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MicroRNA-17-mediated down-regulation of apoptotic protease activating factor 1 attenuates apoptosome formation and subsequent apoptosis of cardiomyocytes

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ABSTRACT

Heart diseases such as myocardial infarction (MI) can damage individual cardiomyocytes, leading to the activation of cell death programs. The most scrutinized type of cell death in the heart is apoptosis, and one of the key events during the propagation of apoptotic signaling is the formation of apoptosomes, which relay apoptotic signals by activating caspase-9. As one of the major components of apoptosomes, apoptotic protease activating factor 1 (Apaf-1) facilitates the formation of apoptosomes containing cytochrome c (Cyto-c) and deoxyadenosine triphosphate (dATP). Thus, it may be possible to suppress the activation of the apoptotic program by down-regulating the expression of Apaf-1 using miRNAs. To validate this hypothesis, we selected a number of candidate miRNAs that were expected to target Apaf-1 based on miRNA target prediction databases. Among these candidate miRNAs, we empirically identified miR-17 as a novel Apaf-1-targeting miRNA. The delivery of exogenous miR-17 suppressed Apaf-1 expression and consequently attenuated formation of the apoptosome complex containing caspase-9, as demonstrated by co-immunoprecipitation and immunocytochemistry. Furthermore, miR-17 suppressed the cleavage of procaspase-9 and the subsequent activation of caspase-3, which is downstream of activated caspase-9. Cell viability tests also indicated that miR-17 pretreatment significantly prevented the norepinephrine-induced apoptosis of cardiomyocytes, suggesting that down-regulation of apoptosome formation may be an effective strategy to prevent cellular apoptosis. These results demonstrate the potential of miR-17 as an effective anti-apoptotic agent.

1. Introduction

Heart diseases such as myocardial infarction (MI) have been the leading cause of death worldwide for many decades [1]. In extremely harsh conditions, such as myocardial ischemia (in which the myocardium is deprived of nutrients and oxygen), individual cardiomyocytes undergo cell death through various mechanisms [2]. Among the different types of cardiomyocyte death, apoptosis is characterized by plasma membrane blebbing, chromatin condensation, nuclear fragmentation, and apoptotic body formation [3]. Apoptosis can be initiated by signals from outside the cell (the extrinsic or death receptor pathway) or from inside the cell (the intrinsic or mitochondrial pathway), and both pathways lead to the

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activation of cysteine proteases called caspases [4]. One of the pivotal events of the intrinsic pathway is the formation of the apoptosome.

Damage to mitochondria causes permeabilization of the outer mitochondrial membrane, which compromises membrane integrity [5]. Compromised membrane integrity, in turn, promotes the release of cytochrome c (Cyto-c) from the mitochondria. This released Cyto-c then binds to apoptotic protease activating factor 1 (Apaf-1) to initiate apoptosome formation [6]. In the absence of Cyto-c, the folded WD-40 domain of Apaf-1 keeps Apaf-1 in its inhibited monomeric state [7]. However, in the presence of Cyto-c and deoxyadenosine triphosphate (dATP), the Cyto-c-bound WD-40 domain is no longer folded, and the interaction between Apaf-1 and dATP exposes the caspase recruitment domain (CARD) [8]. The binding of dATP also enables the oligomerization of Apaf-1 into an Apaf-1 heptamer that facilitates downstream caspase activation [9]. Exposed CARDs in the Apaf-1 heptamer interact with the initiator caspase procaspase-9, and as a result, an apoptosome holoenzyme is formed [10]. Once the apoptosome is formed, procaspase-9 is activated by cleavage, and this step further activates executioner caspases such as caspase-3, triggering caspase-dependent apoptotic signaling cascades [11]. The critical role of the apoptosome in mitochondria-dependent apoptosis has made the apoptosome a potent therapeutic target for the prevention of cardiomyocyte apoptosis [12]. In the present study, we attempted to inhibit apoptosome formation by targeting Apaf-1 with microRNAs (miRNAs) in cardiomyocytes exposed to norepinephrine (NE), which is known to induce cardiac cell apoptosis [13,14].

miRNAs are short, non-coding RNAs that prevent the translation of or induce the degradation of target messenger RNAs (mRNAs) by binding to their 3′ untranslated regions (UTRs) [15]. Because miRNAs are known to be involved in a broad range of biological processes, Apaf-1 could be regulated by miRNAs. In fact, Apaf-1 has been reported to be targeted by a number of miRNAs, including miR-23a/b, miR-27a/b, and miR-24a [16,17]. Nevertheless, the multi-targeting nature of miRNAs strongly suggests that there may be additional Apaf-1-targeting miRNAs. Therefore, in the present study, we screened for Apaf-1-targeting miRNAs based on both miRNA target prediction databases and empirical data. After the selection of an Apaf-1-targeting miRNA, we further investigated whether this selected miRNA could prevent the apoptosis of cardiomyocytes *in vitro*.

