Interferonregulatoryfactor-8(IRF-8) regulates the expression of matrix metalloproteinase-13 (MMP-13) in chondrocytes

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Abstract Low levels of inflammation-induced expression of matrix metalloproteinase (MMP) play a crucial role in articular cartilage matrix destruction in osteoarthritis (OA) patients. Interferon regulatory factor-8 (IRF-8), an important member in the IRF family, plays a key role in regulating the inflammation-related signaling pathway. The aim of this study is to investigate the physiological roles of IRF-8 in the pathological progression of OA. We found that IRF-8 was expressed in human primary chondrocytes. Interestingly, the expression of IRF-8 was upregulated in OA chondrocytes. In addition, IRF-8 was increased in response to interleukin-1 β (IL-1 β) treatment, mediated by the Janus kinase 2 (JAK2) pathway. Overexpression of IRF-8 in human chondrocytes by transduction with lentiviral-IRF-8 exacerbated IL-1 β -induced expression of matrix metalloproteinase-13 (MMP-13) in human chondrocytes. In contrast, knockdown of IRF-8 inhibited IL-1 β -induced expression of MMP-13. Importantly, IRF-8 could bind to the promoter of MMP-13 and stimulate its activity. Additionally, overexpression of IRF-8 exacerbated IL-1 β -induced degradation of type II collagen. However, silencing IRF-8 abrogated the degradation of type II collagen. Taken together, our findings identified a novel function of IRF-8 in regulating articular cartilage matrix destruction by promoting the expression of MMP-13.

Keywords Osteoarthritis · Interferon regulatory factor-8 · Matrix metalloproteinases · Type II collagen

Introduction

Osteoarthritis (OA) is one of the most prevalent joint diseases (Lawrence et al. 2008), affecting millions of people in the world. Efforts have been made in the past decades to characterize its pathological hallmarks; however, the mechanisms mediating the onset and development of OA are still unknown (Goldring et al. 2007). Excessive degradation of articular cartilage caused by low levels of inflammation is one of the most important pathological characteristics of OA (Loeser 2006). Overproduction of proinflammatory cytokines such as interleukin (IL)-1 β and tumor necrosis factor (TNF)- α break the balance between the catabolic and anabolic processes in chondrocytes (Malemud 2015). IL-1 β could induce the expression of matrix metalloproteinases (MMPs), which leads to eventual cartilage matrix (mainly type II collagen and aggrecan) destruction (Sandy et al. 2015). MMP-13 is one of the MMPs that plays an essential role in the pathological progression of OA by degrading the “resident” collagen, especially the type II collagen (Griffin et al. 2000). The regulation of MMP-13 expression is complex. Inhibition of the expression and activity of MMP-13 has become an important therapeutic strategy to impede the development of OA.

Interferon regulatory factor-8 (IRF-8), an important member in the IRF family, plays a key role in regulating the inflammation-related signaling pathway (Waight et al. 2014). IRF-8 has been reported as an essential regulator playing an important role in the pathogenesis of several diseases, including neuropathic pain (Masuda et al. 2012) and multiple sclerosis (Yoshida et al. 2014). IRF-8 upregulation in brain microglia was observed in models of injury, including

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hypoglossal nerve axotomy and kainic acid-induced neuronal injury. Importantly, IRF-8 may activate a program of gene expression that transforms microglia into a reactive phenotype (Cheng et al. 2011). However, little information regarding the physiological function of IRF-8 in chondrocytes and OA has been reported. In the current study, we report that IRF-8 is upregulated in chondrocytes from OA patients. Notably, IRF-8 plays a crucial role in the degradation of type II collagen by regulating the expression of MMP-13.

Materials and methods

Chondrocytes isolation, culture, and treatment

Human subject research protocols were in accordance with the world medical association declaration of the Helsinki Ethical Principles for Medical Research involving human subjects. All of the participants signed a written informed consent. Human OA articular cartilages specimens were obtained from OA patients undergoing total knee arthroplasty ($n = 8$). Normal cartilage specimens were collected from the femoral heads of patients undergoing hip replacement due to femoral neck or distal femoral tumor ($n = 12$). Primary chondrocytes were isolated according to the protocols as previously described (Shaw et al. 2011). Isolated chondrocytes were cultured in monolayer in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin–streptomycin) in a humidified atmosphere of 5% CO₂ at 37°C.

