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Expression of functional μ-opioid receptors in human osteoarthritic cartilage and chondrocytes

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Abstract

There is evidence of effects of morphine on cell proliferation and intraarticular morphine produces analgesia and has an anti-inflammatory effect in chronic arthritis. The effects of opioids are mediated through the G-protein-coupled receptors affecting the cAMP pathway. We demonstrated that human osteoarthritic cartilage and cultured chondrocytes possess the μ-opioid receptor. The presence of the receptor was shown by immunodetection, polymerase chain reaction, and Western blotting. Stimulation of chondrocytes with β-endorphin resulted in decreased phosphorylation of the transcription factor cAMP responsive element binding protein (CREB). The effect was reversed by naltrexone. The obtained results indicate that in human articular chondrocytes opioids affect, via the μ-opioid receptor, the transcription factor CREB which in turn can cause subsequent changes in gene expression.

Keywords: CREB; Human chondrocytes; β-Endorphin; μ-Opioid receptor

Morphine and the endogenous opioids (the enkephalins, dynorphins, and β-endorphin) induce their biological effects by interacting with three major classes of receptors, the δ-, κ-, and μ-opioid receptors. There are some preferences for the different endogenous opioid ligands for the different receptors: β-endorphin for μ, enkephalins for δ, and dynorphins for κ. β-Endorphin binds potently to both the δ- and μ-receptors, but has a relatively lower affinity for κ receptors, suggesting that in peripheral tissues, where β-endorphin is more abundant than enkephalins or dynorphins, it may be an endogenous ligand at the δ- and μ-receptors [1].

β-Endorphin is a part of the pituitary hormone β-lipotropin and is generated by enzymatic processing from the precursor molecule, pro-opiomelanocortin (POMC). The known major sites of POMC biosynthesis are the anterior and neurointermediate lobes of the pituitary gland. The precursor molecules for enkephalins and dynorphins are proenkephalin (ProEnk) and prodynorphin (ProDyn), respectively [2].

The three main types of opioid receptors, μ, κ, and δ, belong to the family of G protein-coupled receptors. Agonists binding to these receptors inhibit adenylyl cyclase and hence decrease the intracellular level of cyclic AMP [3]. However, bimodal opioid regulation of cyclic AMP formation has been suggested, when it was shown that a μ-opioid receptor agonist either increased or decreased the stimulated formation of cyclic AMP in the myenteric plexus [4]. The direction of the opioid modulation of cAMP depended on the concentration of opioid used. The subsequent intracellular signaling pathway is through cyclic AMP dependent protein kinase (PKA), which phosphorylates the cyclic AMP responsive element binding protein (CREB). CREB is a transcription factor that binds to cAMP-responsive elements (CRE) in the promoter region of target genes and modulates their expression. Phosphorylation of Ser^{133} within the kinase-inducible transcriptional activation domain of CREB is required to induce the

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transcriptional activity of the protein, and so CREB can interact with and activate the basal transcriptional machinery [5].

The opioids are usually thought to act centrally due to the presence of opioid receptors in the central nervous system. However, endogenous opioid peptides and opioid receptors are also found in immune cells and skin [6–8]. There is also evidence of β-endorphin binding sites in the articular cartilage of the rat [9] and β-endorphin has been immunolocalized in adult canine cartilage [10].

Intra-articular injection of morphine after knee surgery to patients suffering from osteoarthritis has analgesic effects and that is probably due to activation of peripheral opioid receptors [11,12]. It has also been shown that intra-articular morphine produces analgesia of similar magnitude to dexamethasone and it may have anti-inflammatory actions in chronic arthritis [13].

These facts led us to investigate whether human articular chondrocytes express μ-opioid receptors and to reveal if these receptors are functional in vitro.

