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TNFα-induced downregulation of microRNA-186 contributes to apoptosis in rat primary cardiomyocytes

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ABSTRACT

Progressive loss of cardiac cardiomyocytes is involved in pathogenesis of heart failure. Inflammation is considered as a major risk factor that triggers cardiomyocytes apoptosis or induces cellular damage. Proinflammatory cytokines such as TNFα can directly activate cell apoptosis or promote oxidant production that damages cellular structure eventually. We investigated TNFα mediated apoptosis in cultured rat primary cardiomyocytes. Annexin V/PI staining and apoptosis biomarker expression were used to examine cardiomyocytes cell apoptosis response. We also identified key microRNA that plays a regulatory role in this pathway with genetic and biochemical approaches. Apoptosis Inducing Factor (AIF) expression was found to be upregulated with 10 μg/ml or 50 μg/ml TNFα stimulation for 24 h, which was associated with apoptotic index. Subsequently, miR-186 was identified as direct regulator of AIF in TNFα mediated cardiomyocytes apoptosis from microRNA expression profiling. miR-186 level was downregulated with TNFα treatment that was correlated with AIF induction. Last, in the rescue experiment, miR-186 mimic protected cardiomyocytes against TNFα mediated apoptosis. Collectively, the results suggest TNFα-induced AIF upregulation contributes to apoptosis in rat primary cardiomyocytes through regulating miR-186 expression, which implies miR-186 could be a potential therapeutic target for preventing inflammation associated cardiac damage.

1. Introduction

Cardiac failure is the major cause of human pathological death globally. The pathogenesis of heart failure involves the progressive loss of cardiac cardiomyocytes, which suggests apoptosis as an important mode leading to cell death during heart failure (Haunstetter and Izumo, 1998; Kang and Izumo, 2000; Narula et al., 1996). Inflammation is one of the major risk factors underlining cardiovascular disease. Inflammation causes over-production of proinflammatory cytokines such as TNFα that can directly activate cell apoptosis pathway or that leads to oxidant production to cause tissue damage (Finkel, 2003; Moe et al., 2004).

Studies with TNFα have revealed its important role in apoptosis associated cardiac pathologies such as cardiac hypertrophy and cardiomyopathy (Al-Shudiefat et al., 2013; Lin et al., 2010; Shanmugam et al., 2016). Over-expression of TNFα in mouse model reduced Bcl-2 (anti-apoptotic protein) expression and triggered the downstream caspase cascade, leading to intrinsic apoptotic pathway activation, that eventually contribute to adverse cardiac remodeling in the adult heart (Engel et al., 2004). Clinical study demonstrated that inhibition of TNFα pathway could be effective approach for anti-inflammatory associated with antioxidant benefits (Moe et al., 2004; Sugano et al., 2002). Therefore identifying the downstream effectors or other regulators for TNFα induced apoptosis will inspire new therapeutic strategy, in order to reduce cardiovascular incidents due to inflammation or oxidative stress induced apoptosis. AIF is apoptotic effector protein residing in mitochondria membrane. In response to apoptotic stimulus, AIF translocates from the mitochondria into the nucleus to induce nuclear condensation and DNA fragmentation that resemble key cellular features during cell apoptosis (Daugas et al., 2000). Recent study has revealed AIF translocation and activation are involved in aldosterone induced cardiomyocyte apoptosis that play a part in ALD induced cardiomyocyte injury (Xiao et al., 2013).

MicroRNAs are sequence specific regulators for gene expression and were first discovered in worms in 1990s (Lee et al., 1993). They usually contain 21–25 nucleotides, and fine-tune the expres-

Abbreviations: AIF, apoptosis inducing factor.

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sion of thousands of target mRNAs, with each mRNA targeted by multiple microRNAs. Most microRNAs bind to the complementary sites in 3′-untranslated regions (UTRs) of target mRNAs to suppress gene expression, by either directing mRNAs degradation or protein translation repression (Pillai, 2005). During the last 20 years, microRNAs have been proposed as key regulators in various biological processes, including cell growth, differentiation and apoptosis (Bartel, 2004). Dysregulation of microRNAs in cells may lead to cancerous phenotype (Farazi et al., 2013) or be associated with inflammatory and autoimmune diseases (Dai and Ahmed, 2011). The role of microRNA regulation in cardiovascular diseases is still unclear. Therefore, it will provide more insight for managing cardiovascular diseases by characterizing microRNA candidates involved in cardiomyocytes apoptosis pathway.

In this study, we established a system to study the TNFα mediated apoptosis in rat primary cardiomyocytes. With this approach we established AIF as an apoptotic effector induced by TNFα to cause cell death in cardiomyocytes. We further identified miR-186 as key regulator in this axis. Finally the *in vitro* rescue experiment supported the hypothesis that miR-186 was a potential therapeutic target to confer cardiomyocytes the protection against TNFα mediated apoptosis.

