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Increased Expression of the Collagen Receptor Discoidin Domain Receptor 2 in Articular Cartilage as a Key Event in the Pathogenesis of Osteoarthritis

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Objective. **To investigate the role of the collagen receptor discoidin domain receptor 2 (DDR-2) in the pathogenesis of osteoarthritis (OA).**

Methods. **Histologic and immunohistochemical analyses were performed to characterize femoral head cartilage from 7 patients with OA and 4 patients with fracture, as well as articular cartilage from the knee joints of mice with surgically induced OA. Gene constructs encoding human Raf kinase inhibitor protein (RKIP), DDR-2 lacking the discoidin (DS) domain (ΔDS-DDR-2) or the protein tyrosine kinase (PTK) core (ΔPTK-DDR-2), DDR-2 containing a substitution of tyrosine for alanine at position 740 (Y740A), and luciferase driven by the matrix metalloproteinase 13 (MMP-13) promoter were transfected into human chondrocyte cell lines. Activated and neutralized $\alpha 2\beta 1$ integrin polyclonal antibodies, interleukin-1 receptor antagonist, and the chemical inhibitors SB203580, for p38, and SP600125, for JNKs, were used in cell cultures. Real-time polymerase chain reaction was performed to examine MMP-13 and DDR-2 messenger RNA (mRNA).**

Results. **Increased immunostaining for DDR-2, MMP-13, and MMP-derived type II collagen fragments was detected in cartilage from patients with OA and from mice with surgically induced OA. The discoidin domain and PTK core of DDR-2 were essential for signal transmission and the resulting increased expression of MMP-13 in chondrocytes. Y740A mutation of DDR-2 reduced levels of mRNA for MMP-13 and endogenous DDR-2. The overexpression of RKIP or preincubation with the p38 inhibitor reduced MMP-13 mRNA levels. DDR-2 signaling was independent of the $\alpha 2\beta 1$ integrin and the interleukin-1–induced signaling pathways in chondrocytes.**

Conclusion. **These findings suggest that increased expression of DDR-2, resulting in the elevated expression of MMP-13, may be one of the common events in OA progression.**

Osteoarthritis (OA), the most common form of arthritis (1,2), is considered to be a group of overlapping, distinct diseases associated with different risk factors but with a similar clinical outcome. The pathologic changes during the development of OA are remarkably similar and include proteoglycan degradation at the early stage, followed by type II collagen degradation, leading eventually to localized or complete loss of cartilage matrix (3). The similar pathologic changes suggest that a common molecular chain of events may be responsible for the disease progression. An understanding of these molecular events will not only elucidate

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Dr. Glasson owns stock or stock options in Wyeth and is inventor with Wyeth on a patent for ADAMTS small-molecule inhibitors.

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biomarkers for the early diagnosis of OA, but will also provide novel therapeutic targets for the delay and treatment of OA.

During the last several years, we have used 2 murine models of OA, Col9a1$^{-/-}$ mice, which are deficient in type IX collagen (4,5), and Col11a1$^{cho/+}$ mice, which are haploinsufficient for type XI collagen and are heterozygous for chondrodysplasia (cho/+) (6–8), to investigate molecular events underlying the pathogenesis of OA. Our results demonstrated that the earliest OA-like morphologic changes were the appearance of chondrocyte clusters in the articular cartilage of knee joints and enhanced proteoglycan production in the articular cartilage of temporomandibular joints in both mutant mouse strains at the age of 3 months. The cartilage degeneration became more severe with aging, characterized by increased proteoglycan degradation and collagenases-derived type II collagen fragments. Between the ages of 12 and 15 months, the knee joints in these mice appeared severely damaged, with OA-like changes.

Interestingly, the expression of matrix metalloproteinase 13 (MMP-13), which cleaves type II collagen, was increased in Col9a1$^{-/-}$ and Col11a1$^{cho/+}$ mice at the age of 6 months. At the same time point, the expression of the collagen receptor discoidin domain receptor 2 (DDR-2) was also elevated. DDR-2 is a cell membrane tyrosine kinase receptor that binds preferentially to type II collagen (9–11). Furthermore, our studies demonstrated that the activation of DDR-2 (by plating human chondrocyte cell line or mouse primary chondrocytes on native type II collagen) resulted in increased expression of MMP-13 (8). Based on these observations, we hypothesize that once the proteoglycan is depleted in the matrix, the type II collagen network is exposed to chondrocytes, which results in enhanced contact of the cell membrane with type II collagen fibrils. As a consequence of the interaction of type II collagen with chondrocytes, DDR-2 is activated, resulting in the increased expression of the receptor itself as well as MMP-13. Thus, we propose that the increased expression of DDR-2 may be a common event in the pathogenesis of OA in general.

In this study, we tested our hypothesis by examining articular cartilage from the femoral head of patients with symptomatic OA and patients with fracture for OA morphologic changes and for biochemical changes of DDR-2, MMP-13, and MMP-derived type II collagen fragments. We also examined whether similar changes occurred in articular cartilage from the knee joints of a mouse model of surgically induced OA. We investigated the contribution of the type II collagen–binding domain (discoidin [DS] domain) and the protein tyrosine kinase (PTK) core of DDR-2 to the mechanism by which MMP-13 expression was enhanced by type II collagen–induced DDR-2 and to the signaling pathways involved in chondrocytes. In addition, we investigated whether $\alpha 2\beta 1$ integrin and interleukin-1 (IL-1) were involved in the DDR-2 signaling pathway.

MATERIALS AND METHODS

Histologic assessment of human articular cartilage. Cartilage samples from femoral heads were obtained as surgical waste at the time of hip replacement surgery for clinical OA (n = 7 patients) and for bone fracture without clinical OA (n = 4 patients). Samples were frozen at –80°C until used. Samples were obtained with the approval of the Institutional Review Boards of Beth Israel Deaconess Medical Center and Harvard Medical School.

