Intermedin 1-53 Inhibits Myocardial Fibrosis in Rats by Down-Regulating Transforming Growth Factor-β

Authors' Contribution:
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Background: Myocardial fibrosis is the result of persistent anoxia and ischemic myocardial fibers caused by coronary atherosclerotic stenosis, which lead to heart failure, threatening the patient's life. This study aimed to explore the regulatory role of intermedin 1-53 (IMD1-53) in cardiac fibrosis using neonatal rat cardiac fibroblasts and a myocardial infarction (MI) rat model both *in vitro* and *in vivo*.

Material/Methods: The Western blot method was used to detect the protein expression of collagen I and collagen III in myocardial fibroblasts. The SYBR Green I real-time quantitative polymerase chain reaction (PCR) assay was used to detect the mRNA expression of collagen type I and III, IMD1-53 calcitonin receptor-like receptor (CRLR), transforming growth factor-β (TGF-β), and matrix metalloproteinase-2 (MMP-2). Masson staining was used to detect the area changes of myocardial fibrosis in MI rats.

Results: Results *in vivo* showed that IMD1-53 reduced the scar area on the heart of MI rats and inhibited the expression of collagen type I and III both in mRNA and protein. Results of an *in vitro* study showed that IMD1-53 inhibited the transformation of cardiomyocytes into myofibroblasts caused by angiotensin II (Ang II). The further mechanism study showed that IMD1-53 inhibited the expression of TGF-β and the phosphorylation of smad3, which further up-regulated the expression of MMP-2.

Conclusions: IMD1-53 is an effective anti-fibrosis hormone that inhibits cardiac fibrosis formation after MI by down-regulating the expression of TGF-β and the phosphorylation of smad3, blocking fibrous signal pathways, and up-regulating the expression of MMP-2, thereby demonstrating its role in regression of myocardial fibrosis.

MeSH Keywords: Fibrosis • Matrix Metalloproteinase 2 • Smad3 Protein

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/898522

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Background

Myocardial fibrosis refers to the excess accumulation of collagen fibers, significantly higher concentrations of myocardial collagen, and changes in collagen elements in the normal structure of cardiac muscle. Such pathological changes exist in a variety of cardiovascular diseases. It has been considered that they are closely related to arrhythmia, cardiac dysfunction, or even sudden cardiac death [1]. Myocardial fibrosis is one of the main pathological features of ventricular remodeling. The improvement of cardiac fibrosis can effectively improve cardiac function and inhibit ventricular tissue hypertrophy, and reduce the risk of cardiovascular events [2]. Therefore, it is of great clinical significance to search for ways to effectively inhibit and regress myocardial fibrosis.

Intermedin (IMD) is a new member of calcium gene-related peptide (CGRP) superfamily identified by Roh et al. [3] and others [4], who used phylogenetic analysis to retrieve GenBank data using the primary and secondary structure specific to this superfamily.

IMD is widely distributed in the tissues. It is expressed in tissues and organs such as human cardiac cells, coronary arterial smooth muscle cells, and hypothalamic supraoptic nucleus [3,5–9], which indicates that IMD may be involved in regulating the environmental homeostasis of the body.

The IMD precursor consists of 148 amino acids, with its cleavage site located at Arg100-Arg101 and Arg107-Val108. The two sites produce two polypeptide molecules (preproIMD101-147 and preproIMD108-147, respectively). PreproIMD hydrolyzes at Arg93-Arg94 sites to produce preproIMD95-147, which is IMD1-53 [4]. Previous studies showed that IMD1-53 is a potent vasoactive peptide regulator [5,10]. It can strongly and durably relax blood vessels, lower blood pressure, improve heart function, and increase coronary blood flow [7,11,12]. However, the role and mechanism of IMD1-53 in cardiac remodeling and myocardial fibrosis are not fully clear.