2. Materials and methods

2.1. Isolation of rat cardiomyocytes

All experimental procedures for these animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine and were performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Neonatal rat cardiomyocytes from 1- to 2-day-old Sprague Dawley rat pups were isolated. Briefly, the hearts were minced into small pieces (~1 mm^3) and washed with Dulbecco's phosphate-buffered saline solution (DPBS, pH 7.4, without Ca^{2+} and Mg^{2+}). The tissues were digested with 5 ml of collagenase II (0.8 mg/ml, 262 units/mg) for 5 min at 37 °C. The cells were transferred to a new tube containing cell culture medium (a-MEM, containing 10% fetal bovine serum, Gibco BRL) and centrifuged at 1600 rpm for 3 min at room temperature (RT). The cell pellets were re-suspended in 5 ml of cell culture medium. The processes described above were repeated 7–9 times until most of the tissue was removed. The cell suspensions were collected and incubated in 100-mm tissue culture dishes for 2 h to reduce fibroblast contamination. The non-adherent cells were collected and seeded. The cells were treated with 0.1 μM 5-bromo-20-deoxyuridine (BrdU, Sigma–Aldrich) to increase the purity of the cardiomyocytes. The cells were cultured with 10% FBS α-MEM in a 5% CO_2 incubator at 37 °C. To induce cardiomyocyte apoptosis, the cells were treated with 100 μM NE for 24 h [14].

2.2. Immunoprecipitation (IP)

For IP, cells were lysed in IP lysis buffer (Thermo, Waltham, MA, USA) for 30 min on ice. The cell lysates were centrifuged at 10,000 g for 20 min, and the supernatant was retained. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo). After quantification, the lysates were incubated with an Apaf-1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at 4 °C with rotation. After 1 h, Protein G Dynabeads (Life Technologies, Carlsbad, CA, USA) were added to the lysates, which were incubated overnight at 4 °C. The Dynabeads-antibody-Apaf-1 complexes were centrifuged at 2500 g for 30 min at 4 °C, and the antibody-Apaf-1 complexes were eluted using an elution buffer containing 0.2 M Tris (pH 6.8), 2% SDS, 0.04% Coomassie blue, 40% glycerol and β-mercaptoethanol. The eluted proteins were subjected to Western blotting using primary antibodies specific for Apaf-1 (Santa Cruz Biotechnology) and caspase-9 (Sigma, St. Louis, MO, USA).

2.3. Immunocytochemistry

Cells were cultured in four-well slide chambers, washed twice with PBS, and fixed in 1% paraformaldehyde solution for 10 min. The cells were then washed twice with PBS before permeabilization using 0.1% Triton X-100 for 10 min. Next, the cells were blocked for 1 h in blocking solution (2% bovine serum albumin and 10% horse serum in PBS) and incubated with an Apaf-1 antibody (Santa Cruz Biotechnology) and Cyto-c antibody (Santa Cruz Biotechnology). FITC-conjugated mouse, rabbit, and goat (Jackson ImmunoResearch Laboratories) secondary antibodies were then used. Immunofluorescence was detected by confocal microscopy (LSM710; Carl Zeiss).

2.4. Delivery of microRNA

Transfection with miRNA mimics was performed using the TransIT-X2 system (Mirus Bio LLC, Madison, WI, USA). Mature miRNA mimics and miRNA inhibitors (Genolution Pharmaceuticals, Inc., Korea) were used at final concentrations of 100 nM and 50 nM, respectively. After 4 h of incubation in a CO_2 incubator at 37 °C, the medium was replaced with fresh medium.

2.5. Cell viability assay

To measure cell viability, Cell Counting Kit-8 reagent (CCK-8, Dojindo) was added to each well to a final concentration of 0.5 mg/mL, and the cells were incubated at 37 °C for 2 h. The absorbance at 450 nm was measured using a microplate reader.

2.6. Luciferase reporter assay

The 3′UTR sequence of Apaf-1 was amplified using Apaf-1 primers. The primer sequences were as follows: Apaf-1 sense, 5′-CTA GCT AGC AAA TGA GAG CTC ATT GCG TTA TGC-3′; and antisense, 5′-CCG CTC GAG GGC AAG CGC TCT ACC ACT AGC TA-3'. The 3′UTR fragment containing the miR-17 binding site was then cloned into the pmirGLO vector. Chinese hamster ovary (CHO) cells were plated at a density of 1×10^5 cells/well in a 12-well plate and then transfected with either pmirGLO control vector or pmirGLO vector with Apaf-1 3′UTR using Lipofectamine 2000. After 48 h, the