Lentivirus infection

Human chondrocytes were transduced with the IRF-8 lentivirus system to overexpress IRF-8 or IRF-8 siRNA lentivirus (Applied Biological Materials, Canada) to knock down IRF-8 according to the manufacturer's instructions. Briefly, cells were seeded in a six-well plate at the concentration of 2×10^5 cells per well and incubated for 24 h. A mixture of complete media with polybrene was prepared at a concentration of 8 µg/ml. Normal media was removed from the well and 2 ml of the polybrene-media-mix was added to each well. Cells were infected with a positive GFP control virus or a target virus (IRF-8 or IRF-8 siRNA) with a multiplicity of infection (MOI) of 100:1. After incubation for 12 h, the culture medium was removed and replaced with 1 ml of complete medium. Successful overexpression or knockdown of IRF-8 was determined by western blot analysis.

Quantitative real-time polymerase chain reaction

Extraction of total RNA from primary chondrocytes was performed using Qiazol (Qiagen, USA) according to the protocol

of the manufacturer. The concentration and quality of extracted RNA were evaluated using a Nanodrop spectrophotometer (Nanodrop, USA). One microgram of total RNA was used for reverse transcription PCR with Prime Script reverse transcriptase to synthesize complementary DNA (cDNA). Quantitative real-time PCR was performed on a 7500-real-time PCR system (Applied Biosystems, USA) using the TaqMan method and the data were analyzed using 7500 System SDS Software 1.3.1 (Applied Biosystems). Expression levels were normalized to the values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and then results were presented relative to those of control. The following primers were used in this study: IRF-8, 5'-TGGCTGATCGAGCAGATTGACAGT-3' (forward) and 5'-AAGGGATCCGGAACATGCTCTTCT-3' (reverse); GAPDH, 5'-AGAAGGCTGGG-GCTCATTG-3' (forward) and 5'-AGGGGCCATCCACAGTCTTC-3' (reverse); and β -actin, 5'-ATGGATGATGATATCGCC GCG-3' (forward) and 5'-CTAGAAGCATTGCGGTGGAC-3' (reverse).

Western blot analysis

Primary chondrocytes were washed three times with PBS and then solubilized in cell lysis buffer (Cell Signaling, USA) containing Nonidet-P40, protease-inhibitor cocktail, and phosphatase-inhibitor cocktail. Total protein concentration was determined using a commercial bicinchoninic assay (BCA) kit (#23225, Thermo Fisher Scientific, USA) in accordance with the manufacturer's protocols. Equal amounts of protein (20 µg) were resolved on a 4–12% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE, NuPAGE, Invitrogen) and electroblotted onto a polyvinylidene difluoride membrane (GE Healthcare, USA). Membranes were blocked with 5% non-fat milk and incubated overnight at 4 °C in primary antibody, washed and incubated for 1 h with horse radish peroxidase (HRP)-conjugated secondary antibody at room temperature (RT). Blots were developed using chemiluminescent HRP-substrate (Millipore) and X-ray film (Amersham Hyperfilm ECL, GE Healthcare). The following antibodies were used in this study: rabbit polyclonal antibody to IRF-8 (1:2000, #ab28696, Abcam, USA), rabbit polyclonal antibody to MMP-13 (1:1000, #ab39012, Abcam, USA), rabbit polyclonal antibody to Collagen II (1:1000, #ab34712, Abcam, USA), and rabbit polyclonal antibody to β -actin (1:5000, #ab8227, Abcam, USA).

Immunofluorescence assay

After treatment, human primary chondrocytes were washed in PBS three times and fixed in 4% paraformaldehyde (PFA) for 10 min, followed by permeabilization with 0.4% Triton X-100 on ice for 15 min. Cells were blocked with 5% BSA and 2.5% FBS in PBST. Primary chondrocytes were sequentially probed with the primary anti-IRF-8 antibody (1:500, Abcam, USA)

Fig. 1 Interferon response factor-8 (IRF-8) is expressed in human primary chondrocytes. **a** RT-PCR results indicate that IRF-8 is expressed in human primary chondrocytes at the mRNA level. BV2

cells were used as a positive control; **b** Western blot results indicate that IRF-8 is expressed in human primary chondrocytes at the protein level. BV2 cells were used as a positive control

and TRITC conjugated goat anti-mouse secondary antibody for 1 h at RT (Life technologies, USA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence signals were analyzed using a fluorescence microscope (Olympus DP50, Japan).

Luciferase reporter assay

An upstream 1000 bp fragment of MMP-13 proximal promoter was generated and cloned in pGL3 firefly luciferase reporter (Promega, USA). Plasmids (2.0 μ g) were transfected into chondrocytes using lipofectamine 2000 (Invitrogen, USA) according to the manual instructions. pRL-CMV-Renilla (0.1 μ g) was used to normalize for transfection efficiency (Promega, Madison, WI). Twenty-four hours after transfection, cells were harvested and analyzed for luciferase activity using the dual-luciferase reporter assay system (Promega, USA).

