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CHAPTER 6

COLLAGEN-INDUCED EXPRESSION OF COLLAGENASE-3 (MMP-13) BY PRIMARY CHONDROCYTES IS MEDIATED BY INTEGRIN α1 AND DISCOIDIN DOMAIN RECEPTOR 2: A PROTEIN KINASE C-DEPENDENT PATHWAY

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ABSTRACT

OBJECTIVES. To investigate whether maintaining the chondrocyte's native pericellular matrix prevents collagen-induced up-regulation of collagenase-3 (MMP-13) and whether integrin α1 (ITGα1) and /or discoidin domain receptor 2 (DDR2) modulate MMP-13 expression and which signalling pathway plays a role in collagen-stimulated MMP-13 expression.
METHODS. Goat articular chondrocytes and chondrons were cultured on collagen coatings. Small interfering RNA (siRNA) oligonucleotides targeted against Itgα1 and Ddr2 were transfected into primary chondrocytes. Chemical inhibitors for MEK1 (PD98059), FAK (FAK inhibitor 14), JNK (SP600125) and PKC (PKC412), and a calcium chelator (BAPTA-AM) were used in cell cultures. Real-time polymerase chain reaction was performed to examine gene expression levels of Mmp-13, Itgα1 and Ddr2 and collagenolytic activity was determined by measuring the amount of hydroxyproline released in the culture medium.
RESULTS. Maintaining the chondrocyte's native pericellular matrix prevented Mmp-13 up-regulation and collagenolytic activity when the cells were cultured on a collagen coating. Silencing of Itgα1 and Ddr2 reduced Mmp-13 gene expression and collagenolytic activity by primary chondrocytes cultured on collagen. Incubation with the PKC inhibitor strongly reduced Mmp-13 gene expression levels. Gene expression levels of Mmp-13 were also decreased by chondrocytes incubated with the MEK, FAK or JNK inhibitor.
CONCLUSIONS. Maintaining the native pericellular matrix of chondrocytes prevents collagen-induced up-regulation of MMP-13. Both integrin α1 and discoidin domain receptor 2 modulate Mmp-13 expression after direct contact between chondrocytes and collagen. PKC, FAK, MEK and JNK are involved in collagen-stimulated expression of Mmp-13.

INTRODUCTION

The matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases. They are expressed by a large number of different cell-types and play important roles in various processes such as tumor invasion and tissue remodeling. They have multiple functions in extracellular matrix (ECM) biology: they can degrade most components of the ECM, release neo-epitopes and matrikines from ECM macromolecules, release growth factors and they can modify cell-ECM interactions by acting on cell-surface receptors[1].

MMP-13 (collagenase-3) very efficiently degrades fibrillar collagens such as type I and type II collagen, but it can also degrade type X collagen and aggrecan[2-5]. MMP-13 plays a prominent role in skeletal development since it is required for hypertrophic chondrocyte differentiation[6-10]. MMP-13 is also involved in cartilage pathology. It is highly expressed by chondrocytes in osteoarthritis[11,12], rheumatoid arthritis[13], experimental arthritis[14-16] and a direct correlation between excessive MMP-13 expression and cartilage degradation has been shown[11,17,18].

Despite the alleged importance of MMP-13 in cartilage pathology, the factors that regulate MMP-13 expression by chondrocytes have only partially been identified. Stimulation of MMP-13 expression is best understood: fibronectin fragments bind to integrin α5β1, the nonreceptor tyrosine kinase PYK2 is activated by Protein Kinase C (PKC), and furthermore ERK, JNK and p38 mitogen-activated protein (MAP) kinases are activated, resulting in MMP-13 synthesis[19-21]. In addition to stimulation of MMP-13 expression by fibronectin fragments, it has also been shown that exposing chondrocytes to both type I and type II collagen can induce MMP-13 expression[22-26].

The collagen receptor discoidin domain receptor 2 (DDR2) is associated with osteoarthritis and it has been reported that DDR2 is involved in the collagen-stimulated MMP-13 expression. Silencing DDR2 in the C-28/I2 chondrocyte cell-line reduced collagen-induced MMP13 up-regulation[22]. Furthermore, the MEK/ERK and p38, but not JNK, signalling pathways seemed to be involved in the collagen stimulation of MMP-13 in the C-28/I2 cell-line[22,23]. In addition to DDR2, it has also been reported that integrin α1β1 is involved in collagen-induced MMP-13 up-regulation. Blocking antibodies against α1 and β1 integrin subunits reduced collagen-dependent induction of MMP-13 by MC615 chondrocytes[24]. The MEK/ERK and the JNK pathways were involved in collagen-induced MMP-13 expression by the MC615 chondrocytes[25].