This study's *in vitro* experiment detected the collagen synthesis effects of IMD1-53 on rat cardiac fibroblasts induced by angiotensin II (Ang II) and the function of transforming cardiac fibroblasts into cardiac myofibroblasts. This study's *in vivo* experiment detected the effects of IMD1-53 on cardiac fibrosis using a myocardial infarction rat model and explored its possible mechanism, so as to provide new laboratory data for the prevention and treatment of myocardial fibrosis.

Material and Methods

Culture and identification of cardiac fibroblasts

The heart of 1- to 3-day-old SD rats was taken, and its membrane envelopes were cut. The heart was cut into pieces of 0.5~1.0 mm^3 and digested with 0.1% trypsin. Then it was cultured at 5% CO_2 and 37°C in an incubator for 60 min, and cardiac fibroblasts were obtained by differential adhesion. Morphological observations (Figure 1A) showed that the purity of cardiac fibroblasts was 98%. The second to fourth generations of cardiac fibroblasts were chosen to be used in the experiment. The components of the fibroblast medium were Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), and 1% PS (Gibco, USA). The fibroblasts were treated with IMD1-53 at 1×10^{-7}mmol/L and 100 nM Ang II in serum-free RPMI for 24 hours.

mRNA extraction and Q-PCR analysis

The cultured second generation of cardiac fibroblasts was seeded in a 24-well culture plate. The total RNA was extracted by Trizol, and cDNA was reverse transcribed with the TOYOBO reverse transcription kit (FSQ-101). SYBR Green I real time Q-PCR was used to detect the genes' expression. The target genes were normalized, with GAPDH being an internal reference. Using the relative quantification method, the relative quantification of genes in the sample was calculated by 2$^{-\Delta\Delta Ct}$. Each
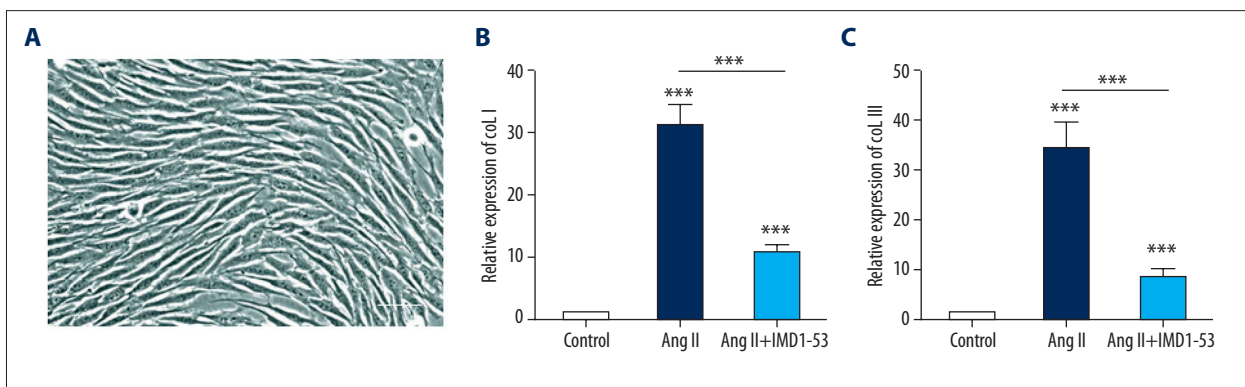
Figure 1. Masson staining shows the area of cardiac fibrosis; the column chart shows the statistical analysis. Scale bar=3 mm, n=5, *** *P*<0.01.

Table 1. The sequence of primers used in this study.

Primer		Sequence
COLI	Forward	5'-GAGGGCCAAGACGAAGACATC-3'
	Reverse	5'-CAGATCACGTCATCGCACAAC-3'
COLIII	Forward	5'-GGAGCTGGCTACTTCTCGC-3'
	Reverse	5'-GGGAACATCCTCCTTCAACAG-3'
α-SMA	Forward	5'-AAAAGACAGCTACGTGGGTGA-3'
	Reverse	5'-GCCATGTTCTATCGGGTACTTC-3'
CRLR	Forward	5'-TCCTGAGGACTCAATTCAGTTGG-3'
	Reverse	5'-CTGTTGCAGTAAACGCCTTCT-3'
TGF-β	Forward	5'-GGCCAGATCCTGTCCAAGC-3'
	Reverse	5'-GTGGGTTTCCACCATTAGCAC-3'
MMP2	Forward	5'-TACAGGATCATTGGCTACACACC-3'
	Reverse	5'-GGTCACATCGCTCCAGACT-3'

sample was detected at least 3 times. Primer sequences are shown in Table 1. PCR conditions were as follows: at 94°C for 4 min, at 92°C for 40 s, at 59.5°C for 45 s, at 72°C for 3 min, 40 cycles, and finally at 72°C for 15 min.

