sST2: A bridge between Sirt1/p53/p21 signal-induced senescence and TGF-β1/Smad2/3 regulation of cardiac fibrosis in mice viral myocarditis

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Research Article

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Abstract

Soluble interleukin 1 receptor-like 1 (sST2) is a novel predictor of poor outcomes, which is involved in inflammatory response and fibrosis of myocarditis. Cardiac fibrosis is a major cause of cardiovascular disease. Cellular senescence is a state of irreversible cell cycle arrest and studies have shown that senescence of myofibroblasts can limit or reduce myocardial fibrosis. Previous studies show that cellular senescence is associated with the progress of myocarditis, and cardiac fibroblasts is closely related to cardiac fibrosis. Therefore, we investigated the role of sST2 on senescence and fibrosis in mice cardiac fibroblasts (MCF). Meanwhile, its pathological role in mice viral myocarditis (VMC) was also investigated. In vitro experiments revealed that sST2 is beneficial to activate MCF by TGF-β1/Smad2/3 signaling and inhibit cell senescence by Sirt1/P53/P21 signaling pathway. CVB3-infected mice exhibited an increased deposition of collagen in the heart and decreased MCF senescence compared to WT mice while these pathological effects are reversed by anti-ST2 mAb. In addition, we found that inhibiting the senescence of MCF is beneficial to cardiac fibrosis and this effect is achieved by paracrine action. The present study reveals a new pathogenic pathway, sST2, which leads to cardiac fibrosis of VMC by inhibiting MCF senescence.

Introduction

VMC is the leading cause of sudden cardiac death in children and adolescents[1]. Many viruses, including enteroviruses, adenoviruses and human herpes virus 6, are associated with VMC[2, 3]. But Coxsackievirus group B type 3 (CVB3), an enterovirus of the picornaviridae family, is known as the main etiological agent in the VMC. At the early stage of CVB3-induced VMC, immune cells infiltrate. In the late stages of CVB3-induced VMC, fibrosis occurs in the heart, which is due to the activation of MCFs leading to increased production of extracellular mechanisms[4–6].

The protein ST2 was encoded by the interleukin 1 receptor-like 1 (IL1RL1) gene belongs to the IL-1 receptor family[7]. The alternative splicing of IL1RL1 results in multiple transcript variants, including the membrane-bound ST2L receptor for IL-33 and a soluble inhibitory decoy receptor, sST2. When the heart is stressed or stimulated, MCF produce large amounts of sST2[8]. The imbalance of sST2 content will greatly accelerate the progression of cardiac fibrosis and cause serious cardiomyopathy, but most of the studies have shown sST2 only as a decoy receptor that prevents IL-33/ST2L signaling[9]. Indeed, sST2 can works through signals independently of IL-33 sequestration[10]. Recent studies have shown that recombinant sST2 promotes mitochondrial fusion in human cardiac fibroblasts, increasing oxidative stress and the secretion of inflammatory cytokines[11]. High levels of sST2 have been found to be associated with poor prognosis of cardiac fibrosis and cardiomyopathy, which can be used as an independent predictor of heart disease[12–14].

Numerous studies have shown that cell senescence and fibrosis are closely related. Cell senescence is a dynamic condition that can lead to irreversible cell cycle arrest[15, 16]. Although most of researches have shown that senescence contributes to the development of fibrosis, several recent studies have also found
that senescence can reduce myocardial fibrosis, hepatic fibrosis, and idiopathic pulmonary fibrosis[17, 18]. MiR-486 increased the expression of P21 and decreased the expression of fibrotic effector genes in the heart after myocardial infarction[19]. TGF-β1 stimulation in idiopathic fibrosis significantly increased the activity of SA-β-gal and the levels of senescence-related proteins P21 and p53 in lung fibroblasts. Mannose lectin limits the progression of liver fibrosis by promoting HSC (hepatic stellate cell) senescence[17]. Therefore, there is an inextricable relationship between cellular senescence and fibrosis.