From the cartilage samples obtained from patients with fracture, 5 areas of full-thickness articular cartilage measuring 0.5×0.5 cm^2 (2 from the medial, 2 from the middle, and 1 from the lateral region) were excised from each femoral head. From the cartilage samples obtained from patients with symptomatic OA, 5 areas of full-thickness articular cartilage were obtained from the residual cartilage. Samples were fixed in 4% paraformaldehyde for 4 hours at room temperature and then embedded in paraffin. Two hundred serial sections (8-μm–thick) were cut for histologic and immunohistochemical analyses. Every fiftieth section was collected for Safranin O–fast green staining and evaluation according to the Mankin histologic and histochemical grading system (12). For immunohistochemistry, successive paraffin sections from one area adjacent to the sections showing histologic changes of OA in each patient were used.

Surgical induction of OA in mouse knee joints and histologic assessment. Experimental procedures in mice were performed following approval from the Wyeth Institutional Animal Care and Use Committee. Surgical destabilization of the medial meniscus of 10-week-old male 129/SvEv mice was performed as previously described (13). Knees not subjected to surgery and knees subjected to sham surgery were used as negative controls. Mice (n = 10 per group) were killed at 2, 4, and 8 weeks postoperatively, and knee joints were harvested and fixed in 4% paraformaldehyde for 24 hours at room temperature. Joints were then decalcified in EDTA for 6 days and embedded in paraffin.

Serial frontal sections measuring 6 μm in thickness were cut through the entire joint and used for Safranin O–fast green staining or immunohistochemistry. Approximately every fifteenth Safranin O–fast green section was scored according to a modification of a published scoring system for mouse joints (14), where 0.5 = loss of Safranin O–fast green staining without structural changes, 1 = roughened articular surface and small fibrillations, 2 = fibrillation down to the layer immediately below the superficial layer and some loss of surface lamina, 3 = mild erosion (<20%), 4 = erosion to the

bone (not a feature of this model), 5 = moderate loss of noncalcified cartilage (20–80%), and 6 = severe (>80%) loss of noncalcified cartilage. The Mankin score was not used for mouse samples because of the thin articular cartilage and the tendency of lesions to rapidly progress from superficial fibrillation to complete loss of noncalcified cartilage. Four areas of the joint (medial tibial plateau, medial femoral condyle, lateral tibial plateau, and lateral femoral condyle) were scored separately. A minimum of 12 sections (~90 μm apart) were scored for each knee joint, encompassing the entire area of articulating femorotibial cartilage. The individual scores were then summed.

For immunohistochemistry, we collected 4 sections from the anterior, middle, and posterior regions of each knee joint from 4 animals (randomly selected from among 10 animals) in the surgical group and 4 animals in the sham-surgical group. The 4 sections were mounted on a glass slide.

Immunohistochemical analysis. Polyclonal antibody against human and mouse DDR-2 (catalog no. sc-7554) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody against mouse MMP-13 (catalog no. AB8120) and monoclonal antibody against human MMP-13 (catalog no. MAB3321) were purchased from Chemicon (Temecula, CA). Polyclonal antibody C1,2C (Ibex, Montreal, Quebec, Canada), recognizing a neopeptide on type II collagen, was a gift from Dr. A. Robin Poole (Shriners Hospitals for Children, Montreal, Quebec, Canada).

For immunohistochemical staining, sections from human femoral heads and from mouse knee joints at 2 weeks and 4 weeks postoperatively were collected. Sections were deparaffinized and quenched for endogenous peroxidase activity. After treatment with chondroitinase ABC (0.25 units/ml, catalog no. C3667; Sigma, St. Louis, MO), the sections were incubated with polyclonal antibodies (1:200 dilution). After washing with phosphate buffered saline, the samples were incubated with biotinylated secondary antibody. Negative controls were prepared by staining without primary antibody.

Construction of expression vectors. Expression vector containing DDR-2 without the discoidin domain (ΔDS-DDR-2) was a gift from Dr. Birgit Leitinger (Imperial College London, London, UK) (15). We generated DDR-2 without the PTK core of the receptor (ΔPTK-DDR-2) by polymerase chain reaction (PCR). The full-length complementary DNA (cDNA) of DDR-2 (8) was used as template. The forward primer was 5′-GCTTGGTACCGAATGATCCTGATTC-3′ and the reverse primer was 5′-GGAATTCTCAGAGTTTCCTGGGGAA-3′ (start and stop codons, respectively, are underlined). The PCR product was subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) at *Kpn* I and *Eco* RI sites. The expression vector was then amplified and purified using the EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, CA). The sequence of the truncated cDNA of DDR-2 was confirmed by DNA sequencing.

To generate the DDR-2 mutant carrying a substitution of tyrosine for alanine at amino acid position 740 (Y740A), we used a QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and the full-length DDR-2 cDNA in the pcDNA3.1 vector as a template, and 2 complementary oligonucleotide primers with a melting temperature of ≥78°C, which contained the desired mutations in the middle of the primers. The forward primer was 5′-GTACAGTGGTGACGCTTACCGGATCCAG-3′ and the reverse primer was 5′-CTGGATCCGGTAAGCGTCACCACTGTAC-3′ (desired mutations are underlined). Each primer was elongated in the opposite direction by DNA polymerase, and parental DNA templates (no mutation) were digested with *Dpn* I. After digestion, the nicked, circular double-stranded DNA was transferred into XL1-Blue cells to join the nicks. Plasmids with the mutation were amplified and isolated. Before transfection, plasmids with the mutations were sequenced to confirm the alteration of the DNA sequence.

The full-length cDNA of human Raf kinase inhibitor protein (RKIP) was obtained by reverse transcription–PCR using total RNAs isolated from human chondrocytes. The cDNA was synthesized using oligo(dT) primer. The primers for the PCR were designed according to the published RKIP sequence. The 5′ end of the forward primer contained an *Eco* RI site, 5′-GGAATTCCATGCCGGTGGACCTCA-3′, and the 5′ end of the reverse primer had an *Xba* I site, 5′-GCTCTAGACCTACTTCCCAGACAG-3′. The PCR product was then subcloned into the expression vector pcDNA3.1 at the *Eco* RI and *Xba* I sites. The sequence of RKIP in the expression vector was confirmed by DNA sequencing.

