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Biochemical and Biophysical Research Communications 333 (2005) 247–252
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Knee joint immobility induces Mcl-1 gene expression in articular chondrocytes ☆,☆☆

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Received 11 May 2005
Available online 31 May 2005

Abstract

The capacity of chondrocytes to synthesize and remodel the extracellular matrix of the articular cartilage is influenced by mechanical forces applied to joints. Either abnormally high or low loads are detrimental to articular cartilage. Experimental work on animals suggests that immobilization can alter proteoglycan synthesis and result in thinning and softening of the articular cartilage. Little is known of the effects of joint immobility on the pattern of genes expressed by chondrocytes. This study focused on the induction of *Mcl-1* gene expression in a rat model of knee joint immobilization by the method of differential display PCR. Increase in *Mcl-1* gene expression in chondrocytes induced by joint immobilization was confirmed by RT-PCR, Northern blotting, and immunohistochemistry. Our results indicate that chondrocytes respond to the complete absence of joint motion by expressing *Mcl-1* gene. This expression may be part of a defense strategy by chondrocytes to overcome the impending chondrocyte death and cartilage degeneration induced by joint immobility.

Keywords: Joint contractures; Articular cartilage; Chondrocytes; Mcl-1; PCR; Immunohistochemistry

The health of an articular cartilage hinges on a small number of chondrocytes scattered in the extracellular matrix (ECM) occupying approximately 5% of the total cartilage volume [1]. Through a pattern of gene expression, chondrocytes synthesize the components of the matrix and maintain a balance between the anabolism and catabolism of the ECM [2]. The major constituents of ECM are fibrils of type II collagen and aggregates of proteoglycans arranged in a dense network. The levels and arrangement of collagen fibrils and proteoglycans determine the mechanical function of the cartilage. When the balance between turnover and degradation of collagen and proteoglycans shifts in the direction of degradation, the result is degeneration of articular cartilage and eventually joint failure.

Factors regulating the synthesis of matrix components by chondrocytes include mechanical stimulation. Both in vivo and in vitro studies indicate that loading and movement are necessary for chondrocytes to synthesize and maintain the ECM of the articular cartilage [3]. Exercise of the knee joint in vivo increases the density of aggrecan in cartilage [4], whereas knee joint inactivity results in decreased aggrecan deposition [5,6]. Deprivation of mechanical stimulation to joints occurs in the lower limb of persons after a complete spinal cord injury (SCI). Absence of both movement and loading of the knees of SCI patients causes a reduction in cartilage thickness [7]. Studies performed with cartilage explants

☆ This work was supported by the Canadian Institutes of Health Research (CIHR) (MOP Grant # 53220 to G.T., O.L., and H.U.).
☆☆ *Abbreviations:* ddPCR, differential display PCR, Mcl-1, myeloid cell leukemia-1; RT-PCR, reverse transcriptase-polymerase chain reaction; OA, osteoarthritis; RA, rheumatoid arthritis; IHC, immunohistochemistry; ECM, extracellular matrix; SCI, spinal cord injury.
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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.05.099

and chondrocyte cultures indicate that chondrocytes enhance matrix production in response to mechanical forces. Compressive and shearing forces applied to cartilage explants led to increased synthesis of collagen and proteoglycans [8]. Many cartilage tissue engineering strategies employ some form of mechanical stimulation to enhance matrix production by chondrocytes in culture [9,10].

A number of animal models have been developed to simulate various aspects of the lack of joint mobility on articular cartilage, including our model of rigid immobilization of the rat knee [11]. Morphological and biochemical changes indicate that the cartilage is degenerating; there is an increase in surface irregularity, minor loss of chondrocytes, and a reduction in proteoglycan content [12,13]. We extracted RNA from chondrocytes of the articular cartilage of mobile and immobile rat knee joints to identify differentially expressed genes. Using the method of ddPCR [14], we observed an increase in the levels of the myeloid cell leukemia (Mcl-1) transcript after a period of 2 weeks of joint immobility compared to sham-operated animals. This observation prompted us to test the hypothesis that chondrocytes express the *Mcl-1* gene in the early phase of the degenerative process. Results indicate that chondrocytes increased both Mcl-1 mRNA and protein levels after the knee joint was deprived of its mobility. The possible role of Mcl-1 in the process of cartilage degeneration induced by joint immobility may be a defense against chondrocyte death.

