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Increased MIG-6 mRNA transcripts in osteoarthritic cartilage

Raluca G. Mateescu [a,*], Rory J. Todhunter [b], George Lust [a], Nancy Burton-Wurster [a]

[a] *Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, NY 14853, USA*
[b] *Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, NY 14853, USA*

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Abstract

The biochemical mechanism for initiation of cartilage destruction in osteoarthritis (OA) is unknown but may involve as yet unidentified cartilage genes. The first evidence that MIG-6, a protein involved in signal transduction, is expressed in articular cartilage came from our recent in vitro microarray experiments using the Affymetrix canine GeneChip. Quantitative RT-PCR (q RT-PCR) confirmed a fourfold increase in MIG-6 mRNA in cartilage in response to mechanical impact in vitro. Our goal is to determine if MIG-6, which responds to mechanical impact, could have a role in the initiation of OA. We determined that mRNA transcript levels of MIG-6 were fourfold higher in degenerated cartilage from dogs with hip osteoarthritis than in disease-free cartilage from unaffected dogs and twofold higher than in the cartilage surrounding the lesion. This is the first report associating MIG-6 with OA. Additional studies will determine what role MIG-6 has in the origin of cartilage degeneration.

Keywords: MIG-6; Gene 33; RALT; Osteoarthritis; Cartilage; Chondrocyte; Mechanical impact

Osteoarthritis (OA) is a frequent cause of disability in humans and in domestic animals. The origins of cartilage degeneration in OA are poorly understood. Instability resulting from cruciate ligament deficiency in the knee and altered distribution of load in hip dysplasia are mechanical factors contributing to the cartilage degeneration associated with OA. We used mechanical impact on cartilage in vitro to mimic osteoarthritic parameters including cellular and matrix responses [1–4] to study early events in the pathogenesis of OA. MIG-6/Gene 33 was one gene with significantly altered mRNA expression in response to mechanical damage which was identified using the Affymetrix canine GeneChip which has 23,836 probe sets. RNA signals for MIG-6 from cartilage disks which had been subjected to impact loading in vitro were fourfold greater in the microarrays than RNA signals for MIG-6 from control cartilage. Confirmation for the up-regulation of MIG-6 in this in vitro model of mechanically damaged cartilage was obtained with q RT-PCR [5].

MIG-6/Gene 33 (also called receptor associated late transducer or RALT) has not been reported in cartilage previously. It is a 53 kDa protein located in the cytoplasm of a variety of cells. It has no catalytic domain, but several domains which bind to molecules known to be involved in cell signaling, including a cdc42/rac interaction and binding domain (CRIB), a src homology-3 binding domain, an epidermal growth factor receptor binding domain, a 143-3 binding domain, and a PDZ binding domain which may be involved in the assembly of ion channels. Thus, MIG-6 is considered a molecular adaptor protein involved in signal transduction [6–9].

In dogs with hip OA, the cartilage degeneration that is observed can be related to dorsolateral displacement of the femoral head (i.e., hip dysplasia) leading to acetabular impingement on the femoral head cartilage at the site of origin of cartilage lesions [10]. The aim of this study was to determine if MIG-6, a gene which responds to mechanical impact, could have a role in the initiation

* Corresponding author. Fax: +1 607 256 5608.
E-mail address: rgm9@cornell.edu (R.G. Mateescu).

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of OA by measuring mRNA transcript levels of MIG-6 in OA hip cartilage and in site-matched cartilage from disease-free dogs.

Materials and methods

Animals. The dogs used in this study came from a colony of Labrador Retrievers and Labrador Retriever/Greyhound crosses maintained at the Baker Institute for Animal Health. Six were F1 backcrosses to the Labrador Retriever and one was a pure Labrador Retriever. Dogs were assigned at 8 months of age to a high or low risk group for development of OA based on the dorsolateral subluxation score (DLS). DLS is a radiographic measure in the weight bearing position developed to permit identification of dogs at risk for hip dysplasia at 8 months of age [11]. Necropsies were performed when the dogs were between 2 and 3 years of age. All four dogs in the high risk group (dogs 1, 2, 3, and 5) had a macroscopically identifiable cartilage lesion in the classic perifoveal site (LES) although the lesion from dog 5 was too small for analysis. All dogs in the low risk group (dogs 4, 6, and 7) showed no lesion in a site-matched perifoveal area (LA, area of lesion predilection). Cartilage was collected in liquid nitrogen at necropsy from these sites as well as from the area surrounding (SA) the LES or LA and it was stored at −80 °C until isolation of RNA. Macroscopically normal cartilage from the shoulders of dogs 1, 2, and 5 was also collected at necropsy and placed into explant culture prior to impact loading.

Cartilage explants. Articular cartilage explants (as 4 mm disks) were obtained under sterile techniques using a 4 mm biopsy punch and a no. 10 scalpel blade. The explants were washed three times with Gey's balanced salt solution (Sigma Chemicals, St. Louis, MO) and transferred to serum-free Ham's F12 medium (Gibco, Carlsbad, CA) for culture. Medium was supplemented (per 100 mL) with 2.5 mL Hepes (1 M), 1.0 mL α ketoglutarate (3 mg/mL), 1.0 mL calcium chloride (4.85 g/mL), 200 μL gentamicin (10 mg/mL), 200 μL penicillin/streptomycin (10,000 U/mL Pen-G, and 10,000 μg/mL streptomycin sulfate) and 400 μL Fungizone (250 μg/mL). After filtering through a 0.22 μm filter, this was supplemented with ITSCR+ premix (Collaborative Biosciences, Bedford, MA) and immediately before changing the media, it was supplemented with 1.0 mL L-glutamine (30 mg/mL) and 1.0 mL ascorbic acid (50 mg/mL). Explants were collected in liquid nitrogen or cultured for 48 h prior to loading at 37 °C, 79% humidity, and 5% CO_2.

