Activation of the Discoidin Domain Receptor 2 Induces Expression of Matrix Metalloproteinase 13 Associated with Osteoarthritis in Mice*†

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Human genetic studies indicate that mutations in type IX and XI collagens result in early-onset osteoarthrosis (OA) with a wide spectrum of osteochondrodysplasia. However, a convincing causal chain of events underlying the role of these collagen mutations in the pathogenesis of OA has not been elucidated. Here we show that the expression of a cell surface collagen receptor, discoidin domain receptor 2 (DDR2), is increased in chondrocytes of the articular cartilage of knee joints in mice that develop OA as a result of a heterozygous mutation in type XI collagen. At the same time point, 6 months, we also found increased expression and activity of matrix metalloproteinase 13 (MMP-13) in the mutant mouse knee cartilage. The expression of both DDR2 and MMP-13 was increased in chondrocytes cultured on plates coated with native type II collagen but not on gelatin, and overexpression of DDR2, but not of a truncated form, was found to induce the expression of MMP-13 when chondrocytes were cultured on type II collagen but not on plastic. The DDR2-induced expression of MMP-13 appears to be specific, since we did not observe induction of MMP-1, MMP-3, MMP-8, ADAMTS-4, ADAMTS-5, and IL-1 transcripts in human chondrocytes or Mmp-3, Mmp-8, Adamts-4, Adamts-5, and Il-1 in mouse chondrocytes. Our data suggest that the defect in the cartilage matrix of mice that are heterozygous for a type XI collagen mutation (cho/+) permits activation and up-regulation of DDR2 in chondrocytes. This could be due to increased exposure of chondrocytes to type II collagen as a result of the decreased amount of type XI collagen in the mutant cartilage. The specific induction of MMP-13 by DDR2 in response to its cartilage-specific ligand, type II collagen, may contribute to cartilage damage in hereditary OA.

OSTEOARTHRITIS (OA),† the most common form of human arthritis (1–3), is considered a group of overlapping distinct diseases with different etiologies but with a similar clinical outcome. Although the causes of OA are diverse, mutations in cartilage specific collagen genes have been identified in human familial OA associated with a wide range of osteochondrodysplasia, from mild to lethal forms (4–7). In particular, mutations in type IX or XI collagens are associated with early-onset OA (8–10). Mutations in genes encoding type IX collagen, COL9A1, COL9A2, and COL9A3, cause multiple epiphyseal dysplasia and intervertebral disc disease in humans. Multiple epiphyseal dysplasia is a milder dysplasia of the epiphyses of peripheral joints that is characterized by cartilage degeneration resulting in early-onset OA. One of the clinical features of intervertebral disc disease is the degeneration of the articular cartilage of the discs. Interestingly, the amount of type IX collagen is reduced in aged people (11), in whom there is high incidence of OA. In humans, mutations in genes for type XI collagen, COL11A1 and COL11A2, have also been identified in several forms of familial OA (12). A reduced amount of type XI collagen is also found in aged people (11). The pathogenetic mechanisms underlying the OA due to these collagen mutations are largely unknown.

We have used a mouse model of chondrodysplasia (cho) to investigate how mutations in type XI collagen cause an OA-like pathology. We found previously that the cho phenotype is due to a single nucleotide deletion leading to frameshift and premature termination of translation of the α1 chain of type XI collagen (13). Electron microscopy of the articular cartilage of knee joints in heterozygous mutant (cho/+ ) mice shows thick collagen fibrils (14), which is one of the early clinical characteristics of human OA (15). The cho/+ mice (homozygous cho/cho mice die at birth) reveal OA-like changes in their knee and temporomandibular joints, starting at the age of 3 months, without other apparent skeletal abnormalities (14). We have also found increased protein expression of matrix metalloproteinase-3 (MMP-3, stromelysin) and MMP-13 (collagenase 3) in knee articular cartilage of cho/+ mice, which is consistent with a recent study of human OA cartilages (16). Although a hereditary defect in type XI collagen is not responsible for most forms of human OA, disruption of matrix composition in cho/+ mice results in the loss of joint stability and associated biochemical alteration that mimics human OA. An additional advantage of the cho/+ mouse is that the degradation of joint structures occurs postnatally with gradual changes throughout the life span of the mouse.