At present, to our knowledge, few studies on sST2 causing cardiac fibrosis through the senescence mechanism have been reported. The aim of this study was to provide a mechanistic assessment of sST2 on activation of MCFs in VMC from the point of view of cellular senescence.

**Materials And Methods**

**Mice**

Male BALB/c (6–8 weeks) mice were obtained from Kavins Laboratory Animal Company (Changzhou, China). All animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals (Ministry of Health, China, 1998).

**CVB3 Infection and Anti-ST2 mAb Treatment**

CVB3 virus (Nancy strain) was maintained by passage through Hela cells (ATCC number: CCL-2). Mice were infected with CVB3 via intraperitoneal (i.p.) injections at the dose of 10^4 50% tissue culture infectious dose (TCID50) of CVB3. Anti-ST2 mAb (CNT03914, Centocor) were given to VMC mice by intraperitoneal (IP) injection the day before and the day after the infection, 100 µg/mouse. Seven days or fifteen days later, the hearts and serum were collected for the experiment.

**Cell Culture**

MCF were purchased from BNCC (Beijing, China) and were cultured with DMEM medium (Gibco) containing 10% FBS in a 5% CO2 incubator. The 3–6 passages of cells were used for experiments. Cells were stimulated with sST2 (50nM, R&D Systems) for 12h or 24 h. The 100uM H2O2 were added for 2 hours prior to the stimulation with sST2.

**Quantitative RT-PCR**

Using an RNA extraction kit (Invitrogen), total RNA was extracted from cells and tissues. Purified RNA was reverse transcribed into cDNA, then amplified by SYBR-Green master mix kit. Real-time PCR primer sequences were as follows: for mouse P53, F:5’-GCGTAACGCTTGGAGATGTT-3’ (forward) and 5’-TTTTTGAGTACTG-3’ (reverse); mouse Sirt1, 5’-ATGACGCTTGGCAGATTGTT-3’ (forward) and 5’-CCGCAAGGCGACAGAT-3’ (reverse); mouse P21, 5’-GTGATTGAGCAGCATGACGT-3’ (forward) and 5’-CTCTTTGCGAAGACCAATC-3’ (reverse); mouse P16, 5’-AGGGCGGTGACGCTGACGT-3’ (forward) and 5’-GGTCTGGTGACGCTGACGT-3’ (reverse). Relative levels
of gene expressions were determined by the relative standard curve method and normalized to GAPDH and β-actin.

**Western Blot**

RIPA buffer was used for preparing whole cell lysates. Protein was separated by SDS-PAGE and transferred to PVDF membranes (Millipore). In order to block the membranes, 1% BSA was added and the membranes were washed three times with TBS-0.1% Tween 20 (TBST). The washed membranes were incubated primary antibodies at 4°C overnight. Afterwards, the secondary antibody (1:3000; Abcam) was incubated at room temperature for 1 h. developed using an ECL chemiluminescence kit. Finally, the antigen-antibody reactions were visualized by chemiluminescence (ECL) kit, and the intensity of protein bands was quantified by using ImageJ software.

**Elisa**

Soluble sST2 levels were quantified in serum according to the manufacturer's instructions. The results were normalized to the control condition. Data were expressed as a fold change relative to the control conditions.

**CCK-8 Assay**

MCF were seeded into 96-well plates at 5000 cells per well 24 hours before treatment and three replicate experiments were performed in each group of cells. At the indicated time points, 10 ul CCK8 solution was added to each well and incubated at 37°C with 5% CO2 for 3 h. Finally, the absorbance was detected using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm. The higher the absorbance in OD450, the more active the cell.

**Examination Of Myocardial Markers**

The levels of lactate dehydrogenase (LDH), creatine kinase-MB(CK-MB) and glutamic oxalacetic transaminase(AST) in serum were measured using the available commercial kits (JianCheng, Nanjing, China), according to the manufacturer's instructions.