Human chondrocyte culture. Transient transfections of constructs containing the full-length DDR-2, ΔDS-DDR-2, ΔPTK-DDR-2, Y740A, and RKIP were performed on the immortalized human chondrocyte cell line C-28/I2. The cell lines obtained after infection of juvenile costal chondrocytes with a neomycin-resistant retroviral vector encoding SV40 virus large T antigen, selection in G-418, and cloning (16) were cultured in Dulbecco's modified Eagle's medium (DMEM)–Ham's F-12 (1/1 volume/volume; Invitrogen) containing 10% fetal calf serum (FCS). The cells were cultured at 37°C in an atmosphere of 5% CO_2 and, at >95% confluence, were passaged at a ratio of 1:8 plates every 5–6 days. In each well of a 6-well plate, 200 ng of plasmid construct, 6 μl of Lipofectamine Plus reagent, and 94 μl of serum-free DMEM–Ham's F-12 were mixed and incubated for 15 minutes at room temperature. Lipofectamine Plus reagent (4 μl) in 96 μl of serum-free medium was then added to each reaction mixture, and incubation was continued for 30 minutes at room temperature. Finally, the transfection mixture was combined with 800 μl of serum-free medium and the lipid–nucleic acid complex was transferred to the washed cell monolayer in each well of a 6-well plate.

After incubation for 4 hours at 37°C, the transfection mixture was diluted with an equal volume of DMEM–Ham's F-12 containing 20% FCS, and incubation was continued on either native type II collagen–coated or uncoated plates (as controls) for 24 hours. For preparation of native type II collagen–coated plates, type II collagen from chicken sternal cartilage (catalog no. C-9301; Sigma) was dissolved in 0.25% acetic acid at a concentration of 1 mg/ml, and then used to coat the 6-well plates (10 μg/well).

Treatment of chondrocytes with inhibitors of p38 and JNK. Human C-28/I2 cells were incubated for 1 hour at 37°C with the p38 α and β inhibitor SB203580 (catalog no. 559389), or the JNKs inhibitor SP600125 (catalog no. 420119) (both from Calbiochem, La Jolla, CA) in 1 ml of DMEM–Ham's

F-12 containing 10% FCS. Final concentration of the inhibitors was 10 μM. The cells were then plated in type II collagen–coated 6-well plates containing 1 ml of culture medium with inhibitors, and incubation was continued for 24 hours, as described above.

MMP-13 promoter activity in human chondrocytes. The pGL2B-MMP-13 promoter construct containing –1,007 to +26 bp of MMP-13 subcloned into the pGL2B (basic luciferase) reporter vector (200 ng) (17) was cotransfected with the pcDNA3.1-DDR-2 (200 ng) or pcDNA3.1 empty vector (control) into C-28/I2 cells in 6-well plates using Lipofectamine Plus, as described above. The transfected cells were incubated for 24 hours to permit sufficient expression of DDR-2 and were then scraped off and transferred to type II collagen–coated plates for a further 24-hour incubation, followed by analysis of luciferase activity. Student's *t*-test was used to detect differences in luciferase activity between the 2 groups at a 5% level of significance.

Treatment of chondrocytes with polyclonal antibodies recognizing $\alpha 2\beta 1$ integrin, IL-1β, and IL-1 receptor antagonist (IL-1Ra). C-28/I2 cells were cultured for 24 hours in type II collagen–coated plates as described above, with JBS2, an activating $\alpha 2\beta 1$ integrin polyclonal antibody (10 μg/ml, catalog no. MAB1967), or with BHA2.1, a neutralizing $\alpha 2\beta 1$ integrin polyclonal antibody (10 μg/ml, catalog no. MAB1998) (both from Chemicon). The cells were plated in type II collagen–coated wells and incubated for 1 hour at 37°C in the presence or absence of recombinant human IL-1Ra (100 ng/ml; Amgen, Thousand Oaks, CA). IL-1β (1 ng/ml, catalog no. 201-LB; R&D Systems, Minneapolis, MN) was added to some dishes and further incubated for 24 hours.

RNA extraction, reverse transcription, and quantitative real-time PCR. Total RNAs were isolated from the cultured chondrocytes using the Total RNA Isolation System (Promega, Madison, WI). The cDNA were synthesized with oligo(dT) primer using a Superscript First-Strand Synthesis system (BD Biosciences Clontech, Mountain View, CA). The conditions and primers for real-time PCR have been described in detail previously (8). The PCR primers for DDR-2 were 5′-AACCTGTACAGTGGTGACTA-3′ (forward) and 5′-ACAAAAGGTGAAAGTCTCCCA-3′ (reverse). Student's *t*-test was used to detect differences in mRNA levels between the control and experimental groups at a 5% level of significance.

RESULTS

Increased expression of DDR-2 and MMP-13 in femoral head cartilage from patients with OA. The morphology of hip articular cartilage from patients with symptomatic OA and patients with fracture was evaluated according to the Mankin scale (Table 1). Histology results showed the appearance of chondrocyte clusters and enhanced Safranin O staining around the clusters in OA cartilage (Figure 1A). In 3 patients with OA (Figure 1A, left, middle, and right), loss of articular cartilage was present in the superficial region, and proteoglycan depletion appeared in the interterritorial matrix in the middle and deep zones of the cartilage samples. The mean ± SD Mankin score was 7.8 ± 1.2 in patients with symptomatic OA and 2.5 ± 0.58 in patients with fracture. The between-group difference was statistically significant at $P < 0.02$ by Mann-Whitney U test.

Table 1. Mankin scores for femoral head cartilage from 7 patients with OA and 4 patients without OA*

Patient group, sex/age	Mankin score
Symptomatic OA	
M/65	6
M/82	8
M/?	10
F/?	8
F/70	7
M/85	8
M/81	8
Fracture without OA	
F/75	2
M/80	3
M/?	2
M/60	3

* Femoral head cartilage obtained at the time of hip replacement surgery for osteoarthritis (OA) or for bone fracture in the absence of OA was scored for histologic changes according to the Mankin scale (12).

