Osteoarthritis and Cartilage 25 (2017) 1912–1921

Osteoarthritis and Cartilage

Lentiviral vector-mediated shRNAs targeting a functional isoform of the leptin receptor (Ob-Rb) inhibit cartilage degeneration in a rat model of osteoarthritis

S.L. Deng [†,a], J. Zhu [‡,a], Q. Huang [†,a], W.L. Fu [†], Q. Li [†], G. Chen [†], X. Tang [†,*], J. Li [†]

† Department of Orthopaedics, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China
‡ Respiratory and Thoracic Surgery Ward, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China

ARTICLE INFO

Article history:
Received 4 March 2017
Accepted 8 August 2017

Keywords:
Leptin receptor functional isoform (Ob-Rb)
RNA interference
Lentiviral vector
Chondrocyte
Osteoarthritis
Cartilage

SUMMARY

Objective: To downregulate the expression of leptin receptor functional isoform (Ob-Rb) on chondrocytes using lentiviral vector-mediated short-hairpin RNA (LV-shRNA) and to determine its effects on cartilage degeneration.

Method: *In vitro*, quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting were performed to select an optimal Ob-Rb LV-shRNA (LV-shRNA3) and to determine its effects on nine OA-related mediators in cultured rat chondrocytes. *In vivo*, an OA model was surgically induced in the right knees of rats, and LV-shRNA3, lentiviral vector-mediated non-targeting control sequence (LV-NTC) or phosphate buffered saline was injected into the joints. Osteoarthritis Research Society International (OARSI) scoring was performed to assess cartilage degeneration, and immunohistochemistry was performed to evaluate OA-related mediator expression in the above groups.

Results: Ob-Rb expression was significantly downregulated by LV-shRNA3 in cultured chondrocytes. In conjunction with Ob-Rb downregulation, the expression levels of pro-inflammatory mediators (TNF-α, IL-1β and IL-6) and catabolic mediators (ADAMTS-5, MMP-9, NOS-2 and COX-2) were also significantly decreased, and the expression levels of anabolic type II collagen were significantly increased. The *in vivo* study results showed that OARSI scores were significantly decreased by LV-shRNA3. Immunohistochemistry analysis demonstrated that Ob-Rb expression levels on chondrocytes were significantly downregulated by LV-shRNA3. In conjunction with Ob-Rb downregulation, ADAMTS-5 and MMP-9 expression levels were also significantly decreased, and type II collagen expression levels were increased.

Conclusion: These results indicate that LV-shRNA3-mediated Ob-Rb downregulation on chondrocytes inhibits cartilage degeneration in a rat model of OA, suggesting that Ob-Rb may be a novel target in the treatment of OA.

Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease characterized mainly by articular cartilage destruction and osteophyte formation and involves the periarticular bones, muscles, ligaments and synovia[1]. OA affects a large population of individuals and results in significant morbidity and disability. OA therapies have continuously improved in recent years; however, their effects remain suboptimal. Current pharmaceutical treatments relieve OA symptoms but do not target the mechanism underlying the disease, i.e., they do not target articular cartilage degeneration. Thus, treatments that can delay, prevent or repair OA-related cartilage degeneration are urgently needed.

Leptin, which serves as a causative link between obesity and OA, is a potential target for which disease-modifying osteoarthritis drugs (DMOADs) are being developed, especially in obese patients[2]. Leptin is a 16-kDa non-glycosylated protein encoded by the obese (ob) gene and is produced mainly by white adipose tissue[3]. Leptin bioactivity is mediated by specific leptin receptors (Ob-Rs), which belong to the class I cytokine receptor family[4]. At least 6 types of Ob-Rs, namely, Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re

*Address correspondence and reprint requests to: X. Tang, Department of Orthopaedics, West China Hospital, Sichuan University, 37 Guo Xue Xiang, Chengdu, Sichuan 610041, China. Fax: 86-02885422430.
E-mail address: tangxin9388@163.com (X. Tang).
[a] These authors contributed equally to this study.

http://dx.doi.org/10.1016/j.joca.2017.08.003

and Ob-Rf, have been found in mammalian cells. All but Ob-Re are transmembrane glycoproteins with cytoplasmic sequences of different lengths. The long isoform of the leptin receptor (Ob-Rb) is the functional receptor and plays a major role in signal transduction. The other isoforms have limited signal transduction ability[4–8].

Accumulating evidence indicates that leptin plays a role in the pathogenesis of OA through Ob-Rb. Extreme obesity caused by impaired leptin signaling does not increase the incidence of knee OA, nor does it increase systemic inflammatory cytokine levels in leptin-deficient (ob/ob) and leptin receptor–deficient (db/db) female mice[9]. Leptin concentrations in SF were significantly higher in patients with OA than in controls and have been shown to vary significantly according to OA severity[10]. Leptin and Ob-Rb are expressed in OA articular chondrocytes and are related to cartilage destruction grades[11]. Moreover, a recent meta-analysis showed that leptin expression levels in plasma and SF were significantly increased in patients with OA compared to control subjects[12]. Furthermore, small-interfering RNA (siRNA)-mediated leptin downregulation significantly inhibited MMP-13 expression in cultured chondrocytes[13]. All these findings indicate that leptin plays a catabolic role in cartilage metabolism. Moreover, these findings indicate that leptin may have adverse effects with respect to the pathogenesis of OA and that it may be therapeutic target in the treatment of OA[14].

