (TGF)-β binding protein 2 (Ltbp2), cartilage oligomeric matrix protein (Comp), and cartilage intermediate layer protein 1 (Cilp). Ltbp2 is part of the LTBP family, which consists of key regulators of TGF-β signaling (9). Comp and Cilp are mainly known for their roles in the binding of specific ECM proteins, such as collagens, in cartilage (10,11). Ltbp2, Comp, and Cilp were upregulated in cultured murine CFB and in the fibrotic regions of TAC hearts, suggesting that their expression is specific to the formation of scar. Furthermore, there was an increase in expression of these proteins in stimulated human CFBs and within the fibrotic regions of heart sections from patients with heart failure, demonstrating their potential as clinical biomarkers for fibrosis. Finally, we show that CILP, specifically the full-length CILP protein, demonstrated a significant difference in circulating levels in the serum of patients with heart failure. The findings in this study introduce potential markers for myocardial fibrosis and support the need to pursue studies on CILP as a possible circulating biomarker for the development of cardiac fibrosis.
METHODS

STUDY APPROVALS. All mouse surgery procedures were carried out with the approval of the University of California, Los Angeles Animal Research Committee or the Institutional Animal Care. The study was approved by an institutional review board (12-001164) and human participants gave written informed consent.

RNA SEQUENCING AND ANALYSIS. CFBs were isolated from murine hearts, as previously described, for RNA sequencing (8). These data are publicly available on Gene Expression Omnibus (GSE51620). Downstream analysis was conducted using the DESeq2, Enhanced Volcano-plot and ggplots R packages (12,13). Detailed strategy for identifying potential gene targets is described in the Supplemental Methods.

MICE. Adult C57BL/6J mice (age 8 to 12 weeks) were used for all experiments. For the in vivo experiments, mice were randomly assigned into sham, TAC, and ischemic reperfusion treatment groups. No phenotypic differences were observed between male and female mice. Details of surgery are described in the Supplemental Methods. All procedures were carried out with the approval of the University of California, Los Angeles Animal Research Committee or the Institutional Animal Care.

CFBs CULTURE AND TGFβ TREATMENT (MURINE AND HUMAN). For mouse explant fibroblast cultures, hearts were collected, digested, and plated as described in the Supplemental Methods. Twelve hours after plating, the floating cells were removed, and the media was replaced. Media changes were done every other day until cells reached 80% confluence, at which point, they were passaged and cultured in serum-free media for 24 h prior to TGF-β treatment (50 ng/ml; Cell Signaling Technology, Danvers, Massachusetts). Throughout the TGF-β treatment, the media was changed daily. Human fibroblasts were cultured according to the company's instructions (Cell Applications, San Diego, California) and similarly passaged for TGF-β treatment (10 ng/ml; R&D Systems, Minneapolis, Minnesota).

RNA EXTRACTION AND REVERSE TRANSCRIPTION-QUANTITATIVE PCR. RNA was extracted from cells using TRIzol LS Reagent (Thermo Fisher Scientific, Waltham, Massachusetts) and following the manufacturer's instructions. RNA was quantified by NanoDrop, and complementary DNA was prepared using the iScript Reverse Transcription Supermix kit (Bio-Rad Laboratories, Hercules, California). Reverse transcription-quantitative polymerase chain reaction (PCR) reactions were prepared using SYBR Green (Bio-Rad) and primers (Integrated Device Technology, San Jose, California) unique for each gene of interest (Supplemental Table 1). The reactions were run on a CFX96 Real-Time PCR Detection System (Bio-Rad) and relative gene expression data were calculated by double delta computed tomography analysis.

IMMUNOCYTOCHEMICAL AND IMMUNOFLUORESCENCE STAINING. Cells were cultured on 8-well chamber slides (Falcon, Fisher Scientific, Thermo Fisher Scientific) and washed with phosphate-buffered saline prior to fixation with 4% paraformaldehyde. For in vivo staining, murine hearts were isolated and fixed with 4% paraformaldehyde overnight prior to being incubated in 30% sucrose and embedded in Optimal Cutting Temperature compound (Fisher). Hearts were sectioned at a thickness of 8 μm in a cryostat, mounted on Colorfrost Plus microscope slides (Fisher), and stored at −20°C until ready to stain. Detailed staining protocol is provided in the Supplemental Methods. Slides were incubated with antibodies outlined in Supplemental Table 2.