Immunohistochemical staining for DDR-2 and MMP-13 was relatively absent in the normal cartilage from patients with fracture (Figure 1B, top and middle). In OA cartilage, DDR-2 and MMP-13 were detected in most of the chondrocytes in the superficial and middle zones. The numbers of cells staining positive for the 2 genes were consistent in each OA sample. Immunolocalization of MMP-13 was also consistent with immunostaining for MMP-derived type II collagen fragments in OA cartilage samples, with localized staining in the middle zone and around chondrocytes in the deep zone (Figure 1B, bottom). These results indicated that the increased amount of type II collagen fragments in OA cartilage was associated with increased expression and activity of MMPs, including MMP-13.

Increased expression of DDR-2 and MMP-13 in knee cartilage from mice with surgically induced OA. To determine whether the expression of DDR-2 and MMP-13 was increased in a nongenetic model of OA, we examined knee articular cartilage from mice in which OA was surgically induced. Control joints had negligible amounts of OA-like changes, as reflected by the mean ± SEM of the summed scores of 2.0 ± 0.5 (maximal score 0.9 ± 0.2). OA was progressive at 2, 4, and 8 weeks, as

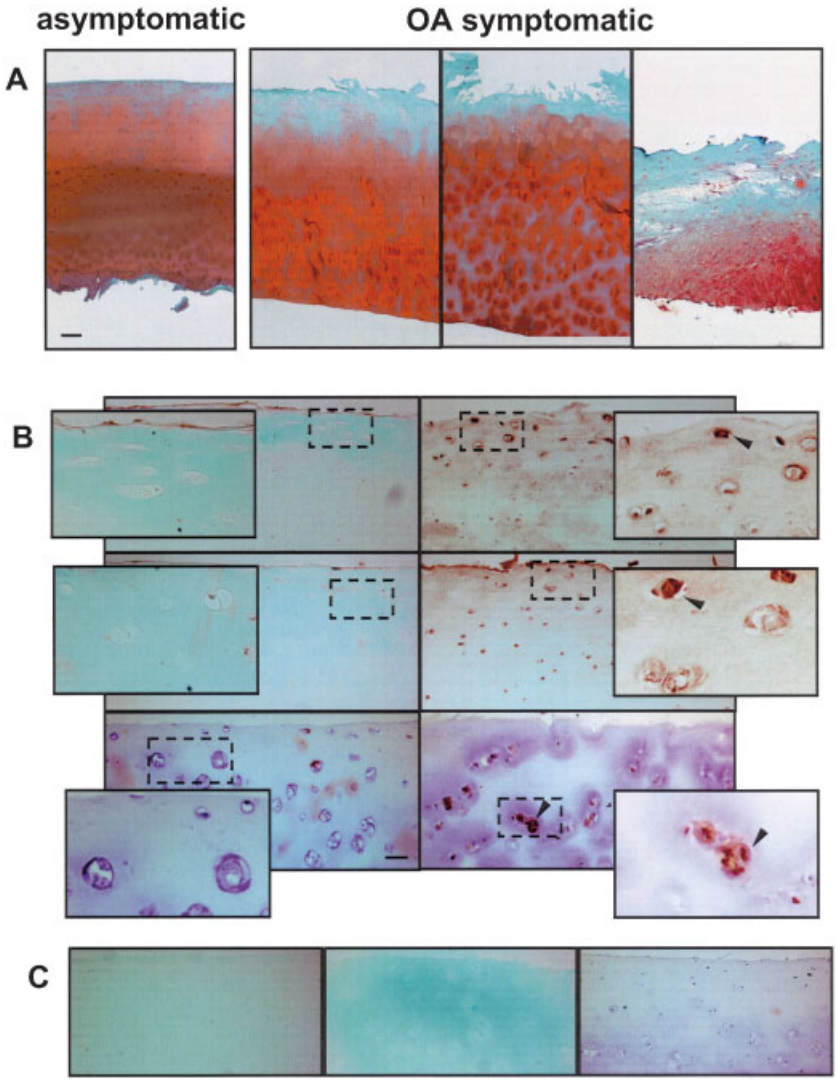

Figure 1. Histologic features and results of immunostaining for discoidin domain receptor 2 (DDR-2), matrix metalloproteinase 13 (MMP-13), and MMP-derived type II collagen fragments in human femoral head cartilage. **A,** Safranin O–fast green staining. Normal (asymptomatic) articular cartilage (left) shows an intact superficial layer with little detectable red staining, a transitional zone with increased red staining, and a deep zone with intense red staining. Red staining indicates proteoglycans; blue staining indicates collagens. Osteoarthritic (OA) cartilage (right) shows a loss of articular cartilage from the superficial layer, with Mankin scores of 6, 8, and 10 (left to right, respectively), in sections from 3 different OA patients. Chondrocyte clusters are present in the transitional and deep zones, and intense red staining appears around chondrocytes (pericellular staining). Bar = 100 μm. **B,** Immunostaining for DDR-2 (top), MMP-13 (middle), and MMP-derived type II collagen fragments (bottom). OA cartilage (right) shows more DDR-2–positive cells (brown staining and **arrowhead** in inset) than does normal cartilage (left). Increased protein expression of MMP-13 is also present in the OA cartilage (brown staining and **arrowhead** in inset), associated with increased amounts of MMP-derived type II collagen fragments (brown staining and **arrowhead** in bottom panel and **inset**). **Insets** are high-magnification views (original magnification $\times$ 40) of the boxed areas shown in the respective main panels. Bar = 50 μm. **C,** Immunostaining of negative controls (without primary antibodies). Negative control experiments were performed for DDR-2 (left), MMP-13 (middle), and MMP-derived type II collagen fragments (right) (original magnification $\times$ 10).

reflected by the mean $\pm$ SEM summed scores of 8.1 $\pm$ 2.2, 24.2 $\pm$ 4.3, and 37.2 $\pm$ 4.9, respectively, with respective maximal scores of 1.6 $\pm$ 0.3, 3.9 $\pm$ 0.3, and 4.5 $\pm$ 0.4.