**Senescence Detection**

A β-galactosidase Staining Kit (Solarbio, Beijing, China) was employed to detect the senescence of MCFs after the cells were treated with sST2 and H2O2. The short answer is that cells were seeded in a twelve-well plate. The medium was removed before the experiment and washed with phosphate-buffered saline (PBS; D8537, Sigma) once. Then, 1 mL stationary liquid was added at room temperature. After 15 min, the stationary liquid was removed and the cells were washed with PBS for three times (3 min/time). Subsequently, 1 mL of β-galactosidase staining working solution was added and incubated overnight at 37°C. Finally, the senescence of the cells was examined were calculated using ImageJ software.

**Histological Examination of the Heart**
The heart tissues were fixed in 4% polyformaldehyde and embedded in paraffin. Then sections were stained with hematoxylin-eosin (H&E). H&E staining was used to analyze the level of inflammation in heart.

**Immunofluorescence Staining.**

Hearts were resected and fixed in 4% paraformaldehyde for 24h and dehydrated with 30% sucrose for 2 h. Then, the specimens were processed into frozen sections. Sections of heart were permeabilized in 0.5% Triton X-100 for 20 min and sealed with 5% BSA for 2 h. Afterwards, the sections were incubated with anti-mouse P21 (Immunoway) and anti-mouse α-SMA (Proteintech) antibodies at 4°C overnight. After being washed, second antibodies (Alexa Fluor 488 anti-mouse and 594 anti-rabbit) were applied. Finally, Nuclei were identified with DAPI (Thermo Fisher Scientific) and representative figures were taken by fluorescence microscope.

**Statistical Analysis**

All data were analyzed using GraphPad Prism 8.0 (GraphPad Software, Inc.). In ImageJ, grayscale scans were performed on Western blot analysis results. The data were expressed as mean ± standard deviation. A t test was used to compare the data between two groups and the differences between multiple groups were analyzed via a one-way analysis of variance. A P value of 0.05 or less was considered statistically significant.

**Results**

**sST2 Increased and Promoted Inflammatory Damage in the Viral Myocarditis**

To investigate the relationship between viral myocarditis and sST2, mice were divided into three groups: Control group, VMC group, and VMC + anti-ST2 mAb group. Firstly, sST2 content in serum and sST2 protein expression in heart tissue of mice were detected. The results showed that VMC can increase the expression and secretion of sST2, while anti-ST2 mAb injection can effectively reduce them (Fig. 1A-B). Since VMC can cause cardiac inflammatory cell infiltration, fibrosis and cardiac injury, we investigated whether sST2 is associated with these pathological phenomena[20]. By HE staining, it was found that anti-ST2 mAb could reduce infiltration of cardiac inflammatory cell, and CK-MB, LDH, AST contents in serum of VMC mice (Fig. 1C-D). In addition, we also detected the genes expression of inflammatory factor in heart tissue by RT-PCR. Consistent with the above results, anti-ST2 mAb decreased the gene expression of IL-1β, IL-6, and TNF-α (Fig. 1E). Together, these results suggest that viral myocarditis increases the protein expression of sST2, and that sST2 may promote cardiac inflammation.

**sST2 Promoted the Activation of MCF and Inhibits its Senescence in Vitro**
Since we have found that sST2 can promote cardiac fibrosis in vivo, we further explored the relationship between sST2 and MCF in vitro. Western Blot analysis was used to detect whether sST2 could promote the expression of fibrosis-related proteins, such as collagen I, collagen III and α-SMA. The results showed that sST2 up-regulated the expression of fibrosis-related proteins in MCF (Fig. 2A). In addition, RT-PCR was used to detect the expression of fibrosis-related genes as evidence (Fig. 2B). Surprisingly, we found that sST2 not only promoted the activation of MCF, but also promoted its proliferation (Fig. 2C), so we wondered whether sST2 could inhibit MCF senescence. Sure enough, Western Blot analysis showed that sST2 significantly inhibited the expression of senescence related proteins P16, P21, P53 in MCF (Fig. 2D). Consistent with the above results, RT-PCR also showed that sST2 significantly inhibited the expression of senescence related genes P16, P21, P53 in MCF (Fig. 2E). These results demonstrate that sST2 can promote the activation of MCF and inhibit its senescence in vitro.