MMPs constitute a group of zinc-dependent proteases that
are involved in remodeling of extracellular matrices in most tissues. MMP-1 (collagenase-1) and MMP-8 (collagenase-2), as well as MMP-3 and MMP-13 function during cartilage development, postnatal growth, and homeostasis to cleave triple-helical type II collagen and degrade proteoglycans in articular cartilage (17, 18). Aggrecanases, particularly ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2), are also important matrix-degrading proteins in the cartilage matrix (19, 20). All of these proteases are expressed at relatively low levels in normal articular cartilage. However, a number of studies have demonstrated that expression of these enzymes is increased in localized regions of human OA articular cartilage and that MMP-13 may be a particularly critical enzyme in the degradation of type II collagen in articular cartilage (21, 22). This is consistent with the OA-like changes in knee joints of mice that constitutively express MMP-13 in cartilage (23). Given the known ability of these enzymes to degrade major components of cartilage matrix, it is likely that their expression in chondrocytes is tightly regulated.

The discoid domain receptors 1 (DDR1) and 2 (DDR2) were identified in humans and mice as cell surface receptor tyrosine kinases (24–27). The mRNA transcripts of the receptors are detected in several human and mouse tissues, such as bone, cartilage, skin, skeletal muscle, brain, and heart. Two research groups reported in 1997 that native collagens were the DDR ligands (28, 29). Interestingly, a synthetic short triple-helical collagen-like peptide (10 repeat of Gly-Pro-Hyp) was not able to activate DDRs. Studies of Ddr1-deficient mice suggest that Ddr1 plays a key role in mammary gland development and arterial wound repair (30, 31). Ddr2-deficient mice exhibit short stature and a delay in the healing of skin wounds (32). Activation of DDR2 by type I collagen increases the level of short stature and a delay in the healing of skin wounds (32).

Four different combinations of primers were tested, forward (nm) / reverse (nm): 200/200, 200/600, 600/200, and 600/600. The optimum concentrations of the primers for each gene were: Mmp-3, 200/200; Mmp-8, 200/600; Mmp-13, 600/600; Adaps-4, 200/200; Adaps-5, 200/ 200; Il-1, 200/200; and Gapdh, 200/200. PCR was performed using 25 μl of 1× PCR buffer containing 50 mM KCl, 3 mM MgCl2, 200 μM dNTPs, each dNTP at 200 μM, and 1 μM of primer, 0.5 unit of TaqDNA polymerase (Qiagen), and 0.5 μl of cDNA. Real-time PCR was performed using the iCycler iQ detection system (Bio-Rad), and the PCR reaction was carried out at 95 °C for 3 min followed by 50 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 2 min. At the end of the PCR cycles, a melting curve, using a temperature range between 55 and 95 °C with +0.5 °C intervals, was achieved to test the specificity of the PCR products. Each experiment was tested in triplicate and each experiment was performed twice. We used Gapdh as an internal control. The efficiency of PCR presented by a standard curve was also tested by plotting the amount of PCR product versus the known amount of a template, 0.001, 0.01, 0.1, 1, and 10 ng. In theory, when the slope of the standard curve is −3.322, the efficiency of PCR is considered 100%. In these experiments, the efficiency reached 90% or higher.

Because type XI collagen is an integral component of the type II collagen fibrils in cartilage, we reasoned that the cho mutation may result in a disruption of the collagen network in cartilage matrix and enhance the interaction between chondrocytes and type II collagen molecules. We report here that the expression of Ddr2, a cell surface receptor interacting specifically with native fibrillar collagen rather than with other matrix components such as fibronectin, proteoglycans, or cartilage oligomeric matrix protein (COMP) is increased in the articular cartilage of knee joints in cho/− mice. Studies in vitro indicate that up-regulation and activation of DDR2 by native type II collagen can specifically induce the expression of MMP-13 by chondrocytes. We also show that the increased expression of Ddr2 and Mmp-13 is associated with type II collagen degradation in adult cho/− mice. Our data suggest a mechanism, involving Ddr2 signaling and increased expression of this receptor tyrosine kinase, for destruction of the collagen network in articular cartilage leading to the age-dependent and progressive OA-like pathology in cho/− mouse joints.