Protein extraction and Western blot

We lysed the cells, centrifuged the lysate, and collected the supernatant. Then the bicinchoninic acid (BCA) method was used to quantify proteins. After sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE), transfer membrane of the gel and blot hybridization were used in type I and III collagen protein. We performed SDS-PAGE with the sampling amount per well of 100 µg of protein. Then we transferred the proteins to a nitrocellulose (NC) membrane and washed them with Tris-buffered saline (TBS), which consists of 5% skimmed milk powder, 20 mmol/L Tris-HCl (pH7. 5), 0.05% Tween20, and 0.6% NaCl at room temperature, and sealed the membrane for 1 h. Primary antibodies were goat anti-rat type I, type III colla-gen monoclonal antibody (abcam, ab32145, ab43212), rabbit anti-rat smad3 monoclonal antibody (cell signaling, #72255), rat anti-mouse phosphorylated smad3 antibody (cell signal-ing, #64214), and goat anti-rabbit antibody MMP-2 (abcam, ab54322), respectively. Then, they were incubated at 4°C over-night and washed 3 times with TBS, each time adding second-ary antibody (horseradish peroxidase-labeled goat anti-mouse polyclonal antibody, mouse anti-rabbit polyclonal antibody) at room temperature for 1 h. They were washed 3 times with phosphate-buffered saline (PBS). Chemiluminescence was uti-lized for color reaction. The results were analyzed by the pro-fessional software National Institutes of Health (NIH) ImageJ to quantify gray values

Rat myocardial infarction model

Twenty 8- to 10-week-old, 250±20 g, male SD rats raised in a specific-pathogen-free (SPF) room were used to construct the myocardial infarction model. The model of left ventricular myo-cardial ischemia was constructed by using 10% chloral hydrate at 0.3 mL/100 g of body weight (bw) intraperitoneal injection for anesthesia. When there was no reaction of tingling limbs in the rats, we fixed them in the supine position on the operating ta-ble, inserted an endotracheal tube, connected it to a respirator machine, removed the chest hair, sterilized the skin with iodine, and cut the skin of the SD rats' left chest between the fourth and sixth ribs. Then, the pectoralis major and pectoralis minor were separated by blunt dissection along the lower edge of pectoralis major, and the intercostal was revealed. Ophthalmic scissors were used to cut along the intercostal, expanding in the front and back without hurting the thoracic artery. Now the beating heart could be seen. A retractor was placed to tear the pericardium until the heart was completely exposed. The left anterior descending cor-onary artery was ligated, after which the chest and skin were su-tured. Rats were divided into three groups: control group (n=6), ischemia group (n=7), and ischemia + IMD1-53 group (n=7). The experimental protocols were approved by the Animal Care and Protection Committee of China Medical University.

Masson staining of heart tissue

The SD rats were killed at 1 week by vertebral dislocation. With PBS perfusion, their hearts were taken. Optimal cutting tem-perature (OCT) embedding and freezing at −80°C were per-formed. A freezing microtome was used for cutting into fro-zen sections of 5 µm/sheet. The Masson staining kit (BOGOO, PT003) was used for staining according to the instruction man-ual, and ImageJ software was used for analysis of fibrosis area.

Statistical analysis

One-way analysis of variance (ANOVA) tests were completed on all quantitative data using the Dunnett post-test to com-pare the experimental groups with the saline control. The level of significance was set as $P < 0.05$. All statistical calculations were computed using GraphPad Prism 4 software.