**sST2 Inhibits MCF Senescence through Sirt1/P53/P21 Signaling Pathway**

In order to further verify whether sST2 can inhibit senescence of MCFs and determine the specific mechanism of sST2 inhibiting senescence of MCFs, MCF was divided into four groups: Control, sST2, sST2 + H₂O₂, H₂O₂. H₂O₂ is a strong oxidizing agent, as the positive control for senescence. CCK8 test showed that pretreatment with sST2 significantly improved the proliferation capacity of MCFs treated with H₂O₂ (Fig. 3A). In addition, SA-β-gal staining revealed that the numbers of SA-β-gal positive staining sST2 + H₂O₂ treatment group were lower than H₂O₂ treatment group (Fig. 3B). It meant that sST2 pretreatment dramatically inhibited MCF senescence. Cellular senescence is mainly mediated by P53/P21-dependent pathway. It has been reported that Sirt1 can regulate this signaling pathway[21]. To explore the underlying signal pathway of sST2 inhibiting MCFs senescence, we detect the expression of senescence related protein p53, P21 and its upstream target protein Sirt1 in activated MCFs treated with or without sST2 by RT-PCR and Western bolt analysis. The results showed that sST2 up-regulated the mRNA and protein expression of Sirt1 and down-regulated the mRNA and protein expression of senescence related protein P53 and P21 (Fig. 3C-G). Together, these data indicate that sST2 inhibits MCF senescence through the Sirt1/P53/P21 signaling pathway.

**sST2 Promotes MCF Activation by Inhibiting Senescence**

A large number of studies have shown that senescence and fibrosis are closely related. Here, we need to investigate whether sST2 promotes MCF activation by inhibiting senescence. We still divide MCF into four groups: Control, sST2, sST2 + H₂O₂, H₂O₂. The expression of MCF activation related proteins was detected by Western Blot analysis. The data showed that sST2 pretreatment significantly increased the protein levels of Collagen-I, Collagen-III and α-SMA (Fig. 4A). In addition, we further investigated the specific ways in which senescence suppressed MCF inhibits its own activation. Therefore, different treated cell supernatants were used to treat MCFs. The results of Western Blot analysis and RT-PCR showed that the protein and gene levels of Collagen-I, Collagen-III and α-SMA in the supernatant of MCFs
pretreated with sST2 were significantly increased compared with the supernatant of MCFs only treated with H$_2$O$_2$. These results suggest that sST2 inhibited MCF senescence and indirectly regulated MCF activation (Fig. 4B-C). Next, it is necessary to understand the specific mechanism leading to MCF activation. TGF-β/Smad pathway is a crucial pathway for fibrosis[22, 23]. We found that sST2 significantly increased the protein and gene expression of TGF-β1 and P-Smad2/3 by western blot analysis and RT-PCR technique (Fig. 4D-E). Together, these findings suggest that sST2 promotes MCF activation by inhibiting senescence and it works by TGF-β/Smad signaling.

**sST2 Inhibits MCFs Senescence and Sirt1/P53/P21 Signaling, Accelerating Cardiac Fibrosis in VMC Mice**

In order to further investigated that whether cell senescence and Sirt1/P53/P21 signaling in MCFs were responsible for initiating cardiac fibrosis and were ameliorated by sST2 neutralizing antibodies (anti-ST2mAb) in vivo. To identify the role of sST2 on cardiac fibrosis, Anti-ST2mAb was injected intraperitoneally into VMC mice. The results showed that VMC group myocardium displaced typical fibrosis characteristics, which was evidenced as the large of extra interstitial extracellular matrix deposition by Masson staining analysis, but not in anti-ST2mAb group (Fig. 5A). Immunofluorescence of Collagen-I and α-SMA was conducted, showing the decrease of the positive region when compared with VMC mice (Fig. 5B-C). And the mRNA expression of fibrosis-related genes also showed the same level (Fig. 5D). In addition, Western blot analysis showed that sST2 decreased the expression of senescence-related genes, such as the decrease of P53 and P21 and the increase of Sirt1 in heart tissue of VMC mice. Compared with sST2 neutralizing antibody treatment group, fibrosis related gene Collagen-I/III and α-SMA was significantly increased (Fig. 5E-F). To further confirm the effect of MCF senescence on cardiac fibrosis, we co-stained the senescence marker P21 with a specific MCF activation marker α-SMA. We found that in anti-ST2mAb group mice P21 was largely expression, but not α-SMA. However, it was significantly reduced in VMC group (Fig. 5G). These results suggested that sST2 accelerated cardiac fibrosis by inhibiting cell senescence and Sirt1/P53/P21 signaling, and promoting collagen deposition.