Materials and methods

Cartilage samples from immobilized rat knees. Knee joint immobilization was performed in adult male Sprague–Dawley rats using an internal fixation system as described before [11]. Essentially, a rigid Delrin plate was screwed at one end to the proximal femur and at the other end to the distal tibia leaving the knee joint, capsule, and cartilage untouched. Sham-operated controls underwent the same operative procedure except that only screws were inserted, and no plate. Experiments were approved by the University of Ottawa Animal Care Committee (protocol #ME-149). The number of animals used in each group was 3 for ddPCR, 4 for RT-PCR, 3 for Northern blot, and 23 for immunohistochemistry. Articular cartilage was harvested after 2 weeks for mRNA. Whole knees were processed after 2 and 4 weeks for histological analysis. Three non-operated rat knees were also used for immunohistochemistry.

RNA isolation and differential display polymerase chain reaction (ddPCR). At the end of the immobilization period, articular cartilage was peeled off the tibial condyles and femur with a scalpel, and stored in RNA Later solution at −80 °C. Total RNA was extracted with Trizol and freed from DNA contamination by incubation with DNase (1 U/sample). RNA integrity was confirmed by ethidium bromide staining after fractionation on a 1% formaldehyde agarose gel and the concentrations were determined by spectrophotometry.

The method of ddPCR [14] was used to compare the profiles of genes expressed by chondrocytes from the cartilage of mobile and immobile knees. Purified total RNA (200 ng) was reverse-transcribed using anchored oligo(dT) 3′ primers and 100 U MMLV. Separate reactions were performed for each of the 3′ primers: H-T_{11}G, H-T_{11}A, and H-T_{11}C. The resulting cDNA was amplified by PCR using arbitrary 5′ primers (H-AP1 to H-AP9) in conjunction with the three original oligo(dT) primers in the presence of [^{33}P]ATP. The PCR cycling parameters used were: 94 °C for 30 s, 40 °C for 2 min, and 72 °C for 30 s for 40 cycles followed by a 5 min period at 72 °C. The resulting 27 sets of amplified cDNA fragments were displayed on a 6% polyacrylamide sequencing gel. Polyacrylamide gels were transferred onto Whatman 3MM paper, dried without fixation, and exposed to auto radiographic films overnight. Films were visually inspected for differentially expressed cDNA bands.

Cloning and sequencing of PCR products. Differentially expressed cDNAs were cloned and sequenced to determine their identity. Complementary DNA bands were excised from the dried gel, reamplified using the QIAquick PCR Purification kit (Qiagen) and H-T_{11}G and H-AP9 primers, and subcloned in the TA vector using T4 DNA ligase (Qiagen). Plasmid DNA was introduced into the JM-109 bacterial strain grown overnight and isolated. Subcloning of the cDNAs was confirmed by restriction digestion before sequencing analysis. Sequencing was performed in both strands using SP6 and T7 primers and the Sequenase v2.0 kit (Amersham). Identity of the cDNAs was obtained by analyzing the obtained sequence against the GenBank database through the blast alignment program.