**EXPERIMENTAL PROCEDURES**

### Analysis of Proteinase Activity in Extracts of Knee Articular Cartilage of cho/− and Wild-type Mice

Articular cartilage samples were dissected from the knee joints of 12 cho/− and 12 wild-type mice at the age of 3 or 6 months. We used an EnzChek gelatinase/collagenase assay kit (Molecular Probes, Inc., Eugene, OR) to assay for metalloproteinase activity (38). The articular cartilage samples were pooled from 12 animals from cho/− or wild-type mice at each age and homogenized in a buffer containing 100 mg (wet weight)/ml. 100 μl of the homogenates, 80 μl of 1× reaction buffer, and 20 μl of DQ gelatin solution were added to each well of a 96-well plate for the analysis. For a negative control, 100 μl of 1× reaction buffer instead of the homogenates, and for a standard curve plot, 100 μl of different enzyme dilutions at 0.05, 0.1, and 0.2 unit/ml instead of the homogenates, were added. Each homogenate was analyzed in triplicate. Fluorescence was measured using the LJJ Analysts microplate reader with excitation at 485 ± 10 nm and emission detection at 530 ± 10 nm. Relative proteinase activity was calculated against the standard plot.

### Examination of mRNA Levels of Matrix-degrading Enzymes in Knee Articular Cartilage of cho/− Mice

Knee articular cartilage from mice was dissected at the ages of 3 or 6 months were collected, and the cartilages from the cho/− or wild-type littermates were pooled together for the isolation of total RNAs using the total RNA isolation system (4°C, Mega, Madison, WI). The cDNA was synthesized with oligo(dT) primer using the Super-Script first-strand synthesis system (Clontech). Real-time PCR conditions were optimized for maximal PCR efficiency by the adjustment of concentrations of the following PCR primers: Mmp-3, CCTACTCTTCTTGTAGAGGAC (forward) and GTCAATTCACAATCGCAAGAG (reverse); Mmp-8, GTAAATGTTAGTCTGTCGTCG (forward) and CATAGGGTCGCTGGCAAGGAG (reverse); Mmp-13, GTTGGAGTTATGATGATGTG (forward) and TGCAGATTCTCAGAATACTGT (reverse); Adaps-4, GGCAACCAGGGGCAGGATAC (forward) and CACTCGTTTTITAGAGG (reverse); Adaps-5, ACGCGTACAGTGCAAGATGG (forward) and GTTGCAAAATGACATGAGGCAGC (reverse); Il-1, GAATCTACATCTGGCTCTTG (forward) and TTATGTCGAGCCACTGGTG (reverse); Gapdh, ACTGAGGACCAGTGTTTACATCAGT (forward) and TGCGATCCATGTATTG (reverse).

### Examination of Degraded Type II Collagen in Articular Cartilage of cho/− Mice

Three knee joints from cho/− or wild-type mice at the ages of 3 and 6 months were collected for cryosection. The samples were fixed in 4% paraformaldehyde at 4 °C overnight, followed by embedding in OCT for sectioning. Sections of 10 μm were cut and incubated with polyclonal rabbit anti-cho/− (IBEX Technologies, Inc., Montreal, QC) that recognizes the neoepitope in degraded type II collagen produced by collagenase cleavage. The samples were incubated with biotinylated secondary antibody for the color detection. A negative control, staining without primary antibody, was also performed.

**Transmission Electron Microscopy**—A detailed experimental procedure has been described in the previous publication (14). Briefly, two knee joints each from 1-month-old cho/− mice and two knee joints each from their normal littermates were obtained. The articular cartilage of the joints was exposed, and specimens were fixed in Karnovsky’s solution with 4% glutaraldehyde overnight at room temperature. The samples were then processed for ultra-thin sectioning. Ultra-thin sections from each sample were examined, and multiple locations in each section were randomly selected for photography.

**Immunohistochemical Staining for Collagen Receptors**—Immunohistochemical experiments were performed to characterize the expression of Ddr1, Ddr2, β1 integrin, and annexin V in the articular cartilage of knee joints from cho/− and wild-type mice. Three knee joints from different cho/− mice and three knee joints from different wild-type littermates at the ages of 3 or 6 months were used. The samples were fixed in 4% paraformaldehyde at 4 °C overnight at 4 °C and embedded in paraffin. Serial sections of 8 μm were cut from the medial side to the lateral side. From each joint, four sets of 12 evenly spaced paraffin sections were then processed for ultra-thin (95 nm) sectioning. Ultra-thin sections from each sample were examined, and multiple locations in each section were randomly selected for photography.
Expression analysis of Ddr2 mRNA Levels in Mouse Articular