Decoy receptor-3 regulates inflammation and apoptosis via PI3K/AKT signaling pathway in coronary heart disease

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Abstract. Coronary heart disease is a disease characterized by coronary artery atherosclerosis lesions caused by vascular cavity stenosis, occlusion, myocardial ischemia, hypoxia or necrosis. Previous studies have demonstrated that decoy receptor-3 (DCR-3) can act as a pleiotropic immunomodulation for enhancing angiogenesis, which may be associated with the progression of coronary heart disease. In the present study, ELISA assay was used to investigate the plasma concentration level of DCR-3 in patients with coronary heart disease. The mRNA and protein level of DCR-3 in myocardial cells were determined by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. The role and molecular mechanism of DCR-3 was also evaluated in myocardial cells in mice with coronary heart disease. The role of small interfering RNA that targeted phosphoinositide 3-kinase (PI3K) in DCR-3 mediated apoptosis was confirmed by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling and immunofluorescence. C57BL/6 mice with coronary heart disease were used to evaluate the efficacy of DCR-3 on inflammation and apoptosis. The data indicated that plasma concentration level of DCR-3 was downregulated in mice with coronary heart disease and that DCR-3 administration improved symptoms of coronary heart disease and prolonged survival of mice with coronary heart disease. In addition, it was demonstrated that DCR-3 treatment suppressed the inflammatory response and apoptosis of myocardial cells. Circulating DCR-3 concentration levels may be identified as a predictor of coronary heart disease and prognosis of coronary heart disease. Notably, it was also demonstrated that DCR-3 inhibited inflammatory factor expression levels by regulation of the PI3K/protein kinase B (AKT) signaling pathway. Taken together, these results indicate that increasing circulating DCR-3 plasma concentration is associated with degree of coronary heart disease, suggesting that DCR-3 may be a promising drug for the treatment of coronary heart disease via regulating inflammation and apoptosis through the PI3K/AKT signaling pathway.

Introduction

Coronary heart disease is a generic term for coronary arteries atherosclerosis lesions caused by a large number of factors, such as vascular cavity stenosis, occlusion, myocardial ischemia, hypoxia, necrosis, inflammation and apoptosis of myocardial cells (1,2). It has been demonstrated that reduced blood pressure and cholesterol are important factors in reducing deaths from coronary heart disease (3). In addition, a large number of proteins are associated with the protective effects for host against myocardial ischemia and reperfusion injury through modulating myocardial apoptosis and inflammation (4,5). Furthermore, inflammation and apoptosis contribute to the initiation and development of coronary heart disease, which have been considered as the prognostic indicators for patients with coronary heart disease following drug treatment in clinical settings (6,7). Therefore, a number of potential strategies to relieve or mitigate the apoptosis of myocardial cells have been proposed to protect the heart against coronary heart disease, such as ameliorating the apoptosis of cardiac cells mediated by endothelial stem cell (8), or activating the signaling pathway to promote the expression of anti-apoptosis proteins and decrease pro-apoptosis proteins to protect the heart (9).

Coronary heart disease is the leading cause of mortality worldwide and is closely associated with metabolism disorders of endogenous substances (10). A growing number of studies have reported that apoptosis of myocardial cells has a crucial role in the progression of cardiovascular diseases (11-13). Apoptosis of leukocytes has been considered as a marker of neutrophil-endotheliocyte interaction in coronary heart disease (14). Expression of apoptosis factors has become an important reference of surgical results following coronary stent implantation in coronary heart disease patients determined by characteristics of neutrophil cluster of differentiation (CD)11b, platelet CD62P, endothelin, monocyte CD11b, and neutrophil.
CD178 in patients with coronary heart disease prior to and following primary coronary stenting (15).

Inflammation as the cause of coronary heart disease has been reviewed previously (16). Expression levels of inflammation cytokines including interleukin (IL)-6, tumor necrosis factor (TNF)-α and IL-1 in patients with coronary heart disease may also be associated with the progression pathogenesis (17). These reports indicate that inhibition of inflammation contributes to the treatment of coronary heart disease. In the present study, the inhibitory effects of decoy receptor-3 (DCR-3) on inflammation were investigated.

It has been demonstrated that DCR-3 can act as a pleiotropic immunomodulator for enhancing angiogenesis, which may be associated with the progression of coronary heart disease (18). DCR-3 is well known as soluble receptor TR6, which belongs to the TNFR superfamily and is constituted by a 300-aa polypeptide (19,20). DCR-3 has been identified as the most common biomarker of tumor deterioration in cancer patients by a previous meta-analysis (21). However, no previous report has focused on the clinical significance of DCR-3 expression levels in coronary and peripheral blood of coronary heart disease.