Results

Intermedin inhibits area of cardiac fibrosis in rats

In vivo results of Masson staining showed that compared with the control group, the area of cardiac fibrosis in rats significant-ly increased after myocardial infarction, while in the LIMD1-53 injection group, the area of cardiac fibrosis of the heart af-ter myocardial infarction was significantly inhibited (Figure 2).
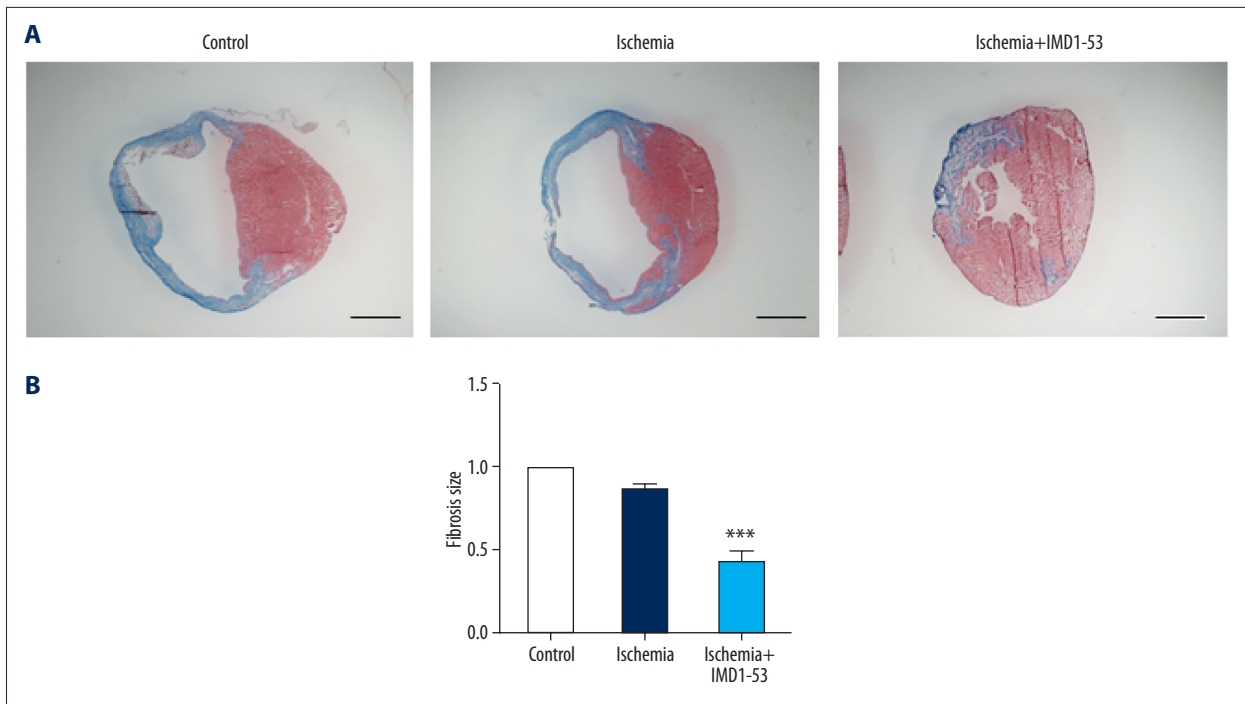
Figure 2. IMD1-53 inhibited the collagen synthesis in the infarcted heart tissue. (**A**) The mRNA level of collagen I and collagen III; (**B**) The protein expression of collagen I and collagen III. n=3, *** $P<0.01$.

Inhibition of collagen synthesis of cardiac tissue after treatment with intermedin

In vivo, after real-time PCR was used to analyze the collagen synthesis of rats' cardiac tissue in myocardial infarction, we found that the collagen expression level of type I and III in myocardial infarction heart was obviously higher than that in the control group ($P<0.01$), while that of the LIMD1-53 injection group was significantly inhibited ($P<0.05$) (Figure 3A, 3B). Detection of protein level also showed that the level of collagen protein type I and III in the LIMD1-53 group was significantly lower than the level of collagen synthesis of cardiac tissue in myocardial infarction (Figure 3C).