Experimental data analysis

Results were presented as mean $\pm$ standard error of mean (S.E.M.). Analysis of Variance (ANOVA) was used to perform the statistical analyses with multiple comparisons. $P < 0.05$ was considered significant.

Results

To the best of our knowledge, the expression pattern of IRF-8 in chondrocytes has not been reported in previous studies. Hence, we sought to find out whether IRF-8 is expressed in chondrocytes or not. IRF-8 has been reported to be expressed in BV-2 cells (Yoshida et al. 2014), so BV-2 cells were used as a positive control. As expected, RT-PCR results in Fig. 1a revealed that IRF-8 was expressed in human chondrocytes, which was confirmed by western blots analysis at the protein level (Fig. 1b). Interestingly, real-time PCR results in Fig. 2a demonstrated that the expression of IRF-8 at the mRNA level was significantly higher in OA chondrocytes compared to those in normal chondrocytes. Consistently, immunostaining results revealed that IRF-8 was mainly expressed in the nucleus of chondrocytes. In addition, elevated expression of IRF-8 at the protein level was found in OA chondrocytes (Fig. 2b).

The proinflammatory cytokine IL-1 β is a key factor in joint cartilage destruction in OA. Therefore, we next evaluated the effects of IL-1 β in regulating IRF-8 in chondrocytes. To address this, normal human chondrocytes were treated with IL-1 β at the concentrations of 5, 10, and 20 ng/ml for 24 h, and the expression of IRF-8 was determined. Real-time PCR results in Fig. 3a and western blots results in Fig. 3b demonstrate that expression of IRF-8 was elevated at both the mRNA level and the protein level in response to IL-1 β in a concentration

Fig. 2 Interferon response factor-8 (IRF-8) is upregulated in Osteoarthritis (OA) chondrocytes. **a** Real-time PCR results reveal that IRF-8 is upregulated in Osteoarthritis (OA) chondrocytes at the mRNA

level; **b** Immunofluorescence results reveal that IRF-8 is upregulated in Osteoarthritis (OA) chondrocytes at the protein level (** $P < 0.01$ vs. control group, ANOVA)

Fig. 3 IL-1 β increased the expression of interferon response factor-8 (IRF-8) mediated by JAK2 pathway. **a** Normal primary chondrocytes were treated with IL-1 β at the concentration of 5, 10, and 20 ng/ml for 24 h, and the gene expression of IRF-8 at the mRNA level was determined by real-time PCR (** P < 0.01 vs untreated group; ** P < 0.01 vs. untreated group); **b** Normal primary chondrocytes were treated with IL-1 β at the concentration of 5, 10, and 20 ng/ml

for 24 h, and the expression of IRF-8 at the protein level was determined by western blot analysis (** P < 0.01 vs untreated group); **c** Normal primary chondrocytes were treated with IL-1 β (20 ng/ml) in the presence or absence of AG490 (5 μ M, 24 h) for 24 h, and the expression of IRF-8 at the protein level was determined by western blot analysis (** P < 0.01 vs. untreated group, # P < 0.01 vs. IL-1 β -treated group)

dependent manner. Notably, the JAK2 inhibitor AG490 (5 μ M, 24 h) inhibited the effects of IL-1 β (20 ng/ml) on the expression of IRF-8 (Fig. 3c), suggesting the possible involvement of the JAK2 pathway.

MMP-13 is a key regulator responsible for the degradation of cartilage matrix. We examined whether IRF-8 had an influence on the expression of MMP-13. Overexpression of IRF-8 in human chondrocytes was carried out by transduction with lentiviral-IRF-8. Successful overexpression of IRF-8 is shown in Fig. 4a. Intriguingly, we found that overexpression of IRF-8 exacerbated IL-1 β -induced expression of MMP-13 in human chondrocytes (Fig. 4b). Furthermore, we knocked down the expression

of IRF-8 (Fig. 4c). In contrast, knockdown of IRF-8 inhibited IL-1 β -induced expression of MMP-13 (Fig. 4d). In order to understand how IRF-8 regulates MMP-13, we detected its effects on the promoter activity of MMP-13. A strong transactivation effect on the MMP-13 promoter is shown in Fig. 5 in response to overexpression of IRF-8.

Since type II collagen is preferentially cleaved by MMP-13, we then examined the effects of IRF-8 on IL-1 β -induced degradation of collagen. As expected, overexpression of IRF-8 promoted IL-1 β -induced degradation of type II collagen (Fig. 6a). However, knockdown of IRF-8 inhibited IL-1 β -induced degradation of type II collagen (Fig. 6b).