In vivo, leptin is secreted systemically by white adipose tissue[3] and locally by articular cartilage, synovia, infrapatellar fat pads and osteophytes[15–17]. Therefore, merely blocking leptin expression on chondrocytes is insufficient to prevent its detrimental effects on articular cartilage. Rather, downregulating Ob-Rb expression on chondrocytes to block the leptin signal transduction pathway may more extensively inhibit the detrimental effects of high-concentration leptin on osteoarthritic articular cartilage.

The discovery of RNA interference (RNAi) may be one of the transforming events in biology of the past decade. RNAi can result in gene silencing or even the expulsion of sequences from the genome. Harnessed as an experimental tool, RNAi has the potential to be used therapeutically, and clinical trials intended to investigate this possibility have already been planned[18]. However, articular chondrocytes are nondividing cells *in vivo*. To date, only viral vectors can be efficiently transfected into chondrocytes *in vivo*. Lentiviral vector-based delivery of RNAi constructs has been performed successfully in cultured chondrocytes and animal OA models and holds promise with respect to the development of OA treatments[19].

Based on these findings, we constructed and screened a lentiviral vector-mediated short-hairpin RNA (LV-shRNA) capable of downregulating Ob-Rb expression and evaluated its effects on cartilage degeneration and OA-related mediators. We expect that LV-shRNA-mediated Ob-Rb downregulation on chondrocytes will inhibit articular cartilage degeneration and OA-related mediator expression.

Materials and methods

Animals

A total of 106 male Sprague–Dawley rats (200–300 g, aged 8–10 weeks, purchased from DOSSY Experimental Animals Co., Ltd., Chengdu, China) were used in this study. The animals were maintained under controlled environmental conditions (24 ± 2°C, 40–60% relative humidity) and provided food and water *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee of Sichuan University and were performed in accordance with the ARRIVE guidelines and the National Guide for the Care and Use of Laboratory Animals.

Design of shRNA template oligonucleotides and lentiviral vector construction

Three siRNA sequences targeting rat Ob-Rb and one non-targeting control sequence were conceived using an RNAi Block-IT Designer (Invitrogen, Carlsbad, CA, USA). These siRNAs were chemically synthesized into single-stranded DNA oligonucleotides (shRNA) [Table I] with a stem-loop-stem structure. The shRNAs were then annealed to form double-stranded DNA oligonucleotides and inserted into pGC-LV shuttle plasmids (GeneChem, Shanghai, China). The positive clones were identified by polymerase chain reaction (PCR) and DNA sequencing. The lentiviral vectors consisted of recombinant pGC-LV shuttle plasmids and two types of helper plasmids (pHelper 1.0 and pHelper 2.0, GeneChem). These plasmids were amplified in DH5α-competent *Escherichia coli* cells and were packaged into lentiviral vectors using 293T cells. The lentiviral vectors were subsequently collected, concentrated and stored at −80°C until further use.

Chondrocyte culture

Articular cartilage was harvested from the knee joints of ten male Sprague–Dawley rats under aseptic conditions. Chondrocytes were isolated following sequential digestion in 0.25% trypsin (Gibco, Grand Island, NY, USA) for 30 min and 0.2% type II collagenase (Sigma, St. Louis, MO, USA) for 2 h and were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 44 mM NaHCO3 (Sigma), 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4, containing the following antibiotics: 100/ml penicillin G sodium, $MgCl_2$ (0.23 mmol/l, Sigma) and 100 μg/ml streptomycin sulfate; and 10% heat-inactivated fetal bovine serum (FBS; Moregate, Bulimba, Australia) under standard conditions (37°C, 5% CO_2). The experiments were performed with third-passage cells. All procedures were performed in triplicate and were repeated three times.

Optimization of transfection conditions

Chondrocytes were seeded into Costar 96-well plates (Costar, Corning, NY, USA) at a density of 5×10^3 cells/well 24 h before lentiviral vector transfection and were allowed to reach ~30% confluence before transfection. Four types of transfection reagents and eight concentration gradients were used to optimize the transfection conditions. The following four transfection reagents were used: (1) DMEM + lentiviral vectors, (2) DMEM with 5 μg/ml Polybrene (GeneChem) + lentiviral vectors, (3) Enhanced Infection Solution (GeneChem) + lentiviral vectors and (4) Enhanced Infection Solution with 5 μg/ml Polybrene + lentiviral vectors. The following eight concentration gradients were used: 0, 10, 20, 30, 50, 60, 80 and 100 multiplicity of infection (MOI). GFP fluorescence intensity and transfection efficiency were assessed using confocal microscopy and flow cytometry, respectively, after 72 h of transfection. Transfection reagent cytotoxicity was detected by Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan).

Selection of the optimal LV-shRNA targeting Ob-Rb

Chondrocytes were seeded in Costar 6-well plates (Corning) at a density of 1.5×10^4 cells/well. Enhanced Infection Solution with 30 MOI lentiviral vectors proved to be the optimal transfection condition, under which five groups of chondrocytes were transfected with PBS (Normal), a lentiviral vector-mediated non-targeting control sequence (LV-NTC), or three LV-shRNAs targeting Ob-Rb (LV-shRNA1, LV-shRNA2 or LV-shRNA3), respectively. The cells