Intermedin affects the collagen synthesis of cardiac fibroblasts and the cell transformation to myofibroblasts

In vitro, after real-time PCR was used to analyze the collagen synthesis of myocardial cells in rats, we found that compared with the control group, the collagen gene expression of cardiac fibroblasts type I and III treated with Ang II was significantly higher ($P<0.01$), while in the IMD1-53 group, compared with the Ang II group, the collagen gene expression of cardiac fibroblasts type I and III was significantly lower ($P<0.01$) (Figure 1B, 1C). The expression of cardiac fibroblast marker α-SMA was obviously inhibited by LIMD1-53 at 1×10^{-7} mmol (Figure 4).

The influence of IMD1-53 calcitonin receptor-like receptor (CRLR) mRNA expression

In vitro, after we further analyzed the gene expression of fibroblasts in the control group, the Ang II group, and the Ang II + LIMD1-53 group, we found that Ang II inhibited CRLR mRNA expression ($P<0.05$), and this inhibition could not be reversed by exogenous IMD1-53 (Figure 5A).

### IMD1-53 inhibits the expression of transforming growth factor-β (TGF-β) in fibroblast and cardiac tissue

In vitro, quantitative PCR showed that Ang II significantly stimulated the expression of TGF-β mRNA ($P<0.01$), while IMD1-53 significantly inhibited the expression of TGF-β induced by Ang II (Figure 5B).