ELISA AND WESTERN BLOT. Protocols for conditioned media and serum sample preparation are described in the Supplemental Methods. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from MyBiosource (San Diego, California) and the manufacturer's protocol was followed. For Western blot, protein concentration was measured by a Pierce BCA Protein assay kit (Thermo Fisher Scientific) and 20 μg was loaded into each well of 4% to 20% Mini-PROTEAN TGX Precast Protein gels (Bio-Rad). After transferring the gel onto a polyvinylidene fluoride membrane, detection of CILP was conducted by incubating the membrane with primary antibody followed by secondary antibody conjugated with horseradish peroxidase (Supplemental Table 2). The signal was developed using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

STATISTICAL ANALYSIS. Continuous data are presented using the mean ± SEM and comparisons between groups were performed using Student’s t-test. A p value < 0.05 was considered statistically significant, and data were analyzed using GraphPad Prism 6 (San Diego, California).

RESULTS

MURINE CFBs EXPRESS Ltbp2, Comp, AND Clip AFTER INJURY. To identify secreted proteins expressed by CFBs in fibrotic hearts, we conducted RNA sequencing on isolated CFBs from female C57BL/6J adult mice (8 to 12 weeks old) that had undergone either sham or TAC surgery (n = 3). CFBs
were isolated 7 days after surgery to observe gene expression changes in the early stages of fibrosis (8). After TAC, many genes were differentially expressed in CFBs (Figure 1A, Supplemental Table 3). Specifically, CFBs from mice that had undergone TAC showed higher expression of various genes associated with fibrosis (Figure 1B). From these, we selected genes that encoded for secreted proteins and then filtered the list to those that were novel in the context of heart failure and had previously reported roles in ECM formation and remodeling. We identified \( \text{Ltbp2} \), \( \text{Comp} \), and \( \text{Cilp} \) as potential candidate biomarkers. These results were further validated by reverse transcription-quantitative PCR (Figure 1C).

The TGF-\( \beta \) signaling pathway is a major component of injury response in CFBs (14). Treatment of fibroblasts in vitro with TGF-\( \beta \) activates and induces proliferation of cultured cells, mimicking in vivo responses (15). To confirm that the TGF-\( \beta \) signaling pathway stimulates a robust increase in the expression of the identified genes, CFBs from uninjured C57BL/6J mice were cultured in media with or without TGF-\( \beta \) for 72 h (Figure 2A). CFBs were isolated by whole explant culture to encompass the entire...
CFBs population in the heart, rather than a subpopulation (16). TGFβ treatment induced expression of fibroblast activation genes, such as periostin (Postn) and α-smooth muscle actin (Acta2) (17), as well as Ltbp2, Comp, and Cilp (Figure 2B). Furthermore, immunocytochemistry confirmed that expression of Ltbp2, Comp, and Cilp were increased at the protein level in cultured CFBs after exposure to TGF-β (Figure 2C). We observed similar patterns of staining for these proteins when CFBs were isolated by fluorescence-activated cell sorting (Supplemental Figure 1) (8). These data confirm that CFBs are a cellular source of Ltbp2, Comp, and Cilp under stimulatory conditions.

**Ltbp2, Comp, AND Cilp ARE LOCALIZED TO FIBROTIC REGIONS.** Although TAC surgery induces fibrosis, it also causes other cardiac pathologies, such as hypertrophy (18). To confirm that the increase in Ltbp2, Comp, and Cilp expression after injury was specific to scar formation, we analyzed the anatomic location of Ltbp2, Comp, and Cilp in the hearts of mice that had undergone TAC surgery. After 7 days, there was visible perivascular and interstitial fibrosis in TAC hearts, compared with sham hearts, which exhibited no fibrosis (Figure 3A). Immunofluorescence staining showed minimal expression of the 3 proteins in sham hearts. In TAC hearts, Ltbp2, Comp, and Cilp expression appeared to colocalize with discoidin domain-containing receptor 2 (Ddr2), a marker for fibroblasts (19), and α-smooth muscle actin (20) within the fibrotic regions of the myocardium in TAC hearts (Figure 3B). Areas of nonfibrinous myocardium in TAC hearts did not stain for any of the target proteins (data not shown), indicating that expression of Ltbp2, Comp, and Cilp are expressed by activated CFBs and localized to regions of fibrosis.