A better understanding of the mechanisms involved in collagen-induced MMP-13 up-regulation could help in the development of a therapeutic target for the inhibition of excess matrix degradation by this enzyme. Furthermore, it can also have an impact on the use of chondrocyte-seeded collagen-based matrices for cartilage repair. Tissue engineering by means of chondrocytes mostly results in too little collagen in the neoconstructs. One of the explanations for such a low level of collagen might be the degradation of newly synthesized collagen by MMP-13 if the chondrocytes are in contact with fibrillar collagens. We hypothesize that the pericellular matrix of chondrocytes *in situ* prevents collagen-

induced up-regulation of MMP-13 as type II collagen is abundantly present in the ECM of healthy cartilage, but this does not seem to stimulate excessive MMP-13 expression by the chondrocytes.

In this study we investigated whether the pericellular matrix present in a chondron prevents a direct contact between the chondrocyte and fibrillar collagens by investigating the response of chondrocytes (cartilage cells without pericellular matrix) and chondrons (chondrocytes with a pericellular matrix) on collagen coatings. Furthermore, we investigated whether discoidin domain receptor 2 and/or integrin α1β1 are involved in collagen-stimulated up-regulation of MMP-13 in primary articular chondrocytes and whether this is regulated through a protein kinase C-dependent pathway as has been reported for fibronectin fragment-induced MMP-13 expression.

MATERIALS AND METHODS

Cell isolation and culture

Cells were isolated from articular cartilage obtained from the shoulders of skeletally mature female Dutch milk goats that were used in other studies (the Animal care and use Committee of the VU University Amsterdam approved the use of goats in these studies). The tissue was dissected and minced. One half (randomly taken) was used for chondrocyte isolation and the other half for chondron isolation.

For chondrocyte isolation, the tissue fragments were subjected to sequential treatments first with Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, UK) supplemented with 1% foetal bovine serum (FBS, HyClone, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B (all from Gibco) and 2.5% (w/v) Pronase E (Sigma, St. Louis, MO) for 1 h, then with DMEM supplemented with 25% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 0.125% (w/v) collagenase (CLS-2, Worthington, Lakewood, NJ) for 16 h at 37°C.

For chondron isolation, minced cartilage was digested with 0.3% (w/v) dispase (Gibco) plus 0.2% (w/v) collagenase in phosphate buffered saline (PBS, Gibco) for 5 h as previously described[27].

The cells were filtered through a 70 µm cell strainer (BD Biosciences, San Diego, CA) and washed. The cells were cultured for 24 h in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 50 µg/ml ascorbate-2-phosphate (Sigma).

For preparation of culture plates, type I collagen from rat tail (BD Biosciences) and type II collagen from chicken sternal cartilage (Sigma) were dissolved in 0.02N acetic acid at a concentration of 1 mg/ml and used to coat 24-well plates. For a coating with denatured collagen, the type I collagen solution (1 mg/ml) and the type II collagen solution (1 mg/ml) were heated at 70 °C for 45 min.

Real-time PCR

Total RNA was isolated from the cells with the RNeasy mini kit (Qiagen, Gaithersburg, MD). DNase I treatment was performed as described by the manufacturer to remove any contaminating genomic DNA. Total RNA (750 ng) was reverse transcribed using 250 U/ml Transcriptor Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany), 0.08 U random primers (Roche diagnostics), and 1 mM of each dNTP (Invitrogen, Carlsbad, CA) in Transcriptor RT reaction buffer at 42˚C for 45 min followed by an inactivation of the enzyme at 80˚C for 5 min. Real-time PCR reactions were performed using the SYBRGreen reaction kit according to the manufacturer's instructions (Roche Diagnostics) in a LightCycler 480 (Roche Diagnostics). The LightCycler reactions were prepared in 20 µl total volume with 7 µl PCR-H_2O, 0.5 µl forward primer (0.2 µM), 0.5 µl reverse primer (0.2 µM), 10 µl LightCycler Mastermix (LightCycler 480 SYBR Green I Master; Roche Diagnostics), to which 2 µl of 5 times diluted cDNA was added as PCR template. Primers (Invitrogen) used for real-time PCR are listed in Table 1. Specific primers were designed from sequences available in the data banks, based on homology in conserved domains between human, mouse, rat, dog and cow. The amplified PCR fragment extended over at least one exon-border (except for 18S). Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz) and 18S were used as housekeeping genes and the gene expression levels were normalized for the normalization factor calculated with the equation $\sqrt{(Ywhaz \times 18S)}$. With the Light Cycler software (version 4), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene using Fit Points method. PCR efficiency was calculated by Light Cycler software and the data were used only if the calculated PCR efficiency was between 1.85 and 2.0.