IMD1-53 inhibits smad3 phosphorylation

In vitro, Ang II could induce the expression of TGF-β, thus contributing smad3 phosphorylation and the expression of downstream genes. In the Ang II + LIMD1-53 group, LIMD1-53 significantly inhibited the expression of the Ang II-induced TGF-β, thereby reducing the level of smad3 phosphorylation and inhibiting cardiac fibrosis (Figure 6A).
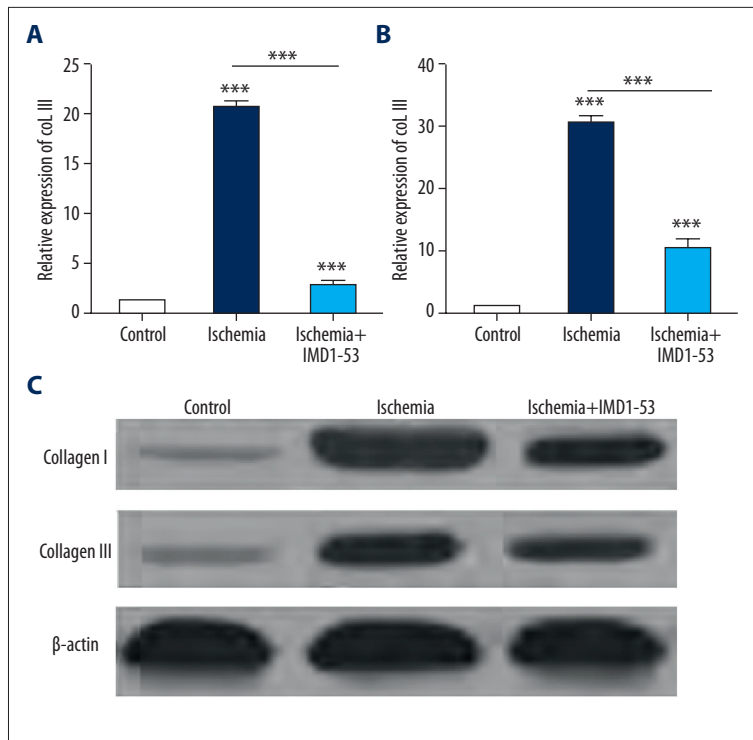
Figure 3. IMD1-53 inhibited the collagen synthesis in the neonatal rat cardiac fibroblasts induced by Ang II. (**A**) The morphology of normal cultured cardiac fibroblasts, scale