Osteoarthritis and Cartilage 25 (2017) 600–610

Osteoarthritis and Cartilage

Hydroxytyrosol modulates the levels of microRNA-9 and its target sirtuin-1 thereby counteracting oxidative stress-induced chondrocyte death

S. D'Adamo † ‡, S. Cetrullo †, S. Guidotti ‡ §, R.M. Borzì §, F. Flamigni † *

† *Dipartimento di Scienze Biomediche e Neuromotorie, Università di Bologna, Bologna, Italy*
‡ *Dipartimento di Scienze Mediche e Chirurgiche, Università di Bologna, Bologna, Italy*
§ *Laboratorio di Immunoreumatologia e Rigenerazione Tissutale, Istituto Ortopedico Rizzoli, Bologna, Italy*

ARTICLE INFO

Article history:
Received 19 July 2016
Accepted 25 November 2016

Keywords:
Oxidative stress
Hydroxytyrosol
microRNA
Cartilage
Chondrocyte

SUMMARY

Objective: Nutraceutical compounds, such as hydroxytyrosol (HT), have been found to exert protective effects in osteoarthritis (OA) by affecting a variety of key molecular and cellular processes in chondrocytes. However, to our knowledge, no relationship has been reported between nutraceuticals and microRNA (miR) network in OA models. Here, we identified a miR that is implicated in HT-mediated chondroprotection following oxidative stress condition by targeting sirtuin-1 (SIRT-1).
Methods: Human primary and C-28/I2 chondrocytes were pre-treated with 100 μM HT 30 min before 100 μM H_2O_2 addition. *In silico* analyses were exploited to select putative candidate miRs able to target SIRT-1 mRNA. Luciferase-based gene reporter assay was employed to demonstrate the direct link between miR-9 and its putative mRNA target. Transient transfection approach was performed to examine the effects of miR-9 levels on caspase activity, cell viability and expression of OA-related genes.
Results: MiR-9 was identified and confirmed as a post-transcriptional regulator of SIRT-1. MiR-9 and SIRT-1 levels showed opposite changes in chondrocytes following H_2O_2 and HT treatment. Moreover mir-9 silencing inhibited cell death induced by H_2O_2 partly through down-regulation of SIRT-1, whereas miR-9 overexpression markedly reduced the protective effect of HT. The manipulation of miR-9 levels also resulted in the modulation of OA-related gene expression, including MMP-13, VEGF and RUNX-2.
Conclusions: These results show that miR-9 is a critical mediator of the deleterious and OA-related effects of oxidative stress in chondrocytes and that modulation of miR expression may be a crucial mechanism underlying the protective action of HT.

Introduction

Current pharmacotherapies for osteoarthritis (OA), a multifactorial degenerative disease[1] are only based on palliative treatments that focus on symptoms, e.g., pain and inflammation, and mainly consist of analgesics and non-steroidal anti-inflammatory drugs (NSAIDs)[2]. Unfortunately they lack efficacy in slowing disease progression and, also, result in several side effects, mostly gastrointestinal and cardiovascular injuries.

MicroRNAs (miRs) are an abundant, evolutionary conserved subfamily of short non-coding RNAs (22–25 nt) that are identified as potent post-transcriptional regulators[3,4]. Recently the great potential of miRs as important regulators of a specific target and/or entire cellular processes has received much attention and also in the field of OA research many investigators are striving to identify the fine crosstalk between deregulated pathways and miRs, thereby discovering new intriguing molecular therapeutical targets[5,6]. Studies on miR deregulation in OA are performed by comparing the expression of these molecules between OA tissue specimens and their normal articular counterpart[7]. Then functional studies allow to shed light on the specific role of a deregulated miR in OA through assessment of the modulation of miR levels and subsequent evaluation of pathological phenotypes in *in vitro* cellular models. In particular miR-140, presently the best characterized miR

* Address correspondence and reprint requests to: F. Flamigni, Dipartimento di Scienze Biomediche e Neuromotorie, Università di Bologna, Via Irnerio 48, 40126, Bologna, Italy.
E-mail address: flavio.flamigni@unibo.it (F. Flamigni).

http://dx.doi.org/10.1016/j.joca.2016.11.014
1063-4584/

implicated in OA, is reduced in OA tissue[6] and targets different factors involved in OA, including HDAC4, a known corepressor of RUNX-2[8], CXCL12, Smad3[9,10] and ADAMTS5, the key aggrecanase in OA development[11]. Moreover, SOX9, the major transcription factor implicated in the differentiation of chondrocyte phenotype and prevention of cellular hypertrophy, can regulate miR-140 levels[12]. Also miR-155, upregulated in OA, can contribute to the autophagy defects in OA by suppression of gene and protein expression of key autophagic regulators[5].

On the other hand several reports suggest that nutraceuticals exert a protective role for degenerative pathologies, including cardiovascular disease, cancer, and OA[13–15]. Some nutraceuticals have shown to exhibit a role not merely as anti-oxidants and ROS scavengers, but also as efficient modulators of gene expression of key factors underlying the OA onset[16,17]. In this regard an interesting candidate molecule is hydroxytyrosol (HT), a phenolic compound endowed with a powerful anti-oxidant action, mainly found in the fruits of olive tree (*Olea europaea* L.) and derivatives, such as olive oil[18–20]. Previously, we showed how HT is able to reduce DNA damage, cell death and the expression of OA markers induced by oxidative stress in chondrocytes[16]. Moreover, we demonstrated that HT mediates the effect of chondroprotection by promoting the autophagy process and cytoplasm-nucleus translocation of sirtuin-1 (SIRT-1), a "longevity factor" and autophagy regulator[21,22].