2. Methods

2.1. Materials and cell culture

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and trypsin were supplied by Gibco (Thermo Fisher, Waltham, MA, USA). Fetal bovine serum (FBS) was supplied from Hyclone (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Recombinant TNFα (cat no. PHC3016) for treatment was obtained from Thermo Fisher. Rabbit monoclonal Anti-AIF antibody [E20] was purchased from Abcam, Cambridge, MA, USA. Both miR-186 mimic and inhibitor were customized by Exiqon. Primary culture of neonatal rat cardiomyocytes was based on the method described previously (Franke et al., 2007).

2.2. NFα treatment

Cardiomyocytes were seeded with 5,000 cells/cm^2 on Day 1. On Day 3, cells were washed with PBS and cultured with serum free medium. After over-night incubation, the cells were stimulated with TNFα in respective concentration for 24 h and then harvested for different assay measurements.

2.3. RT-PCR analysis

Total RNA was extracted from 1×10^6 cells using TRIzol solution (Invitrogen, Carlsbad, CA, USA). 1 μg of total RNA was reverse transcribed into cDNA for 1 h at 50 °C using oligo dT primer and reverse transcriptase in the presence of RNAse inhibitor. Transcribed cDNA template (50 ng) was incubated with 200 nM AIF primers or GAPDH primers (normalization control) in a total volume of 20 μl using KAPA SYBR FAST qPCR kit. Primers used in the study were: AIF forward 5′-CTA TAG GGA GAT CCA GGC AAC TTG-3′, reverse 5′-TAT AGG GAG ACC TCT GCT CCA GCC-3′; GAPDH forward 5′-CGT CTT CAC CAC CAT GGA GAA GGC-3′, reverse 5′-AAG GCC ATG CCA GTG AGC TTC CC-3′.

2.4. Western blot analysis

After treatment, the cells were washed once in PBS and harvested in RIPA lysis buffer. The protein concentration was measure with protein BCA protein assay. 40 μg protein lysate was prepared in SDS sample buffer and separated on an 8–12% SDS-polyacrylamide gel and then transferred to a PVDF membrane. Membranes were washed in TBS-T and then blocked with 5% non-fat milk in TBS-T for 1 h. Membranes are then incubated with indicated primary antibody in 5% non-fat milk prepared for overnight at 4 °C. After washed with TBS-T three times for 10 min each, the membranes were incubated with the corresponding secondary antibodies coupled to HRP (Horse Radish Peroxidase) for 1 h at room temperature. After washed with TBS-T three times for 10 min each, western signals were visualized using Western Pico Super ECL reagent (Pierce, WI, USA). The results shown here were the representatives of at least three times of independent experiments.

2.5. MicroRNA microarray

MicroRNA microarray was customized by Agilent Technologies. Briefly oligos probes for miR-17-5p, miR-20b-5p, miR-106b, miR-75B, miR-455, miR-186, miR-375, miR-20a, miR-93, miR-33, miR-291a-3p, miR-399-5p, miR-1281 and miR-370 were designed and synthesized on SurePrint custom MicroRNA microarray platform. About 100 ng RNAs from each sample were dephosphorylated, labeled with Cyanine 3-pCP and desalted before the hybrodozation (20 h, 55 °C). The MicroRNA expression profile was acquired with Agilent SureScan microarray scanner.

2.6. Luciferase assay

Luciferase reporter gene vectors were transfected into rat primary cardiomyocytes in 6-well plate with TurboFect transfection reagent (Fermentas). Renilla luciferase gene was also co-transfected for all experiments as internal control. After 48 h, the cells were harvested and the luciferase activities were subsequently measured with Dual-Luciferase Reporter assay kit (Promega, Madison, WI, USA). Luciferase activities were normalized to the cell number.

2.7. Flow cytometry analysis

Cardiomyocyte apoptosis was quantified by a flow cytometer. Annexin V/Propidium iodide (PI) Apoptosis Detection Kit for flow cytometry was used to detect the proportion of apoptotic cells. Only those counts showing both Annexin V and PI positive were considered dead cells.

2.8. Statistical analysis

All experimental data were presented as mean ± standard deviation. Experiments were repeated three times. Statistical differences were analyzed by student *t*-test. A P value <0.05 was considered significant.

3. Results

3.1. Dosage effect of TNFα on primary cardiomyocytes

TNFα is a prominent proinflammatory factor that can induce cardiomyocytes apoptosis during cardiovascular inflammation (Bajaj and Sharma, 2006; Nian et al., 2004). In order to elucidate the molecular mechanism that regulates this process, we first sought to measure the dosage response of rat primary cardiomyocytes to TNFα in cell culture. Primary cardiomyocytes were treated with different concentrations of TNFα (2, 10 or 50 ng/ml) for 24 h. Their apoptotic responses were then measured by annexin V-FITC/PI staining. Cells stained with both annexin V-FITC and PI were considered apoptotic cells. As shown in Fig. 1 (A&B), 24 h treatment of TNFα at doses of 10 ng/ml and 50 ng/ml significantly induced