bar=100 μm; (**B**) The gene expression level of collagen I; (C) The gene expression level of collagen III. n=3, *** *P*<0.01.
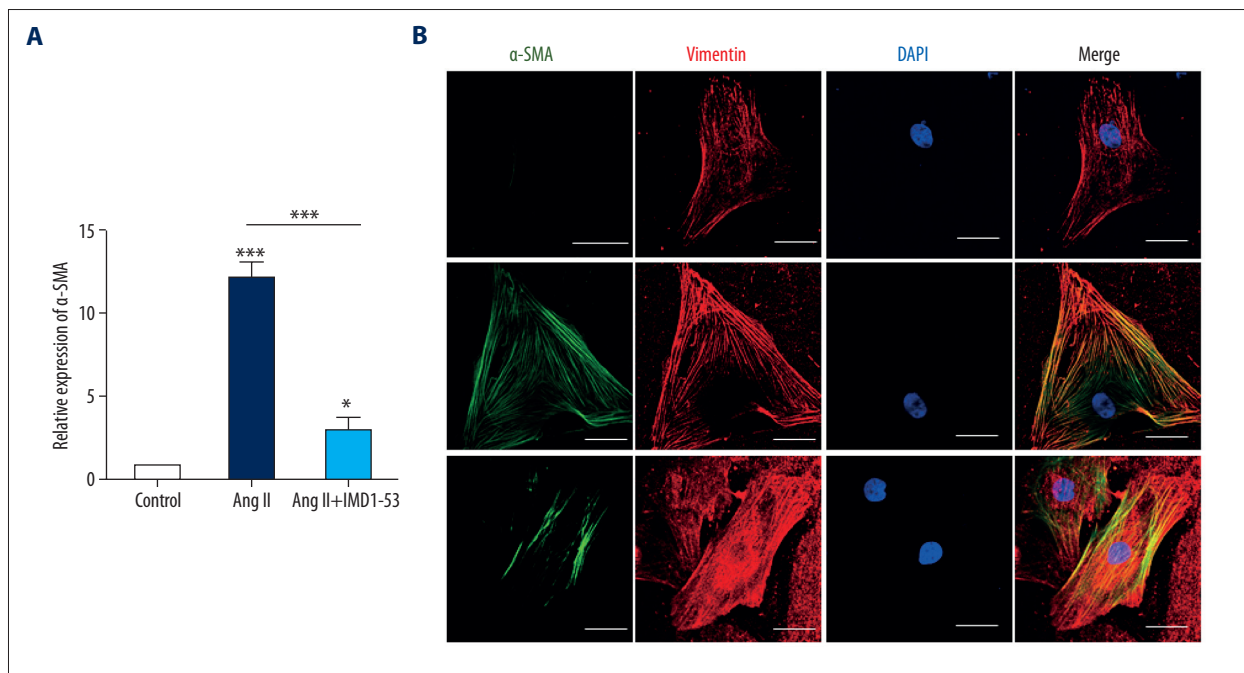
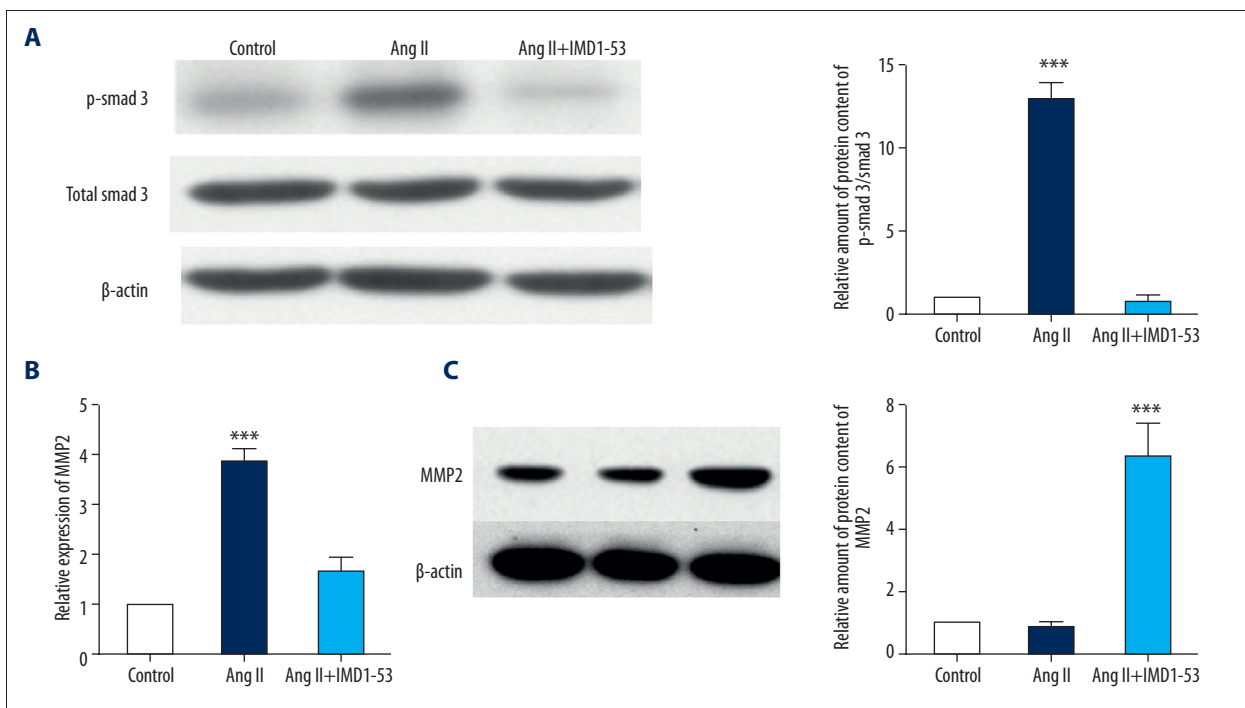**Figure 4.** The expression of cardiac fibroblasts marker α-SMA. (**A**) The gene expression of α-SMA; (**B**) Immunostaining of α-SMA (green), vimentin (red),and nuclei (blue) in cardiac fibroblasts. Scale bar=20 μm, n=3, * *P*<0.05, *** *P*<0.01.
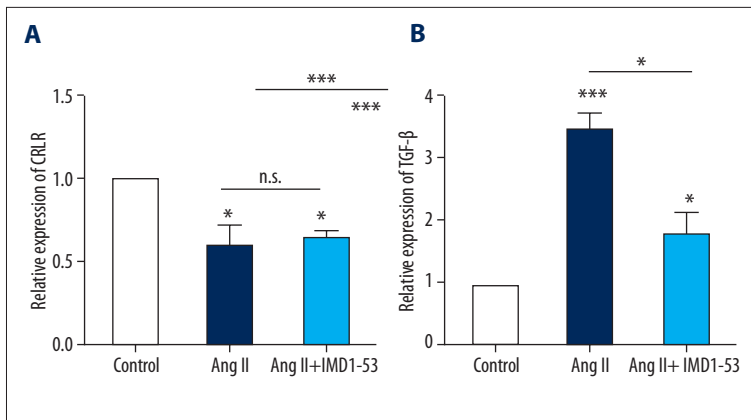
Figure 5. The expression of CRLR or TGF-β by Q-PCR. n=3, * *P*<0.05, *** *P*<0.01.