We next sought to determine whether the expression of these biomarkers is also observed in other types of cardiac fibrosis, such as replacement fibrosis after myocardial infarction. Our findings were confirmed in an ischemic reperfusion injury model in which the hearts exhibited discrete areas of fibrosis, although not to the severity of TAC injury. Ltbp2, Comp, and Cilp were found to be specifically colocalized with discoidin domain-containing receptor 2 and α-smooth muscle actin in hearts that had undergone ischemic reperfusion.
Together, these data suggest that Ltbp2, Comp, and Cilp are expressed by activated CFBs and are localized to regions of fibrosis.

**HUMAN CFBs HAVE INCREASED LTBP2, COMP, AND CILP LEVELS IN RESPONSE TO TGF-β1 TREATMENT.** To confirm the clinical utility of our identified proteins as biomarkers for cardiac fibrosis, we sought to assess their expression levels in human ischemic myocardial tissue. RNA sequencing data of human cardiac tissue from ischemic patients with heart failure in a publicly available database (GSE46224) demonstrated that *LTBP2*, *COMP*, and *CILP* are up-regulated in ischemic hearts (Figure 4A) (21). We next cultured human CFBs and treated them with TGF-β1 to stimulate their in vitro activation (Figure 4B). TGF-β1 treatment led to morphological changes in human CFBs and induced expression of *LTBP2*, *COMP*, and *CILP*, along with fibroblast activation genes (Figure 4C). Immunocytochemistry staining confirmed the up-regulation of LTBP2, COMP, and CILP in response to TGF-β1 treatment, as seen in mouse CFBs (Figure 4D). Conditioned media from cells that had undergone TGF-β1 treatment did not show significant differences in the levels of LTBP2, an increasing trend of COMP levels, and
decreased levels of CILP (Supplemental Figure 3). These results may be due to unknown mechanisms of protein secretion that affect the presence of these proteins in the context of our culture protocol. The results from the in vitro culture of human CFBs mirrored our data from mice, further supporting the potential of these proteins to be biomarkers for cardiac fibrosis.

**LTBP2, COMP, and CILP ARE POTENTIAL BIOMARKERS FOR CARDIAC FIBROSIS.** We used immunofluorescence staining to observe the expression of LTBP2, COMP, and CILP within the myocardium of patients with heart failure compared with the myocardium of healthy hearts. Myocardial tissue from patients with heart failure (with a documented diagnosis of ischemic cardiomyopathy) exhibited significant amounts of fibrosis compared with in healthy hearts (Figure 5A). In healthy hearts, we observed no or minimal positive staining for the candidate markers throughout the myocardium (Figure 5B). However, sections from diseased hearts demonstrated a significant increase in expression of all 3 proteins (Figure 5B). Staining for these 3 proteins were localized to disarrayed regions of the myocardium, indicative of the specificity of these proteins for fibrotic areas.