Fig. 4 IRF-8 regulates the expression of MMP-13 at the transcriptional level. Null, empty virus; Lentiviral-IRF-8, IRF-8 overexpression group. NS, no specific small RNA interference; IRF-8 siRNA, IRF-8 specific small RNA interference. **a** Primary chondrocytes were transduced with lentiviral-IRF-8. Western blot analysis revealed the successful overexpression of IRF-8 (** P < 0.01 vs. EV group); **b** Western blot analysis revealed that overexpression of IRF-8 exacerbated IL-1 β -

induced expression of MMP-13 (** P < 0.01 vs. EV group; # P < 0.01 vs. IL-1 β treatment group); **c** Primary chondrocytes were transfected with IRF-8 siRNA. Western blot analysis revealed the successful knockdown of IRF-8 (** P < 0.01 vs. NS group); **d** Western blot analysis revealed that knockdown of IRF-8 inhibited IL-1 β -induced expression of MMP-13 (** P < 0.01 vs. NS group; # P < 0.01 vs. IL-1 β -treated group)

Fig. 5 Chondrocytes were transfected with an upstream 1000 bp fragment of MMP-13 proximal promoter and transduced with lentiviral-IRF-8. Luciferase assay revealed that IRF-8 activated the promoter activity of MMP-13 (** $P < 0.01$ vs. EV group)

Discussion

In the current study, for the first time, we have provided a new insight into the molecular mechanism whereby IRF-8 plays a critical role in regulating cartilage matrix destruction in human chondrocytes. Firstly, we report that IRF-8 is expressed in human primary chondrocytes. Notably, our results indicate that IRF-8 is upregulated in primary chondrocytes from OA

patients. Furthermore, IL-1 β resulted in an amplification of the expression of IRF-8. Secondly, it was shown that IRF-8 participated in regulating the expression of MMP-13 by promoting its promoter activity. Thirdly, IRF-8 regulates the degradation of type II collagen.

IRF-8, a transcriptional factor playing an essential role in the inflammatory response has been associated with numerous chronic inflammatory diseases (Shaw et al. 2011). Multiple lines of evidence have shown that IRF-8 stimulates the transcription of various genes (Kanno et al. 2005; Levi et al. 2002). In addition, IRF-8 plays a crucial role in promoting neuroinflammation by facilitating the expression of IL-12 family cytokines (Yoshida et al. 2014). IRF-8 exerts a major influence on microglia activation by producing proinflammatory cytokines after peripheral nerve injury (Masuda et al. 2012). Interestingly, IRF-8 activates the transcription of target genes by forming a complex with another IRF family member, IRF-1 (Tamura et al. 2008). Indeed, the upregulation of IRF-1 has been associated with the cartilage damage (Lu et al. 2014). MMP-13, an essential parameter expressed on the surface of cartilage in the knee joints, is related to cartilage matrix destruction in OA (Shibakawa et al. 2003). Actually, MMP-13 has long been considered as the major enzyme involved in OA cartilage erosion, which preferentially degrades type II collagen. The induction of MMP-13 by stimulating IL-1 β has been considered as a tool to identify a potential anti-OA

Fig. 6 IRF-8 regulates the degradation of type II collagen. Null, empty virus; Lentiviral-IRF-8, IRF-8 overexpression group. NS, no specific small RNA interference; IRF-8 siRNA, IRF-8 specific small RNA interference. **a** Primary chondrocytes were transfected with lentiviral-IRF-8. Western blot analysis revealed that overexpression of IRF-8 exacerbated IL-1 β -induced degradation of type II collagen

(** $P < 0.01$ vs. EV group; # $P < 0.01$ vs. IL-1 β -treated group); **c** Primary chondrocytes were transfected with IRF-8 siRNA. Western blot analysis revealed that knockdown of IRF-8 inhibited IL-1 β -induced degradation of type II collagen (** $P < 0.01$ vs. NS group; # $P < 0.01$ vs. IL-1 β -treated group)

pharmacological agent. The inductive effects of IRF-8 on the expression of MMP-13 in human chondrocytes implicate its potential role in the pathological progression of OA. In addition to degrading type II collagen in cartilage, MMP-13 is also able to degrade proteoglycan, types IV and type IX collagen, osteonectin, and perlecan in cartilage (Shiomi et al. 2010). The regulatory effects of IRF-8 on the transcription of MMP-13 imply that IRF-8 might be a novel therapeutic target of OA.

Compliance with ethical standards

Ethics statement Human subject research protocols were in accordance with the world medical association declaration of the Helsinki Ethical Principles for Medical Research involving human subjects. All of the participants signed a written informed consent.

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