Reverse transcriptase-polymerase chain reaction amplification. To confirm the differential expression of cDNAs, we proceeded with reverse transcriptase-polymerase chain reaction (RT-PCR) using primers annealing in the open-reading frame of the *Mcl-1* gene. Total RNA samples were first treated with DNase (1 U) to eliminate genomic contamination. First strand synthesis was carried out using 250 ng RNA and 100 U MMLV reverse transcriptase (Qiagen). PCRs were performed using 2.5 μl of the first strand reaction, 0.3 μM Mcl-1 primers, and 1 U *Taq* DNA polymerase, 10 mM Tris–Cl, 75 mM KCl, 1.5 mM $MgCl_2$, and 200 mM each dNTP. PCR conditions were as follows: 95 °C for 5 min to denature DNA and then subjected to 28 cycles of PCR 94 °C for 1 min, 40 °C for 2 min, and 72 °C for 2 min followed by a final extension at 72 °C for 2 min. The rat Mcl-1 primers (sense 5′-AGATGGCGTAACAAACTGGG-3′ and antisense 5′-AAAGCCAGCAGCACATTTCT-3′) generated a 240 bp fragment. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level was used to control for variations in loading the gels. Rat GAPDH primers (sense 5′-TCCTTGCACCACCAACTGCTTA-3′ and antisense 5′-ACCACCCTGTTGCTGTAGCCA-3′) generated a 523 bp fragment. PCR products were analyzed on a 1% agarose gel and visualized by staining the gel with ethidium bromide. Identity of the PCR products was confirmed by restriction digestion and analysis of the fragments on agarose gels. Primers were synthesized by Invitrogen (Mississauga, Ontario, Canada) and restriction enzymes were obtained from New England BioLabs (Mississauga, Ontario, Canada).

Northern blotting. A third method was used to confirm the differential expression of the *Mcl-1* gene by chondrocytes of the articular cartilage. Total RNA was extracted from cartilage harvested from mobile and immobile knees using Trizol reagent (Life Technologies, NY, USA) and the protocol provided by the supplier. The poly(A)$^+$ RNA was purified from 100 μg of total RNA with a poly(A)$^+$ extraction kit (Oligotex mRNA Kit; Qiagen, Ontario, Canada) in accordance with the instructions provided by the supplier. Briefly, mRNAs were resolved on agarose gels (1%/formaldehyde gels) at 120 V for 3 h and then transferred onto nylon membranes by capillarity. Membranes were hybridized with a ^{32}P-radiolabeled cDNA probe corresponding to the Mcl-1 sequence amplified with primers described in the previous section. Bands were detected by autoradiography.

Immunohistochemistry. Detection of Mcl-1 protein in articular cartilage was carried out by indirect immunofluorescence performed on seven micron-thin sagittal sections from the medial side of the knee joint. Details of the preparation of knee sections and our protocol for immunostaining were published previously [15,16]. The primary antibody against the rabbit Mcl-1 was obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, California, USA) and used at a dilution

of 1:200. The secondary antibody used was a biotinylated goat anti-rabbit IgG antibody obtained from Biogenex (ESBE Scientific, Markham, Ontario, Canada). DAB chromogen visualized the amplified immunologic response. Counterstaining of the slides was conducted with Mayer's hematoxylin for 10 min.

Results

Expression of Mcl-1 gene by articular chondrocytes in vivo

In the course of screening for differences in gene expression between cartilages from mobile and immobile knee joints by mRNA differential display, a PCR fragment originally designated CARG9-1-3 was identified (Fig. 1, arrow). The intensity of this PCR product increased in cartilage from immobilized joints in comparison to cartilage obtained from sham-operated animals or from cartilage of the leg opposite to the immobilized knee. Sequence analysis of both strands of the CARG9-1-3 PCR fragment and a BLAST search of the GenBank databank demonstrated that the PCR fragment corresponded to the 3′ end of the mouse *Mcl-1* gene (NM_009743). The rat Mcl-1 sequence was not identified after BLAST search since the rat Mcl-1 sequence in the databank does not include the 3′ untranslated region (Accession No. AF115380), the region of Mcl-1 transcript we identified by ddPCR.

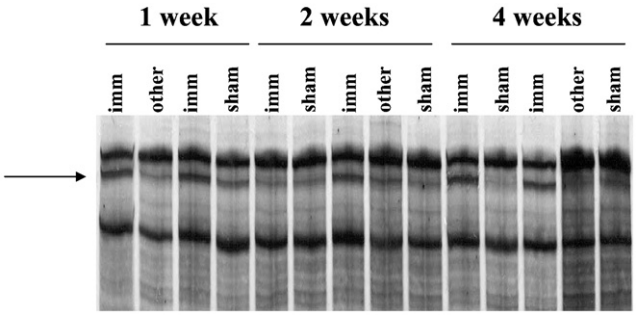

Fig. 1. Differential display-PCR detection of *Mcl-1* gene expression. ddPCR was performed using total RNA prepared from articular cartilages harvested from immobilized (imm) knees at 1, 2, and 4 weeks. Cartilages from 1 week, 2 weeks immobilized, and 4 weeks sham-operated (sham) and the knee contralateral (other) to the immobilized leg were used for comparison. The 150 bp Mcl-1 cDNA fragment indicated by the arrow was amplified by PCR using H-T_{11}G and H-AP9 primers, and subcloned in the TA vector for sequence analysis. Three separate clones were sequenced with T3 and T7 primers, and showed 100% homology to the mouse Mcl-1 cDNA (Accession No. NM_009743).