**FIGURE 4 LTBP2, COMP, and CILP are Up-Regulated in Human Cardiac Fibroblasts in Response to TGF-β**

(A) Ribonucleic acid sequencing data from a public database (GSE46224) show that expression of LTBP2, COMP, and CILP are up-regulated in heart biopsies from patients with ischemic heart failure (HF) compared with biopsies from healthy patients. (B) Schematic of culture and TGF-β treatment of human cardiac fibroblasts. (C) Reverse transcription-quantitative polymerase chain reaction of human cardiac fibroblasts treated with TGF-β normalized to untreated control samples (indicated by dotted line at y = 1) (n = 3). (D) Immunocytochemistry shows increased expression of LTBP2, COMP, and CILP in cultured human cardiac fibroblasts after TGF-β treatment. DAPI (blue) stain was used for nuclei (n = 4). Bar = 100 μm. *p < 0.050 (Student’s t-test). Abbreviations as in Figures 1 and 2.
FULL-LENGTH CILP IS SIGNIFICANTLY DECREASED IN SERUM FROM PATIENTS WITH HEART FAILURE. In addition to increased expression within the fibrotic myocardium, we sought to determine the utility of LTBP2, COMP, and CILP as novel circulating biomarkers for cardiac fibrosis. We measured the protein levels in serum from healthy volunteers and patients with heart failure by ELISA (Supplemental Table 4). We observed no significant differences in the circulating levels of LTBP2 or COMP (Figure 6A). However, serum from patients with heart failure exhibited significantly decreased levels of CILP (Figure 6B). Mice that had undergone TAC injury, compared with sham mice, exhibited a similar trend in decreased levels of circulating Cilp (Supplemental Figure 4). The CILP gene encodes a precursor protein that undergoes cleavage into an N-terminal fragment of roughly 75 kDa and a C-terminal fragment of about 55 kDa. Both of these fragments were shown to inhibit Smad3 phosphorylation, which is normally promoted by active TGFβ signaling. Whereas commercially available ELISA kits target the C-terminal region of CILP (hence detecting both the C-terminal and the full-length fragment), previous work discovered that CFBs secrete the N-terminal fragment as well as the full-length CILP protein.

We specifically used an antibody that spans the cleavage site of the CILP precursor and performed Western blotting to confirm levels of circulating full-length CILP. Our results showed that serum from patients with heart failure had significantly decreased levels of full-length CILP in circulation (Figures 6C and 6D). Together, these data suggest while activated fibroblasts in the fibrotic regions of human myocardium express high levels of CILP, the circulating level of CILP is significantly reduced when compared with healthy individuals with no evidence of cardiac fibrosis.

DISCUSSION

With the increasing prevalence of cardiac disease worldwide, there is significant value in identifying a robust biomarker panel to noninvasively measure the presence and progression of cardiac fibrosis. We hypothesized that, as key participants of the fibrotic response, CFBs may be a source of novel biomarkers for myocardial fibrosis. We performed RNA sequencing of CFBs from TAC and sham murine hearts and identified Ltbp2, Comp, and Cilp to be upregulated in hearts after pressure overload injury. The expression of these proteins by CFBs in response
to injury were validated by in vitro studies in both murine and human CFBs. Additionally, we demonstrated that these proteins localize in fibrotic regions in murine hearts after pressure overload and ischemic reperfusion injury. These findings were further confirmed by high levels of these 3 biomarkers in the fibrotic areas of human ischemic myocardial tissue. Notably, the circulating levels of full-length CILP protein were significantly reduced in the serum of patients with ischemic heart failure compared with the serum of healthy individuals, indicating its potential to be a circulating biomarker.

**LTBP2 AND COMP EXPRESSION IS SPECIFIC TO FIBROTIC REGIONS.** LTBP2 is a member of the latent TGF-β-binding protein family, which consists of key regulators of TGF-β signaling. TGF-β has diverse and pleiotropic effects on various cell types through its binding and activation of TGF-β receptors (14,25). TGFβ is secreted from cells as a multiplex form that is covalently bound to latent TGF-β-binding proteins LTBP1, LTBP3, and LTBP4. These proteins target the latent complex to specific sites for storage within the ECM where it awaits activation. Matrix sequestration of latent TGF-β may serve to regulate its immediate bioavailability while achieving critical threshold concentration at sites of intended function (9,25). However, the functional role of LTBP2 is not well understood. Recent studies suggest that LTBP2 does not bind to latent TGF-β but may interact with other ECM proteins (9). Other studies have additionally reported on the competitive role of LTBP2 with LTBP1 for binding sites on fibrillin-1 within the ECM (26). Our data show strong
support for increased expression of LTBP2 in response to injury and a strong localization of LTBP2 in activated fibroblasts within the fibrotic regions of the myocardium. Whether LTBP2 is merely a surrogate for cardiac fibrosis or is involved in its pathogenesis is not entirely known.

COMP is another ECM protein that is mainly studied in the context of tendons and cartilage (27). The main function of COMP is to directly bind with other ECM components, including collagens and TGF-β1, and to facilitate the stability of the ECM network by the formation of collagen fibrils (28). This role is crucial to maintaining homeostasis of the heart as Comp-knockout mice have been shown to develop dilated cardiomyopathy (29). However, the role of COMP in pathological remodeling is less understood. Studies have shown that COMP is up-regulated in the context of idiopathic pulmonary fibrosis (30) and liver fibrosis (31), although there have been conflicting reports as to whether it can serve as an accurate circulating marker for fibrosis in patients (30,32).