Materials and methods

Immunohistochemistry. Immunohistochemistry was performed using the DAKO EnVision + System Peroxidase (DAB) Rabbit Kit (DAKO, 6392 Via Real, Carpinteria, CA 93013, USA). With informed consent, patients who were subjected to total knee arthroplasty participated in the study, and a piece of macroscopically normal cartilage was taken from their knees during surgery and fixed in 4% paraformaldehyde. After 24–48 h the biopsy was embedded in paraffin and sliced at 5 μm thickness onto positively charged slides. The slides were then warmed at 60 °C for 1 h, deparaffinized with xylene and rehydrated with graded alcohol washes, and immersed in filtrated water. Retrieval was performed with PronaseE (Sigma) and peroxidase-blocking solution was used for the blocking of endogenous peroxidase activity. The cell walls were then perforated by treating with 0.1% polyoxyethylene sorbitane monolaurate (Cat. No. P1379, Sigma) for 5 min. After blocking for unspecific binding with BSA/PBS the sections were then incubated for 30 min with polyclonal rabbit anti-human μ-opioid receptor antibody (Chemicon International) at a dilution of 1:1000. After immersing in PBS the sections were then incubated with HRP (horseradish peroxidase)-labeled polymer conjugated with goat anti-rabbit immunoglobulin (secondary antibody). DAB was added after rinsing in PBS and the sections were also immersed in hematoxylin solution for 1 min. Finally the tissue was dehydrated in graded ethanol washes and mounted with mounting media and cover slides. As positive controls we used sections from the pituitary gland, fixed in 4% paraformaldehyde, and embedded in paraffin before slicing. These histological sections were prepared from normal human pituitary glands obtained from autopsy cases. Negative controls were obtained by omitting primary antibody in the procedure.

The slides were then visualized in a Zeiss Axiophot photomicroscope equipped with phase contrast and micrographs were taken with a digital camera (Nicon Coolpix 4500).

Immunocytochemistry. With informed consent, patients who were suffering from osteoarthritis participated in the study and human articular chondrocytes were obtained from their knees during total knee arthroplasty. Biopsies were taken from macroscopically normal areas and then enzymatically digested before cells were cultured in DMEM/F12 medium, supplemented with gentamicin (Cat. No. G-1264, Sigma) and amphotericin B (Cat. No. A-2942, Sigma) in total concentration of 50 μg/ml, with 10% sterile filtered human serum.

An eight-well chamber slide was coated with fibronectin before cultivated chondrocytes were seeded out in the wells. When the cells were adherent (growing overnight), they were fixed with PBS containing 4% paraformaldehyde for 4 h at 4 °C. The cell walls were then perforated by treating with 0.1% polyoxyethylene sorbitane monolaurate (Sigma) for 5 min. To prevent unspecific binding, the preparation was incubated by blocking solution (6% bovine serum albumin in PBS) for 30 min. Primary incubation was done overnight with rabbit anti-human μ-opioid receptor antibody (Chemicon International) at a dilution of 1:1000. Staining for μ-opioid receptor was performed by use of DAKO EnVision + System Peroxidase (DAB) Rabbit Kit (DAKO, CA, USA). Negative controls were conducted by excluding primary antibody in the procedure.

Total RNA extraction. Total RNA was isolated from cultivated human chondrocytes by the TRIZOL LS Reagent method (Molecular Research Center, USA). Isolation of total RNA from osteoarthritic cartilage was done by a modification of the protocol for Qiagen RNeasy Isolation Midi Kit (Merck Eurolab, Oslo, Norway) [14].

Reverse transcription reaction (RT-PCR). First strand cDNA synthesis was done using Superscript II for RT-PCR (Invitrogen). A mixture of 2 μg RNA, 1 μg of 0.5 μg/μl oligo(dT) 12–18, and 1 μl of 10 mM dNTP-mix (dATP, dGTP, dCTP, and dTTP, 10 mM of each) was incubated at 65 °C for 5 min. The mixture was cooled on ice and 10 μl of 5× First-Strand buffer, 5 μl of 0.1 mM DTT, 1 μl dH_2O, 1 μl of 40 U/μl Recombinant RNasin Ribonuclease Inhibitor, and 1 μl of 200 U/μl Superscript II (reverse transcriptase) were added. Then the reaction was carried out at 42 °C for 50 min and 72 °C for 15 min on a MJ Research PTC-200 DNA Engine thermal cycler (MJ Research, Waltham, MA).