Two independent methods were used to confirm the differential expression of *Mcl-1* gene in the articular cartilage. In the first approach, PCR primers were designed to amplify a section of the coding region of the Mcl-1 mRNA. The identity of the PCR products was confirmed by the size of a 240 bp PCR product, the presence of a *Msc*I restriction site generating fragments of 181 and 59 bp products (Fig. 2A), and 100% sequence homology with the rat Mcl-1 sequence in the databank. The specificity of the RT-PCR was demonstrated by the absence of PCR products in the absence of RT or in the absence of RNA in the reaction (Fig. 2B).

A second independent approach was used to confirm differential expression of *Mcl-1* gene in cartilage. Mcl-1 mRNA was detected by Northern blotting as two bands, 3.7 and 2.3 kb, as described before in rat tissues (Fig. 3). The two transcripts probably resulted from the use of alternative polyadenylation sites in the 3′-untranslated region of the gene [17].

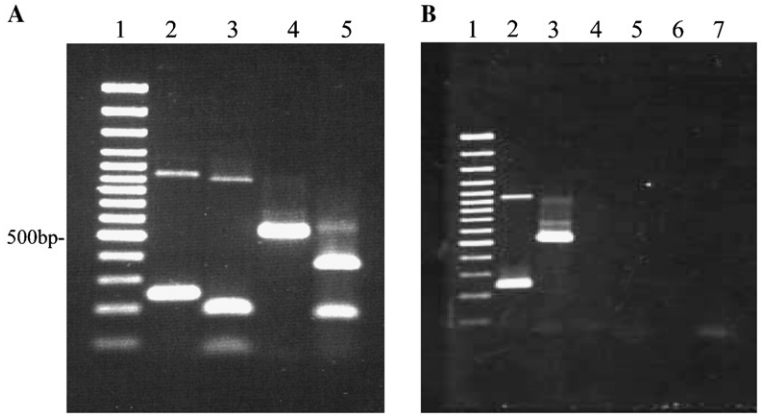

Fig. 2. Reverse transcriptase-PCR detection of Mcl-1 mRNA. RT-PCR was performed on 200 ng of total RNA and primers annealing in the open-reading frame of the Mcl-1 mRNA. GAPDH was used as a control. Resulting PCR products migrated at expected sizes; for Mcl-1 240 bp (A, lane 2 and B, lane 2) and for GAPDH 523 bp (A, lane 4 and B, lane 3). No products were detected in the absence of RT (B, lanes 4 and 5) or in the absence of RNA (B, lanes 6 and 7). Identity of the bands was confirmed by restriction digestion of PCR products generating fragments migrating at expected sizes; Mcl-1 product digestion with *Msc*I: 181 and 59 bp (A, lane 3), GAPDH digestion with *Apa*I: 355 and 168 bp (A, lane 5). Gels are representative of three independent experiments run with samples from three different rats.

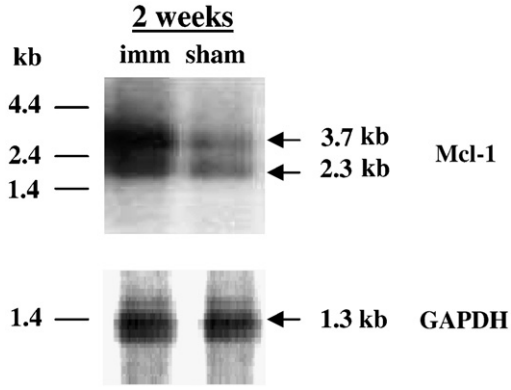

Fig. 3. Northern blot detection of Mcl-1 mRNA. Chondrocytes express the Mcl-1 mRNA. Total RNA was isolated from the articular cartilage of three knees for each of two groups: 2 weeks sham-operated (sham) and 2 weeks immobilized (imm). Five micrograms was subjected to Northern blot analysis and probed with the 241 bp rat Mcl-1 probe generated by PCR. GAPDH was detected with 523 bp rat probe and used as a control. Autoradiographs are representative of three independent experiments run with samples from three different animals.