siRNA design and transfection

The siRNA sequences used for silencing the genes were designed by Block-iTTM RNAi Designer online software (Invitrogen). The siRNA oligonucleotides were designed on sequences available in the data banks, based on homology in conserved domains between human, mouse, rat, dog and cow. Oligonucleotides (Invitrogen) used for siRNA are listed in Table 1. Transfection of siRNA oligonucleotides or the medium GC-content negative control siRNA (Invitrogen) was performed by electroporation using the Microporator Pipet-type Electroporation System (Digital Bio, Hopkinton, MA); freshly isolated chondrocytes were resuspended in T-cell resuspension buffer (Digital Bio) (10000 cells/µl) and 5 pmol/µl siRNA oligonucleotide was added. The electroporation was performed using 3 pulses of 1300 V for 30 ms. Transfected cells were cultured in type II collagen-coated wells for 24 h in DMEM supplemented with 10% FBS and 50 µg/ml ascorbate-2-phosphate (Sigma).

Table 1. Oligonucleotide sequences used for real-time PCR and small interfering RNA.

Target gene		Oligonucleotide sequence	Annealing temperature (°C)	Product size (bp)
Ywhaz	Forward	5' GATGAAGCCATTGCTGAACTTG 3'	56	229
	Reverse	5' CTATTTGTGGGACAGCATGGA 3'		
18S	Forward	5' GTAACCCGTTGAACCCCATT 3’	57	151
	Reverse	5' CCATCCAATCGGTAGTAGCG 3’		
Mmp13	Forward	5' GGAGCATGGCGACTTCTAC 3'	56	208
	Reverse	5' GAGTGCTCCAGGGTCCTT 3'		
Itgα1	Forward	5' AGGACAGTGCCTATAACACC 3’	56	234
	Reverse	5' CGCTGTCACTTGTTGCACTT 3’		
Ddr2	Forward	5' CCTCTGGCATGAAGTACCT 3’	57	341
	Reverse	5' GAGAGTTCTTCCGAGACCAA 3’		
Target gene		**siRNA oligonucleotide sequence**		
Mmp-13	Sence	5' GGU CUG UUG GCU CAC GCU U 3'		
	Antisence	5' AAG CGU GAG CCA ACA GAC C 3'		
Itgα1	Sence	5' ACC AGA ACC AUA GUG AAU U 3'		
	Antisence	5' AAU UCA CUA UGG UUC UGG U 3'		
Ddr2	Sence	5' GCU UAG UGG GUA AGA ACU A 3'		
	Antisence	5' UAG UUC UUA CCC ACU AAG C 3'		
Hprt1	Sence	5' CCA GUC AAC AGG CGA CAU A 3'		
	Antisence	5' UAU GUC GCC UGU UGA CUG G 3'		

Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; Mmp13, matrix metalloproteinase 13; Itgα1, integrin α1; Ddr2, discoidin domain receptor 2; Hprt1, hypoxanthine phosphoribosyltransferase 1.

Hydroxyproline assay

The amount of hydroxyproline released into the culture medium was determined using a modified colorimetric assay[28].

Inhibitors

Primary chondrocytes and chondrons were cultured in the presence of the MMP-inhibitor CT1166 (N1-[1-(S)-(morpholinosulphonylaminoethyl-amino-carbonyl)-2-cyclohexylethyl]-N4-hydroxy-2-(R)-[3-(4-methylphenyl)propyl]succinamide) (Celltech Therapeutics Ltd., Slough, UK) at a final concentration of 10 μM[29].

Primary chondrocytes were incubated for 1 h at 37 °C with a MEK1 inhibitor (PD98059), a FAK inhibitor (FAK inhibitor 14), a JNK inhibitor (SP600125), a calcium chelator (BAPTA-AM) and a PKC inhibitor (PKC412) (all from Tocris Bioscience, Bristol, UK). The final concentration of the inhibitors was 10 μM. The cells were then cultured for 24 h in type II collagen-coated wells containing culture medium with inhibitors.