In the present study, the DCR-3 concentration levels were investigated in patients with coronary heart disease. The efficacy and molecular mechanism of DCR-3 were evaluated in mice with coronary heart disease. Inflammation and apoptosis of myocardial cells were investigated in mice with coronary heart disease following treatment with DCR-3. The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway was also analyzed in DCR-3-treated myocardial cells.

**Materials and methods**

*Ethics statement*. A total of 242 patients (age, 27-56 years; female: male, 1:1.1) with coronary heart disease and 103 healthy volunteers age (28-63 years; female: male, 1:1.1) were recruited from Department of Cardiology, Zhujiang Hospital, Southern Medical University (Guangzhou, China) between January and May 2016 for analyzing serum levels of DCR-3. The patients were classified into four groups. Health group contained participants without angina. Primary group participants who only suffered angina during intense, rapid or prolonged physical activity or exercise. Moderate group were slightly limited in daily activities, as angina occurred following brisk walking, going upstairs, walking in cold air or after mood swings. Severe group were under great limitation and suffered angina after even mild exercise. The present study was approved by the Ethics Committee of Southern Medical University (Guangzhou, China). All patients provided written informed consent prior to experiments. Primary, moderate and severe coronary heart disease was diagnosed using 2014 ACC/AHA Recommendations for the management of NSTE ACS (22).

*Cell culture*. Cardiac fibroblasts were harvested from C57BL/6J mice as described previously (23). A total of 50 female C57BL/6J (age, 8 weeks; weight, 28-32 g) mice were purchased from OrientBio, Inc. (Seongnam, Korea). All mice were given free access to food and water, and housed at 23°C with 50% humidity and a 12-h artificial light/dark cycle. Briefly, mice were sacrificed under 20 mg/kg subcutaneous meperidine anesthesia (24). Harvested hearts were washed with PBS and heart tissues were digested with digestion buffer prepared with 0.05% (w/v) collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 0.06% (w/v) pancreatin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in PBS for 10 min at 37°C. The supernatant was collected and filtrated with a 70-µm filter and collected cells were centrifuged at 100 x g for 4 min at 25°C and resuspended in culture medium; RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) with penicillin-streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C for 12 h. Following washing out digestion buffer with RPMI-1640 medium twice, cells were seeded on cell culture dishes and incubated for 1 h at 37°C. Following incubation, unattached cells were removed and attached cells were cultured with fresh culture medium supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA). Cells were cultured in a 5% CO₂ incubator with a humidified atmosphere at 37°C.

*RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was extracted from cardiac fibroblasts using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.). Quality of extracted mRNA was identified by Spectroscopy Elemental Isotope Analysis using NanoDrop Lite (Thermo Fisher Scientific, Inc.). According to the manufacturer's descriptions, total RNA (1 µg) was reverse-transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). The qPCR reaction was performed with a SYBR-Green detection system (Bio SYBR Green Master Mix; Takara Biotechnology Co., Ltd., Dalian, China). The following primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc.: DCR-3, forward 5'-CTCTTC CTCCCCATGACAC-3' and reverse 5'-CTGGAAAGCCCCAACAAAGTC-3'; and β-actin, forward 5'-CTGGAAAGATG ACCCAGATCA-3' and reverse 5'-CAGGCTGATGGCCTA CGTACA-3'. PCR cycling conditions were performed at 95°C for 30 sec and 45 cycles of 95°C for 5 sec, 56.5°C for 10 sec and 72°C for 10 sec. Relative mRNA expression changes were calculated by the 2^-ΔΔCt method (25). The results are expressed as a fold-change compared with the β-actin control.

*Small interfering RNA (siRNA) transfection*. siRNA to target PI3K (Si-PI3K) and scrambled siRNA (Si-vector) were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences were as follows: Si-PI3K, forward 5'-CCACAACACACGAGAACAAdTdT-3' and reverse 5'-UUUGUUCAGCUUUUGGUdTdT-3'; and Si-vector, forward 5'-UGAAGCGAUAUUGCUAAdTdT-3' and reverse 5'-UUAGGCCAUCCGCUAUdTdT-3'. Cardiac fibroblasts isolated from experimental mice were seeded in 24-well plates and were cultured to 80% confluence. Then 1.25 µl 25 µM Si-PI3K or Si-vector were transfected to cells using Lipofectamine™ RNAi MAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Si-PI3K and Si-vector were from Shanghai GenePharma Co., Ltd. (Shanghai, China). The interval between transfection and subsequent experiments was 24 h.
Syntax score. Syntax score of patients with coronary heart disease were calculated according to a previous study (26). The DCR-3 expression and syntax score were analyzed in patients with coronary heart disease with healthy volunteers as controls.