Effect of joint immobilization on Mcl-1 gene expression

Levels of the two Mcl-1 mRNAs, detected by Northern blot hybridization, are higher in the cartilage obtained from knee joints subjected to a period of 2 weeks of immobilization compared to samples obtained from sham-operated animals (Fig. 3).

Levels of Mcl-1 protein were also assessed with immunohistological staining of whole knee joint preparation from immobilized and sham-operated rats. Staining was observed intracellularly and mainly in the cytoplasm (Fig. 4). Increased intensity of staining and of the number of stained chondrocytes was observed in immobilized specimens compared with sham-operated specimens 2 and 4 weeks after the surgery (Fig. 4). No staining was present in the control experiment when the primary anti-sera step was omitted.

Discussion

Through the use of ddPCR, our study discovered genes of the articular cartilage to be regulated by rigid immobilization of the knee joint. One of these genes was found to be up-regulated when compared to sham-operated animals and sequence analysis revealed a 100% homology with Mcl-1 cDNA. *Mcl-1* gene expression in articular cartilage was confirmed by RT-PCR, Northern blot, and immunohistochemistry.

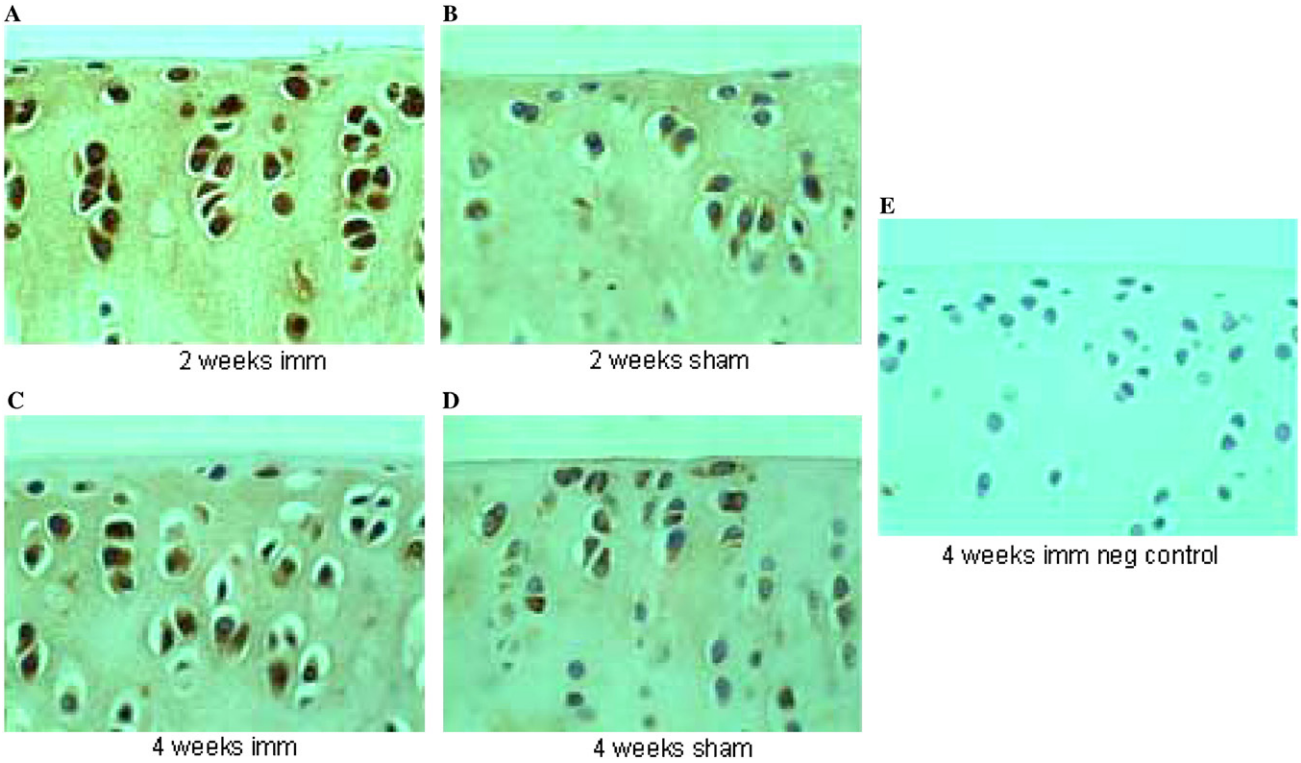

Fig. 4. Immunohistochemistry of Mcl-1. Micrographs of immunohistological staining with Mcl-1 antisera. Batch-stained standardized sagittal section of rat knee joints. (A) Immobilized for 2 weeks: intense cytoplasmic staining in nearly all chondrocytes. (B) Sham-operation 2 weeks: mild cytoplasmic staining in a few chondrocytes. (C) Immobilized for 4 weeks: moderate staining in most chondrocytes. (D) Sham-operation 4 weeks: mild-moderate staining in some chondrocytes. (E) Negative control: omission of the primary antisera step. Counterstain: Mayer's hematoxylin, original magnification 100×.

Mcl-1 protein was detected in the cytosol of chondrocytes of immobilized rat knee joints. This observation indicates that chondrocytes residing in the articular cartilage respond to the complete absence of movement of the rat knee joint by changing their mRNA expression profile.