Polymerase chain reaction (PCR). PCR was performed in a 50 μl reaction mixture containing 1 μl cDNA (from the RT-PCR mixture), 5 μl of 10× reaction buffer (1× is 10 mM Tris–HCl, pH 8.8, at 25 °C, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100), 1 μl of 10 mM dNTP, 1.2 μl of 50 mM primer mixture, 41.3 μl dH_2O, and 0.5 μl DyNAzyme II DNA polymerase. The PCR program was: initial denaturation at 95 °C for 5 min followed by 37 cycles at 95 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s, and then an extension step cycle at 72 °C for 10 min. The primers, amplifying a 441-bp fragment, were designed from the gene encoding the third extracellular loop of the μ-opioid receptor, and these were; primer M1, 5′-GGTACTGGGAAAACCTG CTGAAGATCTGTG-3′ (sense) and primer M4, 5′-GGTCTCTAGT GTTCTGACGAATTCGAGTGG-3′ (antisense) [8]. To exclude the possibility of DNA contamination, the presence of adenine phosphoribosyl transferase (APRT)-gene transcripts was assayed. The primers used for APRT-PCR were 5′-CCCGAGGCTTCCTCTTTGGC-3′ (sense) and 5′-CTCCCTGCCCTTAAGCGAGG-3′ (antisense) corresponding to sequences in Exon 3 and Exon 5 of the APRT gene [15]. These intron-spanning primers allow easy identification of DNA-contamination when generating an 800-bp fragment, while mRNA will generate a 300-bp fragment. The reactions were carried out on a MJ Research PTC-200 DNA Engine thermal cycler (MJ Research, Waltham, MA). PCR products were analyzed by 2% agarose gel electrophoresis and photographed under UV-light.

The bands corresponding to the fragment size were excised from the gel and rapid DNA cleanup was performed with the MinElute Gel Extraction Kit (Cat. No. 28604, Qiagen), according to manufacturer's instructions. These products were analyzed using the Perkin–Elmer ABI Prism 377 DNA sequencer and applying ABI PRISM BigDye Terminators v 3.0 Cycle Sequencing Kit.

DNA isolated from human lymphocytes obtained from fresh blood samples donated by volunteers and using Lymphoprep (Cat. No. 1031966, AXIS-SHIELD PoC AS) served as positive control. As negative control we used sterile water.

Western blotting for μ-opioid receptor protein. Three million chondrocytes were trypsinized (Cat. No. T-3924, Sigma) from the culture flask and, after washing, the cell pellet was lysed in NuPAGE LDS Sample Buffer (Cat. No. NP0007, Invitrogen). Subsequently, the

sample was heated to 70 °C for 10 min, and cooled on ice, before loading onto NuPAGE 10% Bis–Tris Gel (Cat. No. NP0301, Invitrogen) with NuPAGE MOPS SDS Running Buffer (Cat. No. NP0001, Invitrogen). Proteins were then electroblotted on microporous polyvinylidene difluoride (PVDF) membranes. The immunodetection of the μ-opioid receptor protein was then achieved by using WesternBreeze Chromogenic Western Blot Immunodetection Kit (Invitrogen AS, Norway). The following antibody was used: polyclonal rabbit anti-μ-opioid receptor antibody, N-terminal (BioSource, International).

CREB Ser-133 phosphorylation quantified by Western blotting. Human chondrocytes were seeded out in a six-well plate, 1×10^6 cells in each well. After 48 h of culture in DME/F12 medium (University of Tromso, Norway), supplemented with gentamicin (Cat. No. G-1264, Sigma) and amphotericin B (Cat. No. A-2942, Sigma) at a total concentration of 50 μg/ml, with 10% sterile filtered human serum, the medium was aspirated and the cells were washed two times with phosphate-buffered saline (PBS) before adding serum-free medium. The cells were then treated in three separate ways, where one group was stimulated with 600 ng/ml β-endorphin (Cat. No. E-6261, Sigma), the next group was stimulated with 100 ng/ml naltrexone hydrochloride dihydrate (Cat. No. N-3136, Sigma) and 600 ng/ml β-endorphin, and the last group was used as control. After 15 min, the medium was aspirated from wells and cells were washed with PBS and then lysed with NuPAGE LDS Sample Buffer (Cat. No. NP0007, Invitrogen). Following instructions in PhosphoPlus CREB (133) Antibody Kit (Cat. No. 9190, Cell Signaling Technology), extracts were prepared and then subjected to electrophoresis through a NuPAGE 10% Bis–Tris Gel (Cat. No. NP0301, Invitrogen) with NuPAGE MOPS SDS Running Buffer (Cat. No. NP0001, Invitrogen) and proteins were electrotransferred to microporous polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h, washed, and incubated with primary antibodies overnight at 4 °C. After a wash step, immunocomplexes were detected using an anti-rabbit Chemiluminescent Phosphotope-HRP Western Blot Detection Kit. The following rabbit polyclonal antibodies were used: anti-phospho-CREB (Ser133) antibody (Cat. No. 9191, Cell Signaling) which detects CREB only when activated by phosphorylation at Ser 133, and anti-CREB antibody (Cat. No. 9192, Cell Signaling) which detects total CREB levels (phosphorylation state-independent) and used as a control for sample variations. The same membrane was used within each of three single measurements to secure the same protein loading in one sample. Before incubating with the next antibody, the previous antibody was stripped off the membrane by using 0.2 M NaOH for 5 min and then washed for 2×5 min in TBST. A Ponceau staining was performed on each membrane to control the relative level of proteins in samples.