ELISA. In the protein detection assay, serum levels of DCR-3 were measured using an ELISA kit (DY142; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The operating steps were conducted according to the manufacturer’s instructions. The final results were recorded at 450 nm on an ELISA plate reader.

Western blotting. Myocardial cells harvested from mice were lysed in radioimmunoprecipitation assay buffer containing a phosphatase inhibitor and the protease inhibitor cocktail (Pierce; Thermo Fisher Scientific, Inc.). Protein concentrations were determined via bicinchoninic protein assay (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of proteins (40 µg/lane) were loaded and separated by 12% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked for 1 h at 37°C with 10% bovine serum albumin (cat. no. 10735108001; Roche Applied Science, Penzburg, Germany). The following rabbit anti-mouse antibodies were used to incubate protein for 12 h at 4°C: DCR-3 (1:1,000, cat. no. ab8405), cardiac troponin (cTnT) (1:1,000; cat. no. ab45932), cTnI (1:1,000; cat. no. ab210798), IL-6 (1:1,000; cat. no. ab83053), C-reactive protein 1 (CRP-1) (1:1,000; cat. no. ab211631), albumin (1:1,000; cat. no. ab207327), intercellular adhesion molecule (ICAM)-1 (1:1,000; cat. no. ab119817), vascular cell adhesion molecule (VCAM)-1 (1:1,000; cat. no. ab134047), P13K (1:1,000; cat. no. ab40776), AKT (1:1,000; cat. no. ab8805), phosphorylated (p)AKT (1:1,000; cat. no. ab18283) and β-actin (1:2,000; cat. no. ab8226; all Abcam, Cambridge, UK). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:5,000; cat. no. HAF019; Bio-Rad Laboratories, Inc.) was used as a secondary antibody for 2 h at 37°C and protein was detected using a western blotting Luminol reagent (cat. no. 12015218001; Sigma-Aldrich; Merck KGaA) or PBS (Control). The treatments were repeated once daily for 10 days. Following 3 weeks, animals were sacrificed by cervical dislocation under anesthesia with 3% isoflurane (Sigma-Aldrich; Merck KGaA). The present study has been approved by the Committee for Experimental Animal Studies of Southern Medical University.

Histological assay. Cardiac slices isolated from mice with cardiac fibrosis were prepared and fixed in 4% paraformaldehyde for 30 min at 37°C. Cardiac slices were performed using an avidin-biotin-peroxidase technique. Paraffin-embedded tissue sections were prepared, heated at 65°C to melt the paraffin and dewaxed to water with xylene, ethyl alcohol (100, 95, 85 and 75%) and water successively. Epitope retrieval was performed for further analysis. The paraffin sections (4 µm) were incubated with hydrogen peroxide (3%) for 10 min, and were blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 10 min at 37°C. Finally, the sections were incubated with rabbit anti-mouse anti-DCR-3 (cat. no. ab8405; Abcam) diluted with PBS (1:1,000) or anti-major histocompatibility complex I antibodies (cat. no. ab185706; Abcam) diluted with PBS (1:1,000), for 12 h at 4°C following blocking with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA). Following rinsing, sections were incubated in the presence of a biotinylated horse anti-rabbit antibody (cat. no. a0545; 1:500; Chemicon; Merck KGaA) for 2 h at 37°C. Sections were washed and observed (magnification, x10) using fluorescent video microscopy (BZ-9000; Keyence Corporation, Osaka, Japan).

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. The TUNEL assay was used for the analysis of apoptosis of myocardial tissue in experimental mice at 37°C following simvastatin treatment (10 mg/kg/day; Sigma-Aldrich; Merck KGaA) or an equivalent dose of PBS. Procedures were performed as previously described (27). Briefly, 4-µm tissue sections were fixed with 4% paraformaldehyde at 37°C followed by permeabilization with 0.1% Triton X-100 for 1 h at 37°C. Subsequently, tissue sections or myocardial cells (1x10⁵) were stained with TUNEL reaction mixture (Sigma-Aldrich; Merck KGaA) at 37°C for 2 h. Tissue sections and myocardial cells were washed 3 times in TBS-Tween-20. TUNEL assays were conducted using a TUNEL fluorescence FITC kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s instructions. Samples were mounted with neutral gum. Images in 6 fields of view (magnification, x400) were captured using a Zeiss LSM 510 confocal microscope (Zeiss AG, Oberkochen, Germany) at a wavelength of 488 nm.
Statistical analysis. Results are expressed as the mean ± standard deviation. All data were analyzed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). Comparisons between two groups and among multiple groups were conducted by Student’s t-test and one-way analysis of variance followed by Tukey’s post hoc test, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