Statistical analysis

Data are expressed as mean ± SD. Gene expression data are expressed as mean ± SD of treated versus control (T/C) chondrocytes of target gene expression normalized for the equation $\sqrt{(Ywhaz \times 18S)}$. Differences between groups were tested with a two-tailed paired t-test and differences of T/C ratios were tested with a two-tailed t-test for single group mean and compared to 1 (T/C=1, no effect). The level of significance was set at $p<0.05$.

RESULTS

Maintaining the pericellular matrix prevents collagen stimulated MMP-13 up-regulation

Gene expression levels of Mmp-13 were increased when chondrocytes were cultured on type I and type II collagen coatings, but not when they were cultured on denatured type I or type II collagen (Fig. 1A). In contrast, Mmp-13 gene expression levels were not increased by the chondrons under any of these conditions.

The amount of hydroxyproline as a measure of collagen degradation was assessed in conditioned medium. A higher level of this iminoacid was released in the culture medium by the chondrocytes cultured on a type II collagen coating compared to the chondrons. This level decreased significantly in the presence of the MMP-inhibitor CT1166 (Fig. 1B) whereas the release of hydroxyproline by the chondrons was unaffected by CT1166.

Fig. 1

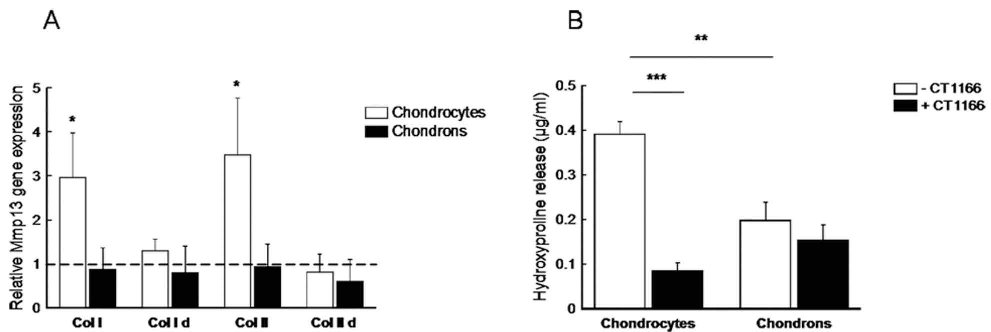

Figure 1: Gene expression levels of Mmp-13 and Mmp-activity of chondrocytes and chondrons cultured on collagen coatings. *A, Real-time PCR was performed on reverse-transcribed RNA isolated from chondrocytes and chondrons from goat articular cartilge after 24 h of culture on type I (Col I), denatured type I (Col I d), type II (Col II) and denatured type II (Col II d) collagen. The results are presented as Mmp-13 gene expression levels relative to gene expression on uncoated plates. B, The amount of hydroxyproline was measured in the culture medium of chondrocytes and chondrons that were cultured for 24 h on type II collagen coatings, without and with the addition of the MMP-inhibitor CT1166. Data are shown as mean ± SD, n = 5. *: p<0.05; **: p<0.01; ***: p<0.001.*

Discoidin domain receptor 2 and integrin α1 are involved in collagen-induced MMP-13 expression

The gene expression levels of integrin α1 (Itgα1) were increased by the chondrocytes when they were cultured on a coating of native type I or II collagen, but not on denatured type I and II collagen (Fig. 2A). The gene expression level of integrin α1 by the chondrons was not affected under any condition. Also the expression levels of discoidin domain receptor 2 (Ddr2) were up-regulated by the chondrocytes when cultured on a type I and II collagen coating, but not on the denatured collagen coatings (Fig. 2B). Again, expression was not affected in the chondrons. Thus, an increased gene expression of Mmp-13 (Fig. 1A) coincided with increased expression levels of both integrin α1 and discoidin domain receptor 2.

To determine whether integrin α1 and discoidin domain receptor 2 are actually involved in the collagen-induced Mmp-13 up-regulation, we transfected the cells with small interfering RNA (siRNA) oligonucleotides targeted against these genes.

First, we assessed whether there was off-target silencing, by including siRNA oligonucleotides against hypoxanthine phosphoribosyltransferase 1 (Hprt1). The gene expression levels of Hprt1 were decreased when a siRNA oligonucleotide against Hprt1 was transfected into the chondrocytes, whereas a transfection with siRNA against Itgα1, Ddr2, Mmp-13 and a combination of Itgα1 and Ddr2 had no effect on the Hprt1 expression levels (Fig. 3A). Silencing of Hprt1 had no effect on the gene expression levels of Mmp-13.