Typical pathologic features of OA were present in mouse knee joints at 4 weeks after surgery, including proteoglycan degradation and loss of noncalcified cartilage to the level of the tidemark. Minimal changes were

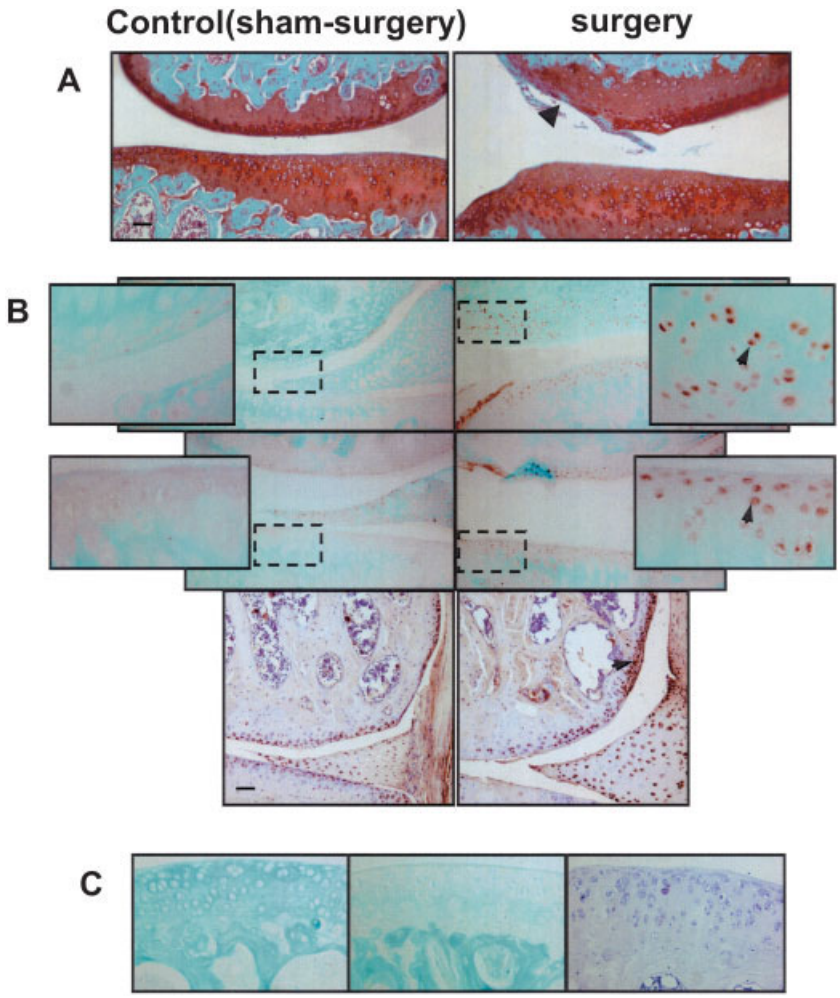

Figure 2. Histologic features and results of immunostaining for discoidin domain receptor 2 (DDR-2), matrix metalloproteinase 13 (MMP-13), and MMP-derived type II collagen fragments in mouse knee cartilage. **A,** Safranin O–fast green staining. Compared with sham-operated control mice (left), knee cartilage from mice with surgically induced osteoarthritis (OA) (right) shows evidence of proteoglycan degradation (**arrowhead**) at 4 weeks after surgery. Bar = 50 μm. **B,** Immunostaining for DDR-2 (top), MMP-13 (middle), and MMP-derived type II collagen fragments (bottom). OA cartilage (right) shows more DDR-2–positive cells (brown staining and **arrowhead** in **inset**) than does control cartilage (left). MMP-13 immunostaining is also increased in OA cartilage (brown staining and **arrowhead** in **inset**), associated with increased amounts of MMP-derived type II collagen fragments (brown staining and **arrowhead**). **Insets** are high-magnification views (original magnification × 20) of the boxed areas shown in the respective main panels. Bar = 50 μm. **C,** Immunostaining of negative controls (without primary antibodies). Negative control experiments were performed for DDR-2 (left), MMP-13 (middle), and type II collagen fragments (right) (original magnification × 10).

observed in cartilage from control knee joints (Figure 2A). Immunohistochemical staining of cartilage sections indicated that the levels of DDR-2, MMP-13, and MMP-derived type II collagen fragments were also increased in the mouse OA samples (Figure 2B, top, middle, and bottom). DDR-2 and MMP-13 were present in the chondrocytes throughout the cartilage, and the number of cells staining positive for the 2 genes was consistent in the samples.

Requirement of the discoidin domain, the PTK core of DDR-2, and Y740 for increased expression of MMP-13 in human chondrocytes. As shown in Figure 3, the endogenous levels of DDR-2 and MMP-13 mRNA expressed in the human chondrocyte line C-28/I2 in response to type II collagen were not reduced in cells transfected with ΔDS-DDR-2. In contrast, in chondrocytes transfected with ΔPTK-DDR-2, the endogenous levels of DDR-2 and MMP-13 mRNA were reduced to

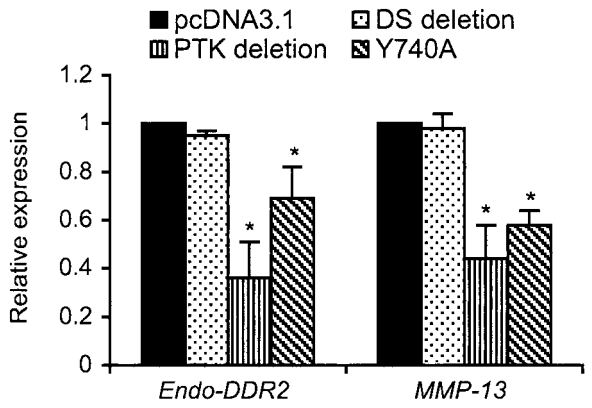

Figure 3. Responses of human chondrocytes to the transient overexpression of discoidin domain receptor 2 (DDR-2) lacking the discoidin (DS) domain (ΔDS-DDR-2), DDR-2 lacking the protein tyrosine kinase (PTK) core (ΔPTK-DDR-2), and DDR-2 containing a substitution of tyrosine for alanine at position 740 (Y740A). C-28/I2 human chondrocytes were transfected with ΔDS-DDR-2 (DS deletion), ΔPTK-DDR-2 (PTK deletion), or Y740A and then cultured for 24 hours on type II collagen–coated plates. Levels of endogenous DDR-2 (Endo-DDR2) and matrix metalloproteinase 13 (MMP-13) mRNA were examined by real-time polymerase chain reaction. The level of endogenous DDR-2 mRNA was not reduced in chondrocytes transfected with ΔDS-DDR-2, but was significantly decreased to ~40% and ~70% by transfection with ΔPTK-DDR-2 and Y740A, respectively. The level of MMP-13 mRNA was not reduced in chondrocytes transfected with ΔDS-DDR-2, but was significantly decreased to ~50% and ~60% by ΔPTK-DDR-2 and Y740A, respectively. Levels in cells transfected with the pcDNA3.1 empty vector alone (control) were set at 1.0. Values are the mean and SEM of 3 experiments. $* = P < 0.05$ versus control.