**Discussion**

The main purpose of this paper is to investigate the effect of sST2 on cardiac fibrosis in the VMC and to elucidate the specific mechanism. The level of sST2 increased significantly and cardiac fibrosis also appeared in the VMC[24]. Our study shows that sST2 can induce MCF activation and collagen secretion, leading to cardiac fibrosis. In addition, sST2 promotes cardiac fibrosis by inhibiting MCF senescence. Therefore, the present study reveals a mechanistic assessment of sST2 on activation of MCF in VMC from the point of cellular senescence.

Most previous studies have emphasized that sST2 plays a harmful role as a soluble decoy receptor by blocking the function of IL-33, which has anti-inflammatory and antioxidant functions[8, 25, 26]. In fact, sST2 can make a difference independently of IL-33. Previous studies have shown that sST2 can promote
cardiac fibrosis, and increase inflammatory molecules production through the production of reactive oxygen species[11]. In the present study, we expand new findings to show that sST2 levels are elevated in the VMC, which can promote cardiac fibrosis by inhibiting MCF senescence. In vivo experiments have been conducted to prove that sST2 can increase deposition of collagen in the heart, increase inflammation and reduce senescence of MCF. We also conducted in vitro experiments to demonstrate that the stimulation of sST2 promotes MCF activation through TGF-β/Smad2/3 signaling and inhibit cell senescence by Sirt1/P53/P21 signaling. Our study further strengthens the idea of sST2 as a predictor of cardiomyopathy and suggests that sST2 can be a therapeutic target for cardiac fibrosis. In addition, we have expanded the research on the mechanism of sST2 causing cardiac fibrosis.

Many signaling pathways are involved in the regulation of cell senescence. P53 and its downstream signaling factor P21 play a crucial role in the regulation of senescence[27–29]. Overexpression of p53 and P21 can increase SA-β-gal activity and induce cell cycle arrest[30]. In this study, we found that sST2 up-regulated the expression of Sirt1 and inhibited the expression of P53 and P21. In addition, the inhibition of MCF senescence by sST2 was verified by β-galactosidase galactosidase staining and CCK8 assay.

TGF-β/Smad2/3 is one of the important mechanisms that cause cardiac fibrosis[31–33]. TGF-β, as a cytokine, promotes MCF activation and extracellular matrix production and plays a key role in cardiac fibrosis[34]. The TGF-β receptor is a heterodimeric receptor complex composed of TGF-β type I and type II receptors[23]. It can phosphorylate Smad2 and Smad3 transcription factors and make them transfer the signal to the nucleus[35–37]. In this paper, we found that sST2 stimulation led to MCF activation and significantly increased expression of TGF-β1, P-smad2, and P-smad3. Furthermore, we also found that sST2 can activate MCF by inhibiting MCF senescence, and this effect can be achieved through paracrine mode. Because the culture supernatant of MCF treated with sST2 and H2O2 can promote the activation of MCF, while the culture supernatant of senescent MCF treated with hydrogen peroxide alone cannot.

In summary, the present study shows that elevated sST2 in the VMC could have a deleterious effect on the heart by inhibiting senescence of MCF, leading to cardiac fibrosis. Therefore, our findings could provide a novel therapeutic target to alleviate cardiac fibrosis.

Declarations

AUTHOR CONTRIBUTION

Jiajia Tan and Jing Wei wrote the manuscript and designed the figures. Hongxiang Lu reviewed and modified the manuscript.