Levels of immunoreactivity were quantified with a Lumi-Imager F1 system (Boehringer–Mannheim) and LumiAnalyst (Version 3.0 for Windows) and Scion Image Beta 4.02 (Version for Windows NT) software.

Results

Immunohistochemistry

Micrographs of cartilage tissue sections showed clearly stained cells in the presence of primary antibody compared to the unstained controls (Fig. 1). Reactions without primary antibody were used as negative controls. This indicates that chondrocytes express μ-opioid receptor in situ. Sections of pituitary gland tissue (positive controls) showed areas of strong straining restricted to groups of cells, consistent with the β-endorphin producing cells in the pituitary gland.

Immunocytochemistry

Micrographs of cultivated chondrocytes showed that cells were stained well in the presence of primary antibody, compared to no staining of negative control (Fig. 2). This indicates that μ-opioid receptor was present on cultured human chondrocytes.

PCR detection of μ-opioid receptor mRNA

We conducted PCR with primers specific for human μ-opioid receptor (M1/M4 primers) on cDNA made by reverse transcriptase-PCR of RNA isolated from human

Fig. 1. Immunohistochemistry of articular cartilage (A) and pituitary gland (C) showing positive staining of cells expressing μ-opioid receptor. Negative control (primary antibody omitted) (B) shows no staining. Magnifications are 40×.

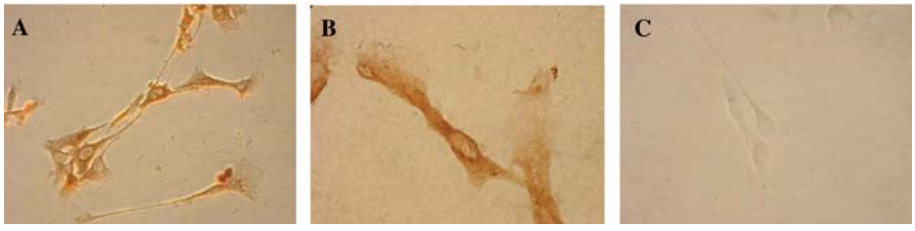

Fig. 2. Micrographs of cultured chondrocytes positively stained with polyclonal rabbit anti human μ-opioid receptor antibody (A,B), and without primary antibody (C) (negative control). Magnifications are 40× (A,C), 100× (B).

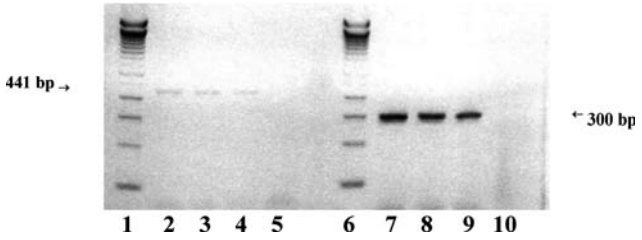

Fig. 3. PCR analysis revealing μ-opioid receptor on mRNA level. Lanes 2, 3, and 4 reflect a 441 bp product from the μ-opioid receptor M1/M4 primers. Lanes 7–9 reflect a 300 bp product from the intron spanning APRT primers that prove non-contamination of cDNA. Positive controls (human monocytes) in lanes 2 and 7. Negative controls in lanes 5 and 10. One hundred base pair ladders are seen in lanes 1 and 6.

articular cartilage. The primers were designed to detect a 441 bp fragment from Exon 3, encoding the third extracellular loop of the receptor. The cDNA detected by gel electrophoresis indicates that mRNA corresponding to 441 bp was present (Fig. 3). The band was excised from the gel, isolated, and sequenced, and after alignment with database (BLAST), there was 99% conformity with the expected sequence. These results demonstrate that chondrocytes in situ express μ-opioid receptor mRNA.