DCR-3 expression levels in myocardial cells of patients with coronary heart disease. In the present study, the plasma concentration levels of DCR-3 in patients with coronary heart disease were analyzed. As presented in Fig. 1A, it was observed that plasma concentration levels of DCR-3 were downregulated in peripheral blood with the severity of the disease increasing, as determined by ELISA. In addition, it was also demonstrated that mRNA and protein expression in myocardial cells were decreased with increasing disease severity, as determined by RT-qPCR and western blotting, respectively (Fig. 1B and C). Furthermore, circulating DCR-3 levels were associated with the Syntax score and severity of coronary heart disease, which was demonstrated by the increase in SXscore along with more serious disease condition (Fig. 1D). Taken together, these results suggest DCR-3 expression levels can predict the events and severity of coronary heart disease.

DCR-3 has benefits for the treatment of mice with coronary heart disease by improvement of coronary lesions. To investigate the efficacy of DCR-3 for coronary heart disease, the therapeutic effects of DCR-3 were analyzed in mice with coronary heart disease. By analyzing the DCR-3 expression in myocardial cells, it was observed that DCR-3 expression in myocardial cells was analyzed. It was observed that DCR-3 expression levels were downregulated in myocardial cells isolated from mice with coronary heart disease (Fig. 2A). It was also observed that plasma concentration levels were downregulated in the coronary heart disease mice (Fig. 2B). In addition, DCR-3 treatment affected the lesions and inhibited the apoptosis of arterial vascular smooth muscle, as determined by histological analysis (Fig. 2C). Furthermore, results indicated that myocardial expression levels of injury-associated proteins cTnT and cTn I were upregulated in myocardium after treatment with DCR-3 (Fig. 2D). Taken together, these results suggest that DCR-3 is beneficial for the remission of coronary heart disease induced by TGF-β1 through improvement of coronary lesions.

DCR-3 inhibits expression levels of inflammatory factors in myocardial cells. A previous study indicated that inflammatory responses are associated with the progression of coronary heart disease (28). Therefore, the inflammatory factor expression was analyzed in myocardial cells and tissues. As presented in Fig. 3A-E, following treatment with DCR-3, IL-6, CRP-1, serum albumin, ICAM-1 and VCAM-1 were all decreased in myocardial cells treated with DCR-3 (Fig. 2D). Taken together, these results suggest that DCR-3 is beneficial for the remission of coronary heart disease induced by TGF-β1 through improvement of coronary lesions.
with DCR-3 (Fig. 3G). Collectively, these results suggest that DCR-3 can inhibit expression levels of inflammatory factors in myocardial cells in experimental mice with coronary heart disease.

**DCR-3 improves inflammatory and apoptosis in myocardial cells through the PI3K/AKT signaling pathway.** It has been demonstrated previously that pretreatment of inflammatory factors by activating the PI3K/AKT signaling pathway contributes to recovery of coronary heart disease-induced myocardial ischemia and injury (29). In the present study, the PI3K/AKT signaling pathway in myocardial cells was analyzed in experimental mice with coronary heart disease treated by DCR-3. As presented in Fig. 4A and B, DCR-3 in myocardial cells upregulated the expression levels of PI3K and p-AKT/AKT, compared with the control group. The results in Fig. 4C demonstrated that endogenous inhibition of PI3K expression by Si-PI3K was successfully achieved in myocardial cells. In vitro assays demonstrated that endogenous inhibition of PI3K expression by Si-PI3K suppressed AKT expression in myocardial cells (Fig. 4D). Furthermore, representative histological images revealed that Si-PI3K also markedly inhibited improvement of DCR-3-mediated apoptosis in myocardial cells, as determined by TUNEL and immunofluorescence (Fig. 4E and F). These findings suggest that PI3K/AKT signaling pathway involves the anti-inflammatory and anti-apoptosis potential of DCR-3 in myocardial cells in the progression of coronary heart disease induced by TGF-β1.

**DCR-3 prolongs survival of mice with coronary heart disease.** The efficacy for the survival of mice with coronary heart disease following treatment with DCR-3 was then determined. As presented in Fig. 5, DCR-3 significantly prolonged the survival of mice with coronary heart disease induced by TGF-β1 in a 90-day observation (n=20 in each group). Survival analysis suggests that DCR-3 may be an efficient agent for the treatment of coronary heart disease.