Mcl-1 is related to the anti-apoptotic protein Bcl-2, which can act as a regulator of cell survival [18]. Mcl-1 and Bcl-2 promote survival of cultured cells under conditions that lead to apoptotic cell death. The presence of Bcl-2 has been documented in skeletal tissue while this is the first evidence for the presence of Mcl-1 in chondrocyte. Evidence indicates that Bcl-2 is part of a signaling pathway involved in the remodeling of skeletal tissue during development [19]. It has been established that Bcl-2, through its anti-apoptotic activity, is involved in a feedback loop that controls maturation of chondrocytes in the growth plate [19]. In addition, in vitro data indicate that Bcl-2 plays a novel role in maintaining the expression of the cartilage matrix protein aggrecan by chondrocytes [20].

The role of Mcl-1 in the physiology of skeletal tissue and cartilage homeostasis remains unclear. A potential role for the increased expression of Mcl-1 in chondrocytes is the prevention of cell death by apoptosis [21,22]. Consistent with this role, chondrocyte numbers remain unchanged after up to 16 weeks of immobilization compared with age-matched sham-operated animals [12]. A modest reduction of chondrocyte numbers is measured only after 32 weeks of immobilization. Although chondrocytes are still present, their capacity to maintain the structure of the matrix is lost; cartilage surface irregularity appears and vascularization can be observed after long periods of immobilization [12]. In agreement with a small decrease in chondrocyte numbers that we observed, cell death by apoptosis or by necrosis does not appear to play a significant role in aging and osteoarthritic (OA) human articular knee cartilage [23]. To explain the reduction in synthetic activities of chondrocytes in OA, a state of cell senescence has been proposed [24]. Whether chondrocytes enter a state of senescence when deprived of stimulation by movement, and as a consequence reduce their capacity to synthesize the cartilage matrix, remains to be determined.

In conclusion, we report that the expression of *Mcl-1* gene is up-regulated in vivo in articular chondrocytes from immobilized rat knee joints. After immobilization, the chondrocytes lose their capacity to maintain cartilage structure and function. In this context, increased expression of Mcl-1 could represent a mechanism to prevent cell death. Thus, chondrocytes from immobilized joints provide a good model to explore the early events of chondroprotection in cartilage that is under threat of degenerating in response to joint immobility.

Acknowledgments

We thank C. Gaudet, J. Courchesne, and S. Kim for their technical support.

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