Western blotting for μ-opioid receptor protein

In the Western blot study of cultured chondrocytes, using the qualitative WesternBreeze Chromogenic Western Blot Immunodetection Kit with rabbit anti-human μ-opioid receptor antibody, bands occurred at approximately 40 kDa (Fig. 4). In vitro translation of full-length μ-opioid receptor cDNA variants from mouse brain showed that the receptor isoforms had molecular weights varying around 40 kDa [16]. This size is nearly consistent with the calculated molecular weight of the human receptor, 44.7 kDa, based on its amino acid sequence, although a recent paper reports a 50 kDa sized human μ-opioid receptor protein [17]. These results indicate that cultured chondrocytes possess μ-opioid receptor.

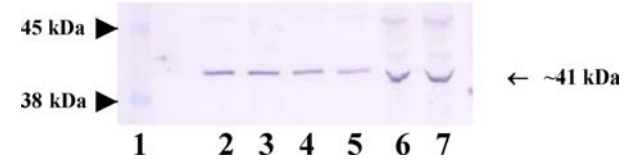

Fig. 4. Western blot showing μ-opioid receptor in chondrocytes. Picture of the membrane after Western blotting with antibody detecting μ-opioid receptor. There are bands (~41 kDa) for the μ-opioid receptor protein. Protein extracts from chondrocyte in lanes 2–5 and from brain tissue in lanes 6 and 7. See Blue Plus 2 pre-stained protein standard ladder in lane 1.

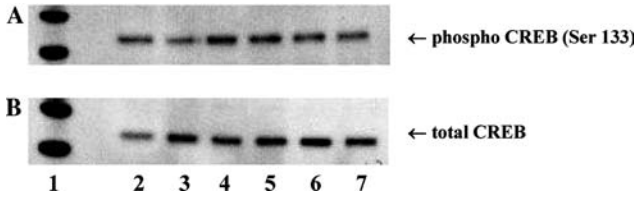

Fig. 5. The membranes from chemiluminescent Western blot with phosphorylated CREB (Ser 133) antibody (A) and total CREB antibody (B) of cells stimulated with β-endorphin (lanes 2–3), β-endorphin and naltrexone (lanes 4–5), and non-stimulated cells (lanes 6–7). Magic Mark Western Standard ladder in lane 1. Bands are seen at approximately 43 kDa. The results from densitometric analysis are seen in Fig. 6 and Table 1.

CREB Ser-133 phosphorylation quantified by Western blotting

In Western blot study of cultured chondrocytes using rabbit anti-phospho-CREB (Ser133) antibody and anti-CREB antibody, bands occurred at 43 kDa as expected (Fig. 5). The levels of immunoreactivity were quantified and when cultured chondrocytes were incubated with 600 ng/ml β-endorphin for 15 min, a decrease of CREB phosphorylation was observed (Fig. 6 and Table 1). Chondrocytes incubated with 100 ng/ml naltrexone 5 min before and during the 15 min period of incubation with 600 ng/ml β-endorphin, showed no alteration of CREB phosphorylation. Analysis of the phosphorylation-state independent total CREB served as an internal control. These results indicate that the μ-opioid receptor in chondrocytes is functional.

Discussion

The results from the present study indicate that a μ-opioid receptor exists in human articular chondrocytes both at the mRNA and at the protein levels. RNA was isolated from cartilage in osteoarthritic knees, and reverse transcriptase PCR, amplification of DNA, and sequencing revealed a 441 bp DNA fragment identical to the μ-opioid receptor from human brain [18]. Immunohistochemistry and immunocytochemistry using antibody against μ-opioid receptor resulted in strong staining of chondrocytes and Western blot analysis of cultured chondrocytes discovered the presence of the receptor protein. Also previous observations in animals have suggested the presence of an opioid system in articular cartilage. A high density of opioid binding sites was found in inflamed canine joint tissue [9], and autoradiographic evidence of β-endorphin binding sites on chondrocytes from the distal femur of rats has also been reported [10].