**Discussion**

DCR-3 acts as a soluble receptor of Fas ligand (FasL), TNF superfamily member 14 (LIGHT) and TNF superfamily member 15 that is reported highly expressed in cancer cells, but is expressed less in myocardial cells (30). A previous study has indicated that expression of DCR-3 is notable in chronic liver disease (31). However, the significance of expression of DCR-3...
in myocardial cells in progression of coronary heart disease has not been reported in a previous study (32). The present study investigated the molecular mechanism of DCR-3 in myocardial cells in the progression of coronary heart disease. Although previous reports have reported that plasma DCR-3 levels may indicate the severity of coronary heart disease (33),
that apoptosis of leukocytes acts as a disease marker of neutrophil-endotheliocyte interaction in patients with coronary heart disease (14). Liu et al (15) have previously investigated the expression levels of inflammatory and apoptosis factors in coronary stent implantation in coronary heart disease patients, and demonstrated that downregulation of inflammatory and apoptosis factors is beneficial to the recovery of coronary heart disease. DCR-3, as a pleiotropic immunomodulator for enhancing angiogenesis, has been observed to downregulate cell apoptosis by binding with its receptor of FasL and LIGHT, and subsequently improved survival of many types of cell, such as human IPF fibroblasts (44) and mouse lymphocytes (45). Furthermore, DCR-3 also suppressed Fasl-induced apoptosis via extracellular signal-regulated kinase 1/2 activation in various cancer cells (46-48). Furthermore, a number of studies have reported DCR-3 function in lymphocytes and dendritic cells (45,49). The present study indicated that DCR-3 could inhibit apoptosis of myocardial cells in mice with coronary heart disease, which contributed to protect myocardial cells against injury in the process of coronary heart disease. Notably, injury-associated protein levels of cTnT and cTn I were markedly upregulated in myocardium by the treatment of DCR-3. These results suggest that inhibition of apoptosis is the essential function of DCR-3 for the treatment of coronary heart disease. However, the present study did not use a healthy or sham control to identify the role of DCR-3 for the treatment of coronary heart disease, which is a limitation of this research.

The PI3K/AKT signaling pathway is involved in the progression of myocardial infarction in the process of coronary heart disease (50). Hu et al (51) presented the protective effect of proanthocyanidins on anoxia-reoxygenation injury of myocardial cells and results have indicated that the PI3K/AKT/glycogen synthase kinase-3β pathway mediates mitochondrial ATP-sensitive potassium channel to regulate oxidative stress. It has also been indicated that the PI3K/AKT pathway-regulated recruiting T cells could attenuate myocardial ischemia-reperfusion injury in a mice model (52). In the present study, by investigating the association between DCR-3 and the PI3K/AKT signal pathway in myocardial cells in mice with coronary heart disease, it was observed that DCR-3 mediated the improvement of myocardial ischemia via the PI3K/AKT signaling pathway. Notably, it also demonstrated that endogenous inhibition of PI3K expression by Si-PI3K abolished DCR-3-mediated anti-inflammation and anti-apoptosis in myocardial cells in mice with coronary heart disease.

In conclusion, the present study investigated the efficacy of DCR-3 in the progression of coronary heart disease and the molecular mechanism of DCR-3-mediated anti-inflammation and anti-apoptosis in myocardial cells in mice with coronary heart disease. Results have demonstrated that patients and mice with coronary heart disease have lower DCR-3 expression levels in peripheral blood. DCR-3 can inhibit inflammatory factor expression and apoptosis of myocardial cells and that the PI3K/AKT signaling pathway is associated with anti-inflammation and anti-apoptosis in the process of coronary heart disease. These results confirm that DCR-3 treatment may be beneficial for the recovery of coronary heart disease by anti-inflammation and anti-apoptosis in myocardial cells through the PI3K/AKT signaling pathway, suggesting that DCR-3 may be able to predict the extent and severity of cardiovascular diseases.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XJC, RHW and WC designed the experiments. XJC and RHW performed the experiments and analysis. RHW and LL helped analyze the data. RHW, WC and ZLL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee and the Committee for Experimental Animal Studies of Southern Medical University (Guangzhou, China). All patients provided written informed consent. The present study was approved by the Ethics Committee and the Committee for Experimental Animal Studies of Southern Medical University (Guangzhou, China). All patients provided written informed consent.

Patient consent for publication

All patients provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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