AVAILABILITY OF DATA AND MATERIALS

The data used or analyzed during the current study are available from the corresponding author on reasonable request.
Declaration of Competing Interest

We confirm that there are no conflicts of interest pertaining to this paper.

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References


Figures
Figure 1

sST2 increased and promoted inflammatory damage in the viral myocarditis.

(A) The generation of sST2 was determined using ELISA. (B) Western Blot analyses of protein expression of sST2. (C) H&E stain were used to evaluate the degree and grade of cardiac inflammation. (D) LDH, CK-MB, and AST levels in the serum were determined using a fully automatic biochemical analyzer. (E) RT-
PCR analyses of the mRNA levels of IL-1β, IL-6, TNF-α. Data were represented as mean ± S.D. Compared with control group, *p<0.5; **p<0.01; ***p<0.001.

**Figure 2**

sST2 promoted the activation of MCF and inhibits its senescence in vitro. (A) Western Blot analyses of protein expression of P16, P21, P53. (B) RT-PCR analyses of the mRNA levels of P21, P53, Sirt1.
(C) Western Blot analyses of protein expression of Collagen-I, Collagen-III, α-SMA. (D) RT-PCR analyses of the mRNA levels of Collagen-I, Collagen-III, α-SMA. (E) CCK8 assay was used to detect proliferation ability of MCFs. Data were represented as mean ± S.D. Compared with control group, *p<0.5; **p<0.01; ***p<0.001.

Figure 3
sST2 inhibits MCF senescence through Sirt1/P53/P21 signaling pathway. (A) Western Blot analyses of protein expression of P21, P53, Sirt1. (B-D) RT-PCR analyses of the mRNA levels of P21, P53, Sirt1. (E) CCK8 assay was used to detect proliferation ability of MCFs. (F) SA-β-gal stain was used to detect the senescence of MCFs. Data were represented as mean ± S.D. Compared with control group, *p<0.5; **p<0.01; ***p<0.001.
sST2 promotes MCF activation by inhibiting senescence. (A) Western Blot analyses of protein expression of Collagen-I, Collagen-III, α-SMA. (B) Western Blot analyses of protein expression of Collagen-I, Collagen-III, α-SMA. (C) RT-PCR analyses of the mRNA levels of Collagen-I, Collagen-III, α-SMA. (D) RT-PCR analyses of the mRNA levels of TGF-β1, Smad2, Smad3. (E) Western Blot analyses of protein expression of TGF-β1, P-Smad2, P-Smad3. Data were represented as mean ± S.D. Compared with control group, *p<0.05; **p<0.01; ***p<0.001.
sST2 inhibits MCFs senescence and Sirt1/P53/P21 signaling, accelerating cardiac fibrosis in VMC mice. The heart tissues were collected on day 15 for pathological examination. (A) Representative micrographs of paraffin-embedded heart stained histochemically for Masson’s trichrome (Masson). (B-C) Immunofluorescence images showing the fibroblast activation in heart tissue, n = 5 per group. Collagen-I staining is showed in green, α-SMA is showed in red (Scale bar = 20μm). (D) RT-PCR analysis for Collagen-I/III mRNA in the heart at 15d after VMC. The gene expression level was normalized with that of GAPDH (n = 5). (E) Western blotting for Sirt1, Collagen-I/III, P53, α-SMA, P21 and P16. GAPDH was used as the loading control. Protein expression relative to β-actin was assessed by densitometric analysis. (F) RT-PCR analysis for Sirt1/P53 mRNA in the heart at 15d after VMC. The gene expression level was normalized with that of GAPDH (n = 5). (G) Representative micrographs of cells immunofluorescently stained for P21 and α-SMA, with DAPI staining the nucleus. Data were represented as mean ± S.D. Compared with control group, *p<0.5; **p<0.01; ***p<0.001.

Elevated sST2 in the VMC could have a deleterious effect on the heart by inhibiting senescence of MCF, leading to cardiac fibrosis.