~40% and ~50%, respectively, of the levels in chondrocytes transfected with the empty vector. We also found that the endogenous levels of DDR-2 and MMP-13 mRNA were reduced to ~70% and ~60%, respectively, in chondrocytes transfected with Y740A.

Reduced expression of MMP-13 induced by overexpression of RKIP or by a chemical inhibitor of p38. We previously found that PD98059, a chemical inhibitor of MEK-1, could specifically inhibit the increased level of MMP-13 mRNA in human chondrocytes cultured on type II collagen, which suggested a role of the MEK/ERK pathway in the DDR-2–mediated response (8). To confirm our observation, we overexpressed RKIP in human chondrocytes. As shown in Figure 4A, the level of MMP-13 mRNA in chondrocytes overexpressing RKIP was ~50% of that in chondrocytes transfected with the empty vector.

To determine whether other MAPK pathways (p38 and JNK) were also involved in the increased expression of MMP-13 in human chondrocytes, we treated chondrocytes with 2 protein kinase inhibitors, SB203580, which is selective for p38 α and β isoforms, and SP600125, which inhibits JNKs 1, 2, and 3. The results indicated that MMP-13 mRNA in chondrocytes treated with SB203580 was reduced by ~80% compared with control levels. The inhibitory effect was similar to that reported previously using the MEK-1 inhibitor PD98059. SP600125 had no appreciable effect on MMP-13 mRNA in human chondrocytes (Figure 4B).

We also investigated whether DDR-2 signaling was independent of $\alpha 2\beta 1$ integrin and IL-1β signaling, since both $\alpha 2\beta 1$ integrin and IL-1β are involved in the induction of MMP-13 expression in chondrocytes (18,19). As shown in Figure 4C, the level of DDR-2 mRNA was not affected by incubation with either JBS2 (activating $\alpha 2\beta 1$ integrin polyclonal antibody) or BHA2.1 (neutralizing $\alpha 2\beta 1$ integrin polyclonal antibody) as compared with chondrocytes cultured on type II collagen without antibody (control). However, the level of MMP-13 mRNA increased ~2.2-fold in chondrocytes treated with the activating antibody JBS2, but not in the presence of the neutralizing antibody BHA2.1 (Figure 4C).

To determine whether IL-1β could account for the enhanced expression of MMP-13 due to type II collagen–induced DDR-2, chondrocytes were cultured for 24 hours in type II collagen–coated plates in the absence (control) or presence of IL-1β, either alone or together with IL-1Ra. IL-1Ra prevented MMP-13 induction by IL-1β, indicating that IL-1Ra could effectively block the IL-1 receptor in our system. However, IL-1Ra did not reduce the level of MMP-13 mRNA induced by DDR-2 in response to type II collagen (Figure 4D).

Increased MMP-13 promoter activity in chondrocytes as a result of DDR-2 signaling. To examine whether the increased level of MMP-13 mRNA in chondrocytes was the result of increased gene transcription, we cotransfected the pGL2B-MMP-13 construct with the pcDNA3.1-DDR-2 expression vector into C-28/I2 cells. The transfected cells were then cultured in type II collagen–coated plates. As shown in Figure 5, the activity of the MMP-13 promoter was elevated ~2-fold by the overexpression of DDR-2.

DISCUSSION

Numerous studies have indicated that MMP-13 may be one of the major enzymes that degrades type II