Figure 6. The phosphorylation level of smad3 and the expression of MMP-2. (**A**) Western blot showed the phosphorylation level of smad3; (**B**) Q-PCR or Western blot showed the expression level of MMP-2. n=3, *** *P*<0.01.

Down-regulation of TGF-β leads to the up-regulation of expression of matrix metalloproteinase (MMPs)

Q-PCR data showed that in the Ang II + LIMD1-53 group, down-regulation of TGF-β was accompanied by the up-regulation of expression of MMP-2 *in vivo*, which indicates that LIMD1-53 can also perform one of the mechanisms of anti-cardiac fibrosis (Figure 6B).

Discussion

IMD plays a lot of roles in the body. The role in the cardiovascular system is particularly evident, mainly in terms of relaxing

blood vessels and lowering blood pressure [13]. IMD can weaken ischemia/reperfusion injury caused by myocardial apoptosis [14]. At the same time, it can increase myocardial contractility and coronary flow [15,16] and regulate neonatal rat cardiomyocyte hypertrophy [17–20]. Through the experiment, we found that IMD1-53 reduced collagen synthesis of cardiac fibroblasts in the rat and inhibited the transformation of cardiac fibroblasts into myofibroblasts. The studies showed that IMD1-53 can improve the area of cardiac fibrosis in rats after they died because of myocardial infarction. The results of fluorescence Q-PCR and Western blot showed the reduction of collagen synthesis in myocardial tissue, and IMD1-53 CRLR did not change significantly in the myocardial infarction group and the IMD1-53 group. It was found that the TGF-β expression

levels of IMD1-53 were significantly lower than those of the myocardial infarction group, and the expression levels of MMP-2 were significantly increased. We further found that the protective effect could be achieved by inhibiting the expression of TGF-β, thereby blocking the TGF-β-smads signaling pathway.

Cardiac fibrosis is an extremely complex process, involving multiple paths that interact with each other, but TGF-β plays a central role in cardiac fibrosis. Domestic and international studies have reported that during the formation of scar tissue, TGF-β is believed to promote scar generation [1,8]. It also has the physiological function of promoting fibroblast growth and the expression of extracellular matrix (ECM), and inhibits the degradation of ECM. In addition, it can promote the transformation of fibroblasts into myofibroblasts [1]. After cardiac muscle has been affected by pathological factors, substances such as endocrine TGF-β, Ang II, and endothelin directly stimulate the proliferation of fibroblasts and the production of collagen [18,21]. Ang II can inhibit the synthesis of myocardial cells and secretion of IMD1-53, and exogenous IMD1-53 significantly inhibits Ang II induction of the proliferation of cardiac fibroblasts [12,22]. This study found that the gene expression of TGF-β was significantly decreased with IMD1-53, and smad3 phosphorylation levels were significantly decreased, suggesting that the anti-fibrosis effect of IMD1-53 is realized by inhibiting TGF-β expression so as to block smad3 phosphorylation, while the IMD CRLR did not change significantly, indicating that IMD1-53 inhibition of myocardial fibrosis may not related to the number of receptors.

ECM is a dynamic network structure composed of macromolecules such as collagen, proteoglycans, and glycoproteins [8], whose synthesis and degradation are influenced by many factors. The basic characteristics of tissue fibrosis showed the alterations of organizational structure and excessive deposition of ECM [23]. Matrix metalloproteinases (MMPs), a group of highly homologous zinc-dependent peptide enzymes, can degrade various ECMs. Their activity is closely related to the expressions of the specific tissue inhibitors of MMPs (TIMPs) in the tissue [24]. Studies have shown that the key to maintaining normal metabolism of ECM is the secreted balance between MMPs and TIMPs, and their imbalance is one of the important factors in a variety of types of tissue fibrosis [2,25]. TGF-β inhibits the activity of MMPs by inducing the specificity of matrix TIMPs, thereby inhibiting the degradation of ECM [26]. This study found that the down-regulation of TGF-β leads to the higher expression of MMP-2, in which TGF-β has played a role in the degradation of extracellular matrix.

This study defines IMD1-53 regulation of cardiac fibrosis, but more research is needed on its mechanism. The direct targets of IMD1-53 in regulating the TFG-β/smad3 pathway need to be identified, and the exact mechanism of how it influences the expression of MMPs is unclear and requires further study.

Conclusions

In brief, IMD1-53 can inhibit the collagen synthesis of cardiac fibroblasts, and the down-regulation of TFG-β expression is one of the mechanisms of inhibition of myocardial fibrosis. IMD is very likely to be a new endogenous substance that has a role in regression of myocardial fibrosis, with potential clinical application.

Statement

Conflict of interest

The authors declare that they have no conflict of interest.

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