Cyclic impact loading. Cyclic loads were applied to the central 2 mm of the 4 mm cartilage explants by means of our mechanical loading machine, which has been described in Chen et al. and Farquhar et al. [1,2]. The loading machine allows pneumatically controlled testing of samples in triplicate whilst housed in an incubator. Labview 6 programming software (National Instruments, Austin, TX) enables load control and data acquisition through a computer equipped with a PCI-M10-16E4 Data Acquisition Board. Explants were loaded in stainless steel chambers and were held in place by stainless steel rings. The stainless steel indenters were non-porous. Loading chambers were filled with Gey's balanced salt solution for the duration of the loading. Loading was for 120 min at a magnitude of 5 MPa. The frequency of loading was 0.3 Hz, of which active loading lasted for 1.0 s per cycle. Loading in this square waveform produced a stress rate of 60 MPa/s. Control cartilage was kept in an identical metal chamber, but without loading. After loading, disks were placed in culture for 24 h and then harvested by freezing in liquid nitrogen in preparation for RNA extraction. Loaded and corresponding control disks were pooled separately for RNA extraction.

Isolation of RNA. RNA was isolated as described in MacLeod et al. [12] but with two modifications. First, the pulverization step was omitted and cartilage was transferred directly to the homogenizer. This minimizes losses due to the small sample size. Second, the preparation was digested with RNase-free DNase while bound to the RNAeasy columns (Qiagen). This results in a high quality, DNA-free RNA preparation. For quantitation, RNA was measured in a fluorimetric assay using SybrGreen II.

Real-time quantitative RT-PCR. In order to validate microarray data for MIG-6 gene, q RT-PCR was performed using β-actin as an endogenous control. Canine-specific sequences for MIG-6 and β-actin were obtained from Affymetrix and NCBI web sites:

http://cbsusrv01.tc.cornell.edu/users/affy/canine.aspx
http://www.ncbi.nlm.nih.gov/genome/guide/dog/

PCR primers and TaqMan probes (Applied Biosystems, Foster City, CA) were designed using Primer Express version 1.0 software (Applied Biosystems) and are presented in Table 1. TaqMan probes were labeled with a 3′,6-carboxy-tetramethylrhodamine (TAMRA) label as a quencher dye and a 5′,6-carboxyfluorescein label (6-FAM for MIG-6 and VIC for β-actin) as a reporter dye.

The 63 bp β-actin PCR product and the 67 bp MIG-6 PCR product were cloned into pCR II using Invitrogen's TA cloning kit. The plasmids were subsequently transfected into TOP10 competent cells (Invitrogen, CA). Positive transformants were selected on the basis of kanamycin resistance. The canine cDNA structures for MIG-6 and β-actin were confirmed by sequencing. Large scale plasmid DNA purification was carried out using a Qiagen Plasmid Kit. Both vectors were linearized by *Bam*HI and the cloned canine cDNAs were transcribed with T7 RNA polymerase at 37 °C for 1 h using the reagents supplied with the Ambion's MaxiScript Kit to produce sense MIG-6 and sense β-actin RNA. The concentration was calculated by measuring absorbance at 260 nm.

A two-step real-time q RT-PCR method was employed and real-time PCR was performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). In the first step, sample total RNA or reference RNA (10 ng) was reverse-transcribed in a volume of 10 μL containing TaqMan reverse transcription (RT) buffer, 5.5 mM $MgCl_2$, 500 μM each deoxynucleotide, 2.5 μM random hexamers, 0.4 U/μL RNase inhibitor, and 1.25 U/μL MultiScribe reverse transcriptase at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. In the second step, real-time PCR was carried out in a MicroAmp Optical 96-well plate (Applied Biosystems) using TaqMan Gold PCR reagents. Each well contained 1 μL of reverse-transcribed cDNA, TaqMan buffer A, 5.5 mM $MgCl_2$, 200 μM each dATP/dCTP/dGTP, 400 μM dUTP, 900 nM each of forward and reverse primers, 250 nM TaqMan probe, 0.01 U/μL AmpErase UNG (Applied Biosystems), and 0.025 U/μL AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 25 μL. The thermal cycling conditions were the following: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of melting (95 °C for 15 s), followed by annealing/extension (60 °C for 60 s). In each q RT-PCR run, a standard curve for the target and the endogenous control gene was generated using a serial dilution reference RNA. Fivefold serial dilutions ranged from 0.001 to 0.16 pg of the standard cDNA fragment. Each sample was assayed in duplicate. Absolute expression levels were determined by relating the measured threshold cycles (C_t) to the standard curve. Threshold cycles were defined as the number of PCR cycles at which the fluorescent signal reached a fixed threshold signal, being directly proportional to the amount of input.

Table 1
Sequences of primers used in real-time PCR

Gene	Primer sequence (5′–3′)	Amplicon size (bp)
MIG-6 forward	CCGGCGAGATTGGGACAGAG	63
MIG-6 reverse	GGGTCGGAACAGCAAAATCA	
β-Actin forward	ATGAACTCCCAGTCCTACGGG	67
β-Actin reverse	TCCATGTCGTCCCAGTTGGT	