Fig. 2

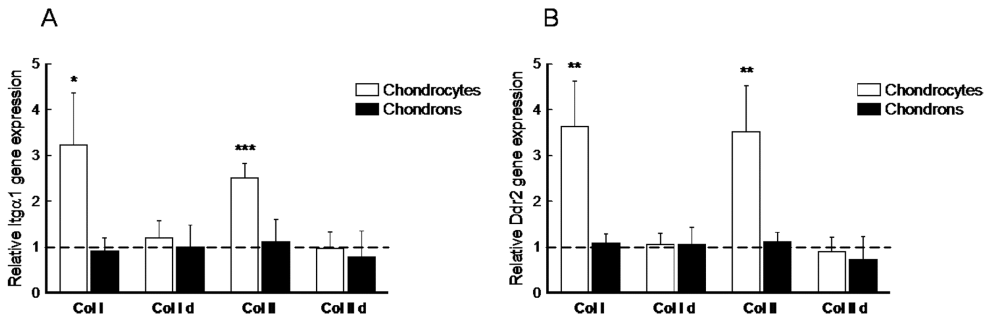

Figure 2: Gene expression levels of integrin α1 and discoidin domain receptor 2 by chondrocytes and chondrons cultured on collagen coatings. *Real-time PCR was performed on reverse-transcribed RNA isolated from chondrocytes and chondrons from goat articular cartilge after 24 h of culture on type I (Col I), denatured type I (Col I d), type II (Col II) and denatured type II (Col II d) collagen. The results are presented as integrin α1 (Itgα1, A) and discoidin domain receptor 2 (Ddr2, B) gene expression levels relative to gene expression on uncoated plates. Data are shown as mean ± SD, n = 5. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.*

Tranfection of siRNA targeted against Mmp-13 down-regulated gene expression levels of Mmp-13 (Fig. 3B). Also silencing of Itgα1, Ddr2, or the combination of Itgα1 and Ddr2 resulted in decreased gene expression levels of Mmp-13.

Silencing Ddr2, or the combination of Ddr2 and Itgα1, resulted in a down-regulation of discoidin domain receptor 2 gene expression levels (Fig. 3C). No decreased discoidin domain receptor 2 gene expression levels were observed when Mmp-13 (or Hprt1) expression levels were silenced with an oligonucelotide. However, transfecting a siRNA oligonucleotide against Itgα1 resulted in decreased discoidin domain receptor 2 gene expression levels.

Gene expression levels of integrin α1 were down-regulated when Itgα1 or a combination of Itgα1 and Ddr2 were silenced (Fig. 3D). No effect was observed on integrin α1 gene expression when Mmp-13 (or Hprt1) was silenced. Silencing Ddr2 also down-regulated integrin α1 gene expression levels.

Silencing Mmp-13 decreased the amount of hydroxyproline that was released (Fig. 4). Silencing Itgα1, Ddr2 or Itgα1 and Ddr2 together also resulted in a decreased amount of hydroxyproline that was released into the culture medium, whereas silencing Hprt1 had no effect.