However to date, few nutraceuticals have been reported as modulators of specific miRs and no one in OA physiopathology. In the present work primary OA and C-28/I2 chondrocytes were employed to address the question whether HT could exert its protective effect against oxidative stress by modulating specific miRs. We show that HT protects from cell death, reduces the expression of some OA markers and rescues protein levels of SIRT-1 by impairing the up-regulation of miR-9 by H_2O_2.

Methods

Cell cultures and treatments

C-28/I2 chondrocytes[23], kindly provided by Dr. Mary Goldring, are a human cell line representative of primary chondrocytes that has been used extensively as a reproducible "*in vitro*" model to study chondrocyte physiopathology in experiments requiring large numbers of cells. With local Ethics Committee approval, primary cultures of chondrocytes were used and prepared from fragments of articular cartilage obtained from adult OA patients undergoing knee arthroplasty. The number of different primary cultures used for each experiment is detailed in Supplementary Table I. The use of the human cells in this study was in accordance with the Helsinki Declaration of 1975. Both C-28/I2 and primary chondrocytes were grown in DMEM medium supplemented with 10% fetal bovine serum as previously detailed[21].

The cells were incubated in the absence or presence of 100 μM H_2O_2 for 4 or 24 h as indicated in the various figures; 100 μM HT (from Cayman Chemical) was added 30 min before H_2O_2, on the basis of previously published studies[16,21]. Control cells received the corresponding volume of vehicle. Viable cells were directly counted following the trypan blue exclusion test. Dead cells including the dye were reported as a percentage of the total number of cells. Caspase activity was measured by the cleavage of the fluorogenic peptide substrate Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) as previously mentioned[24]. Since the sequence DEVD represents a substrate for caspase-3 and other effector caspases, this activity has been referred to as caspase 3-like activity. Caspase activity was expressed per mg protein, and normalized to untreated controls.

Cell transfection

Primary chondrocytes and C-28/I2 cells were seeded in 6-well plates at a density of 2×10^5 cells/well and in 96-well plates at a density of $1{,}28 \times 10^3$ cells/well without antibiotics. The next day, Ambion® Anti-miR™ miRNA Inhibitors, negative control #1 (anti-miR-NC) (50 nM) and antimiR-9 (50 nM), and Ambion® Pre-miR™ miRNA Precursors, negative control #1 (premiR-NC) (50 nM) and premiR-9 (Life Technologies) (50 nM) were transfected into cells by using Lipofectamine RNAiMAX (Invitrogen) for 24 h. Anti-miR miRNA Inhibitors are single stranded nucleic acids designed to specifically bind to and inhibit endogenous miR molecules. Pre-miRNA precursor molecules are small, chemically modified double stranded RNA molecules designed to mimic endogenous mature miRs.

C-28/I2 and primary cells were co-transfected with either antimiR-NC or antimiR-9 and either ON-TARGETplus Human Sirt1 siRNA (25 nM) or ON-TARGETplus non-targeting pool (25 nM) (Dharmacon) by Lipofectamine® 3000 Reagent in Opti-MEM® Medium (Life Technologies) according to manufacturer's instructions. The cells were incubated for 24 h before evaluating cell viability and caspase 3-like activity.

Western blotting

In western blotting procedure equal amounts of cell extract were subjected to electrophoresis in 10% gels, blotted onto nitrocellulose membranes essentially as previously described[25,26], and probed with primary antibody at 4°C overnight. The following antibodies were used: anti-β-actin (A5316, Sigma–Aldrich), anti-SIRT-1 (sc-74465, Santa Cruz Biotechnology). After washes, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (Santa Cruz Biotechnology) IgG for 1 h. The chemiluminescent signals were detected using an ECL system (LuminataTM Crescendo, Millipore). β-actin was used as loading control. A representative image of visualized immunoreactive bands and densitometric analysis show the relative intensity of protein expression.

Bioinformatics prediction of miR candidates targeting SIRT-1

TargetScan and miRWalk databases were used to predict miR candidates targeting SIRT-1 by detecting the complementarity between their seed sequences and specific sequences in SIRT-1 mRNA.

RNA isolation, cDNA synthesis and real-time PCR

Total cellular RNAs were extracted with 700 μl TRIZOL (Invitrogen), according to manufacturer's instructions. The RNA pellets were treated with DNAse (DNA-free, Ambion, Austin, TX) and, after buffer exchange, were quantified by using RiboGreen RNA quantitation reagent (Molecular Probes, Eugene OR). The same quantity of total RNA (100 ng) was reverse transcribed by using random primers and the reagents provided with the Superscript VILO System for RT-PCR (Invitrogen). The cDNA mixture (2 μl) was used in Real time PCR analysis in a LightCycler Instrument (Roche Molecular Biochemicals) by means of the QuantiTect SYBR Green PCR kit (TaKaRa, Japan) with the following protocol: initial activation of HotStart Taq DNA polymerase at 95°C for 10 s, followed by amplification (40 cycles: 95°C for 5 s followed by appropriate annealing temperature for each target as detailed below kept for 20 s). The protocol was concluded by melting curve analysis to check amplicon specificity. The following primers (from Invitrogen) were used: GAPDH (NM_002046) 579–598F and 701–683R; MMP-13 (NM_002427) 496–511F and 772–756R; RUNX-2 variant