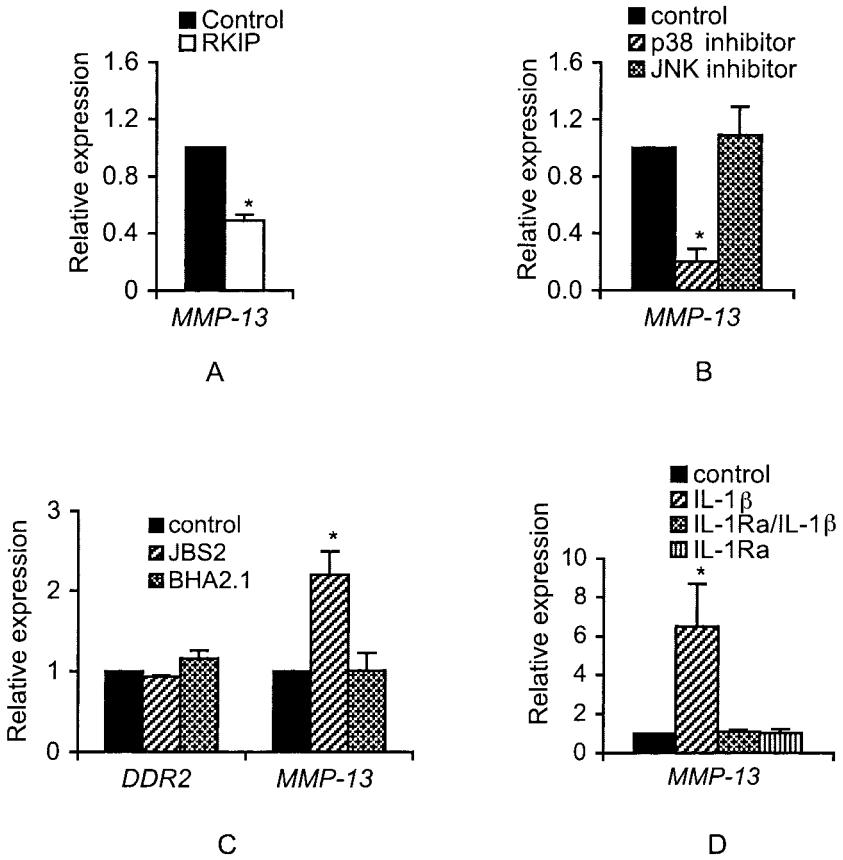

Figure 4. Effects of the overexpression of Raf kinase inhibitor protein (RKIP) and treatment with protein kinase inhibitors, $\alpha 2\beta 1$ integrin polyclonal antibodies, and interleukin-1 receptor antagonist (IL-1Ra) on the levels of matrix metalloproteinase 13 (MMP-13) and discoidin domain receptor 2 (DDR-2) mRNA in human chondrocytes cultured in type II collagen–coated plates. **A,** C-28/I2 cells were transiently transfected with RKIP or empty vector (control) and incubated for 24 hours. The level of MMP-13 mRNA was decreased by ~50% compared with control. There was no difference in the MMP-13 mRNA level due to the overexpression of RKIP in cells plated onto plastic (data not shown). **B,** C-28/I2 cells were treated for 1 hour with the p38 inhibitor SB203580 or the JNK inhibitor SP600125 and cultured for 24 hours on type II collagen–coated plates. The level of MMP-13 mRNA in cells treated with SB203580 was ~20% of the control (type II collagen–induced) level in the absence of inhibitor. The level of MMP-13 mRNA in cells treated with SP600125 was about the same as that in the control. **C,** C-28/I2 cells were cultured for 24 hours on type II collagen–coated plates with either JBS2 (activating $\alpha 2\beta 1$ integrin polyclonal antibody) or BHA2.1 (neutralizing $\alpha 2\beta 1$ polyclonal antibody). The level of DDR-2 mRNA was not affected by incubation with either JBS2 or BHA2.1 as compared with control cells without antibody treatment. The level of MMP-13 mRNA was increased ~2.2-fold by incubation with JBS2 as compared with control cells without antibody treatment, but was not appreciably affected by incubation with BHA2.1. **D,** C-28/I2 cells were treated with interleukin-1β (IL-1β), with or without IL-1 receptor antagonist (IL-1Ra) (see Materials and Methods for details). Treatment with IL-1Ra blocked the IL-1β–induced increase in MMP-13 mRNA, but not the type II collagen–induced expression of MMP-13. Values are the mean and SD of 9 experiments. $* = P < 0.05$ versus control.

collagen in OA articular cartilage (20,21). Fibronectin fragments, IL-1, and tumor necrosis factor α (TNFα) can induce the expression of MMP-13 in chondrocytes. However, fibronectin fragments may not be present until there is significant degradation of the articular cartilage matrix, and IL-1 and TNFα may not be involved inter-

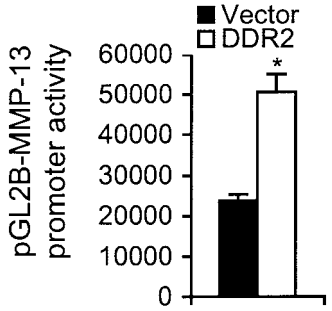

Figure 5. Matrix metalloproteinase 13 (MMP-13) promoter activity in human chondrocytes cultured in type II collagen–coated plates. The pGL2B-MMP-13 reporter vector, containing –1,007 to +26 bp of MMP-13, was cotransfected with the pcDNA3.1-DDR-2 construct or with the pcDNA3.1 empty vector (control) into C-28/I2 cells. After 24 hours of culture, luciferase activity was elevated ~2-fold in cells transfected with discoidin domain receptor 2 (DDR-2) as compared with control ($* = P < 0.05$). Values are the mean and SD of 9 experiments.

mittently during episodes of inflammation. One question remained: Which molecules, if any, can stimulate chondrocytes to synthesize and release MMP-13 in articular cartilage prior to the significant degradation that occurs during the early stage of OA?

In previous studies, we found that the activation of DDR-2 resulted in increased expression of MMP-13 in chondrocytes in vitro (8). We also observed that the levels of DDR-2 and MMP-13 mRNA and proteins were elevated in articular cartilage from the knee and temporomandibular joint of 2 mutant mouse strains, Col9a1$^{-/-}$ and Col11a1$^{cho/+}$ mice, in association with the early onset of OA. These results suggested that DDR-2 may play a role in the early stage of OA, at least in the genetic forms of OA-like pathology. In the present study, findings of increased DDR-2 and MMP-13 in articular cartilage from the femoral heads of patients with OA and from the knees of mice with surgically induced OA suggest that DDR-2 may be one of the key factors in the pathogenesis of OA. Although the femoral head cartilage from OA patients was obtained at the time of joint replacement surgery, which might have been at the end stage of the disease, the remaining cartilage analyzed in our study revealed the characteristic morphology of OA progression. With regard to the knee cartilage from mice with surgically induced OA, we found that immunodetectable DDR-2 and MMP-13 were increased when the proteoglycan degradation became evident at 4 weeks after surgery. This suggests that the increased expression of DDR-2 and MMP-13 occurs after the proteoglycan degradation. This is consistent with our observation in Col9a1$^{-/-}$ and Col11a1$^{cho/+}$ mouse models. In contrast, some clinical studies have suggested that the proteoglycan degradation occurs prior to the breakdown of type II collagen in articular cartilage (22,23).

Taken together, the results lead us to speculate that exposure of the collagen network to chondrocytes under any circumstances, for example, after the depletion of proteoglycans, will permit the interaction of type II collagen with chondrocytes and result in the activation of DDR-2. The activated DDR-2 will induce the expression of MMP-13. This provides an example of a substrate, such as type II collagen in the extracellular matrix, that can regulate its own proteolysis via its cell membrane receptor. Furthermore, type II collagen molecules are present in territorial and interterritorial locations in the extracellular matrix of normal articular cartilage, and there are few or no type II collagen molecules around chondrocytes in the pericellular region (24). This suggests that there is little or no contact between chondrocytes and type II collagen molecules in mature articular cartilage under normal conditions. Thus, the increased expression of DDR-2 resulting from the interaction of chondrocytes with type II collagen may be one of the common steps in OA progression.