Fig. 3

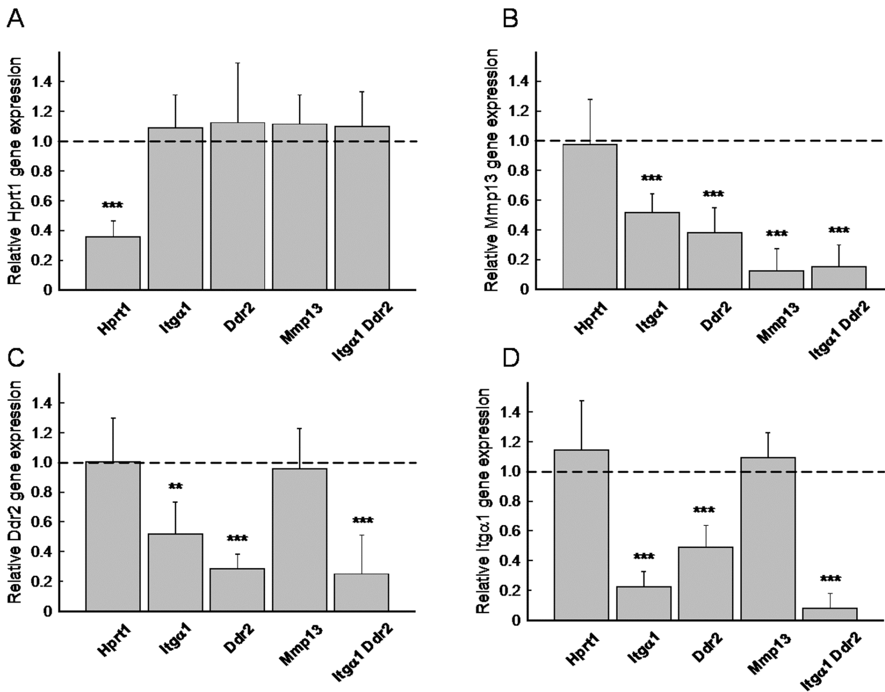

Figure 3: Collagen-induced Mmp-13 up-regulation is dependent on discodin domain receptor 2 and integrin α1. *Real-time PCR was performed on reverse-transcribed RNA isolated from goat articular chondrocytes that were transfected with small interfering RNA (siRNA) oligonucleotides targeted against hypoxanthine phosphoribosyltransferase 1 (Hprt1), integrin α1 (Itgα1), discoidin domain receptor 2 (Ddr2), collagenase-3 (Mmp-13) and both integrin α1 and discoidin domain receptor 2 (Itgα1 Ddr2) after 24 h of culture on type II collagen coatings. The results are presented as hypoxanthine phosphoribosyltransferase 1 (Hprt1, A), collagenase-3 (Mmp-13, B), discoidin domain receptor 2 (Ddr2, C) and integrin α1 (Itgα1, D) gene expression levels relative to the gene expression after transfection with a scrambled siRNA oligonucleotide. Data are shown as mean ± SD, n = 7. **: $p<0.01$; ***: $p<0.001$.*

Inhibition of protein kinase C suppresses collagen-stimulated MMP-13 induction

To examine the potential signalling pathways involved in collagen-induced Mmp-13 up-regulation, we examined the effects of a MEK1 inhibitor (PD98059), a FAK inhibitor (FAK inhibitor 14), a JNK inhibitor (SP600125), a calcium chelator (BAPTA-AM) and a PKC inhibitor (PKC412).

Treating chondrocytes with the PKC inhibitor resulted in a strong down-regulation of Mmp-13 gene expression levels (Fig. 5A). Gene expression levels of Mmp-13 were also decreased by chondrocytes treated with the MEK, FAK or JNK inhibitor.

Treatment with the calcium chelator had no effect on the gene expression levels of Mmp-13. Gene expression levels of integrin α1 were decreased when the chondrocytes were treated with the FAK inhibitor, whereas the other inhibitors had no effect (Fig. 5B).

Treatment with the PKC inhibitor down-regulated discoidin domain receptor 2 gene expression levels (Fig. 5C). None of the other inhibitors had an effect on expression levels of this gene.

Fig. 4

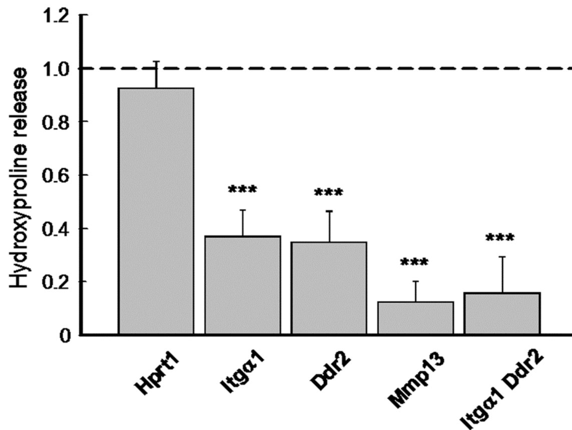

Figure 4: Mmp- acticity is dependent on discodin domain receptor 2 and integrin α1 expression. *The amount of hydroxyproline was measured in the culture medium of goat articular chondrocytes that were transfected with small interfering RNA (siRNA) oligonucleotides targeted against hypoxanthine phosphoribosyltransferase 1 (Hprt1), integrin α1 (Itgα1), discoidin domain receptor 2 (Ddr2), collagenase-3 (Mmp-13) and both integrin α1 and discoidin domain receptor 2 (Itgα1 Ddr2) after 24 h of culture on type II collagen coatings. The results are presented as the amount of hydroxyproline relative to the amount of hydroxyproline after transfection with a scrambled siRNA oligonucleotide. Data are shown as mean ± SD, n = 7. ***: $p<0.001$.*

DISCUSSION

In this study we have shown that maintaining the native pericellular matrix of chondrocytes prevents collagen-induced MMP-13 up-regulation. Direct contact between chondrocytes and fibrillar collagens stimulates MMP-13 expression[22-26] and the pericellular matrix prevents such a direct contact. It is likely that this is also the case in native cartilage. The pericellular matrix forms a transition and buffer between chondrocytes and the native ECM which is very rich in type II collagen.

Fig. 5

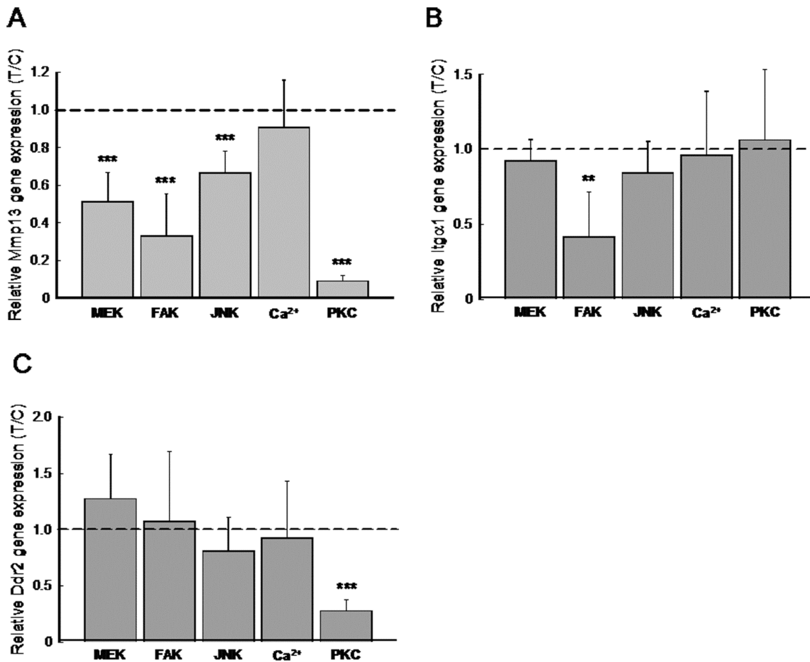

Figure 5: Collagen-induced Mmp-13 up-regulation is dependent on activity of MEK, FAK, JNK and PKC. *Real-time PCR was performed on reverse-transcribed RNA isolated from goat articular chondrocytes after 24 h of culture on type II collagen in the presence of the MEK-inhibitor PD98059 (MEK), FAK-inhibitor 14 (FAK), the JNK inhibitor SP600125 (JNK), the calcium chelator BAPTA-AM (Ca^{2+}) and the PKC inhibitor PKC412 (PKC). The results are presented as collagenase-3 (Mmp-13, A), integrin α1 (Itgα1, B) and discoidin domain receptor 2 (Ddr2, C) gene expression levels relative to gene expression after treatment with the vehicle. Data are shown as mean ± SD, n = 10. ***: $p<0.001$.*

As a consequence of direct contact between chondrocytes and collagens, cell-ECM interactions result in activation of collagen receptors such as integrin α1 and discoidin domain receptor 2[22-24]. We have shown now that expression of Mmp-13 is modulated by each of these collagen receptors; silencing integrin α1, discoidin domain receptor 2, or integrin α1 and discoidin domain receptor 2 together resulted in decreased Mmp-13 gene expression levels.

To determine whether the activation of chondrocytes by fibrillar collagen also resulted in an increased collagenolytic activity, we assayed the amount of hydroxyproline released in the culture medium. Silencing Mmp-13, integrin α1, discoidin domain receptor 2, or integrin α1 and discoidin domain receptor 2 together not only decreased Mmp-13 gene expression levels, but it also decreased collagen breakdown as shown by the decreased

release of hydroxyproline in the culture medium. The degradation of the collagen proved to depend largely on the activity of MMPs as shown with the selective MMP-inhibitor. Given these findings we assume that the digestion was mediated by MMP-13.

Discoidin domain receptor 2 and integrin α1 recognize native fibrillar collagens[30]. This explains why there was no effect on Mmp-13, integrin α1 and discoidin domain receptor 2 gene expression levels when chondrocytes were cultured on the denatured fraction of type I and II collagen.

The fact that chondrocytes respond to native collagen by an up-regulation of MMP-13 may also have an impact on chondrocyte-seeded collagen matrices that are used for cartilage repair. As excessive collagen degradation is undesirable, especially that of the collagen that is deposited in the neoconstruct, it might be useful to prevent direct contact between chondrocytes and fibrillar collagen in scaffolds used for cartilage repair. This can be accomplished by seeding chondrons in the scaffolds instead of chondrocytes. The problem is not limited to collagen scaffolds; an up-regulation of Mmp-13 was seen also when chondrocytes were cultured in alginate beads[31], which might be due to direct contact of the chondrocytes with the type II collagen that is produced by them. The pericellular matrix apparently plays an essential role in the modulation of the activation of chondrocytes. This suggests that the pericellular matrix is important in cartilage catabolism and this is supported by the finding that one of the earliest identifiable matrix changes associated with cartilage catabolism are changes in the pericellular matrix[32-34]. Type VI collagen might play an important role in this, as it is the major component of the pericellular matrix.

As overproduction of collagenases, in particular MMP-13, plays a central role in collagen degradation in arthritic cartilage, it is important to determine signalling pathways that regulate collagenase expression by chondrocytes. MMP-13 production may be stimulated not only by cytokines like interleukin-1[54,36], but also by ECM components[19-23]. This study showed that upon interaction of collagen with integrin α1 and discoidin domain receptor 2, PKC plays an important role as Mmp-13 gene expression levels were strongly decreased by treatment with the PKC inhibitor. For fibronectin fragment-stimulated MMP-13 expression it has been reported that upon activation of integrin α5β1, the nonreceptor tyrosine kinase PYK2 is activated by PKC[20]. Although we did not determine PYK2 phosphorylation by PKC in this study, it is apparently an important step in collagen-stimulated MMP-13 expression. As PKC is involved both in fibronectin-fragment and in collagen-stimulated MMP-13 expression, it might be a potent target for the development of a therapeutic agent for the inhibition of excess matrix degradation by MMP-13. Besides PKC, also FAK plays a role in collagen-induced MMP-13 expression. It is known that integrins can signal both through PKC and FAK[36] and this makes it very likely that in this case, integrin α1 signals through both. The fact that BAPTA-AM had no effect on Mmp-13 expression levels, suggests that the signalling pathway is calcium independent.

MAP kinase activation takes place downstream of the PYK2 signalling pathway[20,37-41]. Our results suggest that at least both the MEK/ERK and the JNK pathway

are involved in collagen stimulation of MMP-13. It is very plausible in this pathway that the Grb2-Sos complex is involved in coupling PYK2 to activation of ERK and p130CAS and Crk to activation of JNK[42]. With respect to chondrocytes, Xu et al.[22,23] reported that the MEK/ERK and p38 pathways, but not the JNK pathway, were involved in collagen-stimulated MMP-13 expression in the C-28/I2 chondrocyte cell-line. Other data, however, have shown that apart from the MEK/ERK also the JNK pathway was involved in collagen-induced MMP-13 up-regulation by MC615 chondrocytes[24]. Our results support the latter data. The fact that inhibiting the JNK pathway had no effect on MMP-13 gene expression levels in the C-28/I2 chondrocyte cell-line[23] might be due to differences between this cell-line, MC615 and primary chondrocytes.

The receptors and pathways we identified in collagen stimulated MMP-13 expression are very similar to those involved in the collagen-stimulated expression of N-cadherin by human pancreatic cancer cells[43], which promotes tumor growth, invasion and metastasis. As MMP-13 also plays a critical role in the invasive and metastatic potential of cancers, inhibiting these pathways might also be helpful in comprehending cancer.

Integrin α1 and discoidin domain receptor 2 gene expression levels are up-regulated upon interaction with fibrillar collagen. That silencing of integrin α1 had an effect on discoidin domain receptor 2 gene expression levels and vice versa, suggests that the gene expression of both proteins is linked to each other. This coupling was not at the level of PKC since PKC inhibition only had an effect on the expression of discoidin domain receptor 2, but not on integrin α1; gene expression levels of integrin α1 were decreased after treatment with the FAK inhibitor. Where the association between integrin α1 and discoidin domain receptor 2 takes place, still remains to be investigated.

The present study showed that direct contact between chondrocytes and collagen increases both Mmp-13 gene expression and collagenolytic activity. Maintaining the native pericellular matrix of chondrocytes prevents collagen-induced up-regulation of MMP-13. Both integrin α1 and discoidin domain receptor 2 modulate Mmp-13 expression upon direct contact between chondrocytes and collagen. Upon an interaction of the receptors with collagen, PKC plays a very important role, probably in activating PYK2. Furthermore, we have shown that both the MEK/ERK and the JNK pathway are involved in collagen-stimulated expression of MMP-13. The expression of discoidin domain receptor 2 is mediated by PKC and the expression of integrin α1 by FAK.

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