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

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
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 mm³ and digested with 0.1% trypsin. Then it was cultured at 5% CO₂ 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 1×10⁻⁷mmol/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⁻ΔΔCt. Each

![Figure 1](image-url)

**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'-GAGGGCCAAGCAGAAGACATC-3'         |
|        | Reverse: 5'-CAGATCACGCATCGGCAACAG-3'         |
| COLIII | Forward: 5'-GGAGCTGCGCTCTCTGCG-3'            |
|        | Reverse: 5'-GGGAACATCGCTTCAACAG-3'           |
| α-SMA  | Forward: 5’-AAAAGACAGCTACGTGGTGA-3’          |
|        | Reverse: 5’-GCCATGTTCATCCGGCTACTTC-3’        |
| CRLR   | Forward: 5’-TCTGGAGGACATCAATGCATTGGG-3’      |
|        | Reverse: 5’-CTTGGCACTAACACGCCTCTT-3’         |
| TGF-β  | Forward: 5’-GGGCGATCTGGTCAAGGCG-3’           |
|        | Reverse: 5’-GTTGGTTCCACATGCCACAC-3’          |
| MMP2   | Forward: 5’-TACAGGATCGTGGCTACACACAC-3’       |
|        | Reverse: 5’-GGTCACATCGCTCCAGACT-3’           |

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-polyacrylamide 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 collagen monoclonal antibody (abcam, ab32145, ab43212), rabbit anti-rat smad3 monoclonal antibody (cell signaling, #72255), rat anti-mouse phosphorylated smad3 antibody (cell signaling, #64214), and goat anti-rabbit antibody MMP-2 (abcam, ab54322), respectively. Then, they were incubated at 4°C overnight and washed 3 times with TBS, each time adding secondary 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). Cheliuminescence was utilized for color reaction. The results were analyzed by the professional 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 myocardial infarction 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 table, 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 pericardials major and pectoral 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 coronary artery was ligated, after which the chest and skin were sutured. Rats were divided into three groups: control group (n=6), ischemia group (n=7), and ischemia + LIMD1-53 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 temperature (OCT) embedding and freezing at −80°C were performed. A freezing microtome was used for cutting into frozen sections of 5 μm/sheet. The Masson staining kit (BOGOO, PT003) was used for staining according to the instruction manual, 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 compare 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 significantly increased after myocardial infarction, while in the LIMD1-53 injection group, the area of cardiac fibrosis of the heart after myocardial infarction was significantly inhibited (Figure 2).
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 LIMD1-53 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.
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-smad3 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 be 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 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 TGF-β/sm3d 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 TGF-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

The authors received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that they have no conflict of interest.

References:

1. Park JY, Ryu SK, Choi JW et al: Association of inflammation, myocardial fibrosis and cardiac remodelling in patients with mild aortic stenosis as assessed by biomarkers and echocardiography. Clin Exp Pharmacol Physiol, 2014; 41(3): 185–91
2. Guo H1, Sa Y, Huang J et al: Urethral Reconstruction with small intestinal submucosa seeded with oral keratinocytes and TIMP-1 siRNA transfected fibroblasts in a rabbit model. Urol Int, 2016; 96(2): 223–30
3. Roh J, Chang CL, Bhalla A et al: Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor-activity-modifying protein receptor complexes. J Biol Chem, 2004; 279(8): 7264–74
4. Martinez-Alvarez RM, Volkoff H, Cueto IA, Delgado MI: Molecular characterization of calcitonin gene-related peptide (CGRP) related peptides (CGRP, amylin, adrenomedullin and adrenomedullin-2/intermedin) in golden fish (Carassius auratus): Cloning and distribution. Peptides, 2008; 29(9): 1534–43
5. Kindt F, Wiegand S, Löser C et al: Intermedin: A skin peptide that is down-regulated in atopic dermatitis. J Invest Dermatol, 2007; 127(3): 605–13
6. Pan CS, Yang IH, Cai DY et al: Cardiovascular effects of newly discovered peptide intermedin/adrenomedullin 2. Peptides, 2005; 26(9): 1640–46
7. Takey Y, Inoue K, Ogoshi M et al: Identification of novel adrenomedullin in mammals: A potent cardiovascular and renal regulator. FEBs Lett, 2004; 556(1–3): 53–58
8. Yang X, Zhang H, Jia Y et al: Effects of intermedin1-53 on myocardial fibrosis and cardiac remodeling in patients with mild aortic stenosis as assessed by biomarkers and echocardiography. Clin Exp Pharmacol Physiol, 2014; 41(3): 185–91
9. Chauhan M, Balakrishnan M, Blesson CS, Yallampalli C: Adrenomedullin2 (ADM2)/intermedin (IMD) in rat ovary: Changes in estrous cycle and pregnancy and its role in ovulation and steroidogenesis. Biol Reprod, 2015; 92(2): 36
10. Hashimoto H, Hyodo S, Kawasaki M et al: Adrenomedullin 2 (AM2)/intermedin is a more potent activator of hypothalamic oxytocin-secreting neurons than AM possibly through an unidentified receptor in rats. Peptides, 2007; 28(5): 1104–12
11. Qi YF, Teng X, Song JQ et al: Inhibition of endoplasmic reticulum stress by intermedin1-53 protects against myocardiac ischemia/reperfusion injury through CRLR/RAMPs-Akt signalling pathway. Regul Pept, 2010; 164(1): 34
12. Xiao F, Wang LJ, Zhao H et al: Intermedin restricts vessel sprouting by inhibiting the loosening of endothelial junction. Biochem Biophys Res Commun, 2015; 458(1): 174–79
13. Zhao Y, Bell D, Smith LR et al: Differential expression of components of the cardiomyocyte adrenomedullin/intermedin receptor system following blood pressure reduction in nitric oxide-deficient hypertension. J Pharmacol Exp Ther, 2006; 316(3): 1269–81
14. Yang JH, Pan CS, Jia YX et al: Intermedin1-53 activates L-arginine/nitric oxide synthase/nitric oxide pathway in rat aortas. Biochem Biophys Res Commun, 2006; 341(2): 567–72
15. Yang JH, Xie YX, Pan CS et al: Effects of intermedin1-53 on cardiac function and ischemia/reperfusion injury in isolated rat hearts. Biochem Biophys Res Commun, 2005; 327(3): 713–19
16. Jia YX, Yang JH, Pan CS et al: Intermedin1-53 protects the heart against isoproterenol-induced ischemic injury in rats. Eur J Pharmacol, 2006; 549(1–3): 117–23
17. Ha T, Hua F, Li Y et al: Blockade of MyD88 attenuates cardiac hypertrophy and decreases cardiac myocyte apoptosis in pressure overload-induced cardiac hypertrophy in vivo. Am J Physiol Heart Circ Physiol, 2006; 290(3): H985–94
18. Lu WW, Zhao L, Zhang JS et al: Intermedin1-53 protects against cardiac hypertrophy by inhibiting endoplasmic reticulum stress via activating AMP-activated protein kinase. J Hypertens, 2015; 33(8): 1676–87
19. Dong J, Chen X, So Y et al: [Intermedin (IMD) gene expression in hypertrophic cardiac myocyte of renal vascular hypertension rats and the intervention of Valsartan, Amlodipine and Enalapril in the expression]. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi, 2009; 26(5): 1082–87 [in Chinese]
20. Yang JH, Ma CG, Cai Y et al: Effect of intermedin1-53 on angiotensin II-induced hypertrophy in neonatal rat ventricular myocytes. J Cardiovasc Pharmacol, 2010; 56(1): 45–52
21. Zhao L, Peng DQ, Zhang J et al: Extracellular signal-regulated kinase 1/2 activation is involved in intermedin1-53 attenuating myocardial oxidative stress injury induced by ischemia/reperfusion. Peptides, 2012; 33(2): 329–35
22. Yamac AH, Bacaksiz A, Ismailoglu Z et al: Implication of plasma intermedin levels in patients who underwent first-time diagnostic coronary angiography: A single centre, cross-sectional study. BMC Cardiovasc Disord, 2014; 14: 182
23. Kandilci HB, Gumusel B, Lippton H: Intermedin/adrenomedullin-2 (IMD/AM2) relaxes rat main pulmonary arterial rings via cGMP-dependent pathway: Role of nitric oxide and large conductance calcium-activated potassium channels (BKCa). Peptides, 2008; 29(8): 1321–28
24. Di Gregoli K, George SJ, Jackson CI et al: Differential effects of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 on atherosclerosis and monocyte/macrophage invasion. Cardiovasc Res, 2016; 109(2): 318–30
25. Johnson KM, Crocker SJ: TIMP-1 couples RhoK activation to IL-1beta-induced astrocyte responses. Neurosci Lett, 2015; 609: 165–70
26. Matsuda A, Itoh Y, Koshikawa N et al: Clusterin, an abundant serum factor, is a possible negative regulator of MT6-MMP/MMP-25 produced by neutrophils. J Biol Chem, 2003; 278(38): 36350–57

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS] [Index Copernicus]