Activation of the G_i-coupled receptor is known to decrease CREB phosphorylation at serine 133 [19,20],

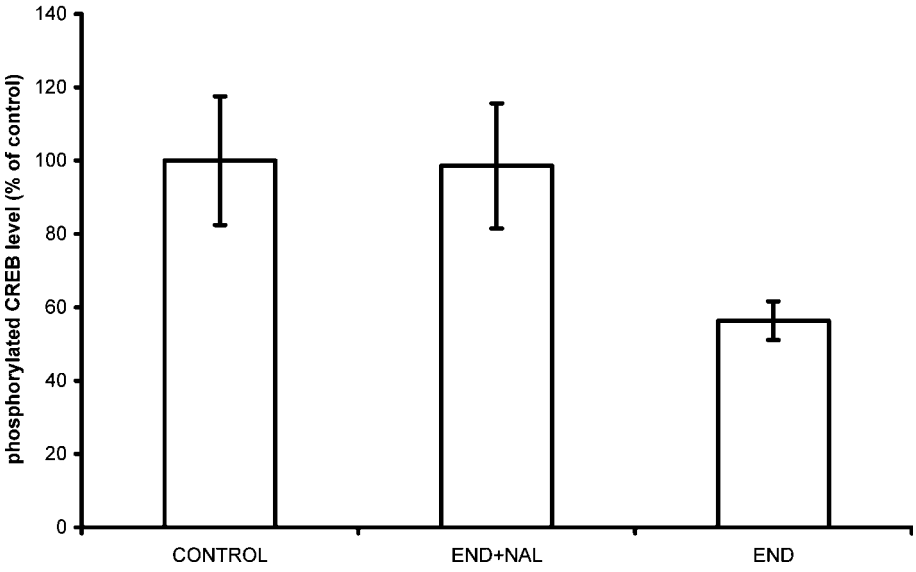

Fig. 6. β-Endorphin stimulated chondrocytes contain lower levels of phosphorylated CREB in comparison with control and naltrexone treated cells. (END) β-endorphin (600 ng/ml; 15 min of incubation) treated group. (END + NAL) β-endorphin treatment under the same conditions as previous, plus naltrexone (100 ng/ml) added 5 min before. (CONTROL) Control group, where cells were kept as they were in the other groups before stimulation. Data are expressed as means ± SEM from three independent experiments (phospho-CREB to total CREB ratio shown as % of control).

Table 1
The quantity of phosphorylated CREB decreases after β-endorphin stimulation: (A) phospho-CREB/total CREB ratio measured in chondrocyte cultures from three different patients; (B) ratio values as means, standard deviations (STDEV), and standard errors of the means (SEM)

	END	END + NAL	CONTROL
(A)			
Culture I	0.62	1.52	1.42
Culture II	0.59	0.82	0.77
Culture III	0.79	1.16	1.36
(B)			
Mean	0.66	1.16	1.18
STDDEV	0.11	0.35	0.36
SEM	0.06	0.20	0.21

β-Endorphin stimulated (END) groups showed lower levels of phosphorylated CREB in comparison with control and naltrexone (NAL) treated group. There seems to be a 50% decrease of phosphorylated CREB after β-endorphin stimulation. Furthermore, it appears that there is no difference between the other two groups, speaking in favor of the specific pharmacological effect of β-endorphin in the first group.

and in this study β-endorphin decreased CREB phosphorylation at serine 133 in cultured chondrocytes by approximately 50% compared to non-stimulated chondrocytes. By adding naltrexone before and simultaneously with β-endorphin, this effect was blocked and CREB phosphorylation did not decrease. The reduced CREB phosphorylation after β-endorphin treatment therefore seems to be a specific opioid effect, since naltrexone is a well-known opioid antagonist for μ-, κ-, and δ-receptors. These data show that the μ-opioid receptor in cultured chondrocytes is functionally active.

Agonist binding to the G-protein coupled opioid-receptor is primarily known to give pain relief when binding to receptors in the central nervous system [2] or on peripheral terminals of sensory neurons [21]. However, the opioid receptor is also located on non-neuronal cells, such as immune cells and keratinocytes [6,8], and it has also been shown that intra-articular morphine may have anti-inflammatory actions in chronic arthritis [13]. Opioids from immunocytes interact with receptors on sensory nerves and inhibit nociception in inflammation [22]. It is reported that morphine has an effect on cell proliferation [23] and a paper described that chronic morphine stimulation of a clonal cell line with transfected opioid receptor gene inhibited cell proliferation [24]. A recent paper also stated that serum taken from patients anesthetized with opiates was less effective in the support of chondrocyte growth in vitro [25]. These facts open the question as to whether endogenous opioids and stimulation of opioid receptors on chondrocytes play a role in the process of articular chondrocyte proliferation and differentiation.

From these results we conclude that the human articular cartilage in an osteoarthritic knee expresses functional μ-opioid receptors. Further studies are needed to reveal if endogenous opioids contribute to the regulation of chondrocyte growth and cartilage tissue generation.

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