Our results suggest that LTBP2 and COMP both have a strong potential for being markers for cardiac fibrosis as the expression of these proteins are specific to scar formation. However, our data does not support their use as circulating biomarkers after cardiac injury. Although these proteins are known to be secreted, it is possible that they remain within the ECM and participate in the process of fibrosis and scar formation. Further research is warranted to investigate the specific functional contributions of LTBP2 and COMP to the development of cardiac fibrosis. Due to their known roles in other organ systems, it is possible that these proteins may be markers for general fibrosis and not specific to cardiac fibrosis (33).

**DECREASED LEVELS OF CILP MAY BE INDICATIVE OF HEART FAILURE.** The exact function of CILP within cartilage is still unknown, but it has been implicated in cartilage remodeling and maintenance of the ECM (31,34). The up-regulation of CILP has been found in various disease models including osteoarthritis, idiopathic pulmonary fibrosis, and ischemic heart disease (34–37). However, the contribution of CILP to the development of cardiac fibrosis remains unknown. Whereas most studies suggest that CFBs are the major source of CILP expression in the heart, a recent study has shown evidence of cardiomyocytes being another a major contributor (38,39). Although we did not explore the expression of CILP in cardiomyocytes, our data support the claim that CFBs are a major cellular source of CILP. Several studies have reported that cardiac injury causes an up-regulation of CILP in CFBs but the potential for CILP to be a potential biomarker for fibrosis had not been previously explored (24,39).

The **CILP** gene encodes for a precursor protein containing a furin cleavage site. The precursor is first synthesized and processed by furin proteases intracellularly prior to being secreted (23). The N-terminal fragment has been shown to directly interact with TGF-β, suppressing TGF-β signaling in CFB, whereas the C-terminal fragment is homologous to a porcine nucleotide pyrophosphohydrolase, which has been reported to have limited enzymatic activity (23,40). In contrast to the 2 fragments, the functional role of full-length CILP protein has not been well studied. The full-length Cilp has been shown to inhibit TGF-β signaling, similarly to the N-terminal fragment, most likely due to the common thrombospondin-1 domain, which has been shown to bind to TGF-β (24). However, further studies to determine any functional differences between the N-terminal fragment and the full-length CILP are required. Our data specifically demonstrate that circulating levels of the full-length CILP are attenuated in patients with heart failure but show an abundance of expression in the fibrotic myocardium. A possible mechanism for this paradox is that full-length CILP is sequestered to the ECM by its binding to TGF-β, therefore reducing circulating levels. Studies have reported that while injury induces increased expression of TGFβ in the myocardium of patients with heart failure (41), circulating TGFβ is reduced (42,43). Due to the inhibitory role of full-length CILP in TGF-β signaling, it is possible that increased levels of CILP may reside in the ECM and promote a negative feedback mechanism to suppress CFB activation (24,44). Further studies on the dynamics of CILP turnover in the ECM are required to elucidate the significance of both circulating and interstitial CILP.

**STUDY LIMITATIONS.** Our data suggests that circulating levels of full-length CILP are reduced in patients with heart failure. However, our study consists of several limitations. The small sample size limits the statistical power of our analysis and supports a need to conduct additional validation studies on a larger cohort of patients. Furthermore, we did not explore levels of CILP throughout a variety of heart failure etiologies. Conducting larger studies with more patients will provide valuable information to better determine the clinical implications of CILP as a biomarker for the presence of cardiac fibrosis.
CONCLUSIONS

The present study confirms the potential for LTBP2, COMP, and CILP as novel markers of cardiac fibrosis in both mouse and human heart failure models. Most notably, we discovered a significant reduction in serum levels of full-length CILP in patients with heart failure. Our results suggest that LTBP2, COMP, and CILP are worthy of future investigation as participants in cardiac fibrosis and as biomarkers for the development of ischemic heart failure.

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**KEY WORDS** biomarker, cardiac fibrosis,
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**APPENDIX** For supplemental methods, ta-
bles, and figures, please see the online version of
this paper.