To understand the activation mechanism of DDR-2, we investigated the role of the discoidin domain (the type II collagen–binding domain) and the PTK core of the receptor in the increased expression of MMP-13 and the receptor itself in chondrocytes. Our results suggest that the direct interaction of type II collagen with DDR-2 is required for signal transmission. DDR-2 without the type II collagen–binding domain did not affect the expression of endogenous DDR-2, whereas DDR-2 without the PTK core interfered with endogenous DDR-2 signaling.

Although there is no direct evidence to explain the different effects of the absence of the type II collagen–binding domain and the PTK core of the receptor on the endogenous level of DDR-2 in chondrocytes, one plausible explanation is that DDR-2 molecules are monomers in the cell membrane (25), and following binding to type II collagen, these monomers form dimers by ligand-induced dimerization. In our study, we overexpressed DDR-2 without the type II collagen–binding domain in chondrocytes. The mutated DDR-2 may not have been able to bind to type II collagen, and thus, there was no dimerization between endogenous DDR-2 and mutated DDR-2. Hence, the

dimers on the cell membrane are those with 2 endogenous DDR-2 monomers. However, in chondrocytes transfected with DDR-2 without the PTK, 3 kinds of DDR-2 dimers may be formed: 2 endogenous DDR-2 molecules, 1 endogenous DDR-2 with 1 truncated DDR-2 molecule, and 2 truncated DDR-2 molecules. Since we predominantly expressed the truncated DDR-2 molecule, the majority of the dimers may be heterodimers consisting of either 1 normal DDR-2 and 1 truncated DDR-2 molecule or 2 truncated DDR-2 molecules. Under this condition, the DDR-2 signaling is impaired, and as expected, the type II collagen–induced expression of MMP-13 and DDR-2 is down-regulated.

We also investigated the significance of Y740 in type II collagen–induced DDR-2 activation and induction of MMP-13 expression. Contrary to the prediction that Y740 phosphorylation should induce autophosphorylation of other tyrosine residues on the DDR-2 cytoplasmic domain, one group of researchers reported that Y740 inhibited DDR-2 autophosphorylation, since the substitution of tyrosine at position 740 with phenylalanine (Y740F) could stimulate DDR-2 autophosphorylation (26). However, our experiments demonstrated that Y740A prevented the increased expression of MMP-13 and DDR-2, suggesting that Y740 phosphorylation positively regulated the activation of DDR-2. Thus, the Y740F mutation may be inappropriate for investigating the signaling properties of DDR-2, since the substitution of tyrosine with phenylalanine in one of the receptor tyrosine kinases, human epidermal growth factor receptor, has been shown to have no effect on the signaling properties of the receptor (27).

To determine the signaling pathways involved in the up-regulation of MMP-13 and DDR-2 in chondrocytes by type II collagen–induced DDR-2, we overexpressed RKIP in chondrocytes or treated chondrocytes with 2 chemical protein kinase inhibitors. RKIP has been shown to bind Raf kinases and MEK-1/2 kinases to prevent phosphorylation (28). The results from our RKIP experiment confirmed our previous observation that the ERK pathway is implicated in the increased expression of MMP-13, but not in the up-regulation of DDR-2 expression. Data from the experiments with the chemical protein kinase inhibitors indicated that the p38 pathway, but not the JNK pathway, was also involved in the increased expression of MMP-13. Interestingly, other groups of researchers have reported that the phosphorylation of ERK, p38, and JNK is involved in the increased expression of MMP-13 in chondrocytes induced by the activation of $\alpha 2\beta 1$ integrin (18). In this study, we demonstrated that DDR-2 signaling is independent of $\alpha 2\beta 1$ integrin, since activating integrin antibody and type II collagen showed a synergistic effect on the expression of MMP-13 in chondrocytes. IL-1 can also increase MMP-13 expression, but via activation of both p38 and JNK signaling (29). Our results showed that IL-1Ra does not prevent the DDR-2–dependent up-regulation, suggesting that IL-1 does not act as an intermediate in the DDR-2 signaling pathway. Inhibition of the ERK, p38, or JNK pathway does not affect the up-regulation of DDR-2 mRNA due to the receptor activation, although other signaling pathways may be involved in this process.

A study by Verzijl et al (30) indicated that the half-life of cartilage collagen was 117 years. This suggests that chondrocytes may have a limited ability to produce type II collagen in mature articular cartilage once the collagen becomes degraded. Thus, the breakdown of type II collagen in articular cartilage may be a "rate-limiting" step in the progression of OA. Identification of a drug that can prevent the degradation of type II collagen is important, since such a drug may be able to delay the progression of OA and reduce the need for joint replacement. Although data from our study demonstrate that activation of ERK and p38 increases the expression of MMP-13 in chondrocytes by type II collagen–induced DDR-2, we do not think that ERK and p38 are appropriate therapeutic targets for the treatment of OA progression, since they have broad biologic effects in different cells. A common chondrocyte-specific downstream effector of ERK and p38, such as one of the mitogen kinases, may be a good target for the treatment of OA. Furthermore, the transcription activity of the MMP-13 promoter is at least partly responsible for the up-regulation of MMP-13 expression in chondrocytes by type II collagen–induced DDR-2. This provides another site in the search for a therapeutic target for the inhibition of MMP-13 up-regulation, for example, a chondrocyte-specific transcription factor that activates the MMP-13 promoter. In addition, a chondrocyte-specific inhibitor of DDR-2 may also be a target for the treatment of OA.

In summary, our findings suggest a unique mechanism common to OA in humans and in mouse models that involves the activation of DDR-2 and the up-regulation of MMP-13 in the absence of inflammation and without prior collagen damage. This process may be one of the common events in the progression of OA.

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AUTHOR CONTRIBUTIONS

Dr. Xu and Li had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Xu, Goldring, Li.
Acquisition of data. Xu, Peng, Glasson, Lee, Hu, Ijiri, Goldring, Li.
Analysis and interpretation of data. Xu, Peng, Lee, Hu, Ijiri, Olsen, Goldring, Li.
Manuscript preparation. Xu, Glasson, Goldring, Li.
Statistical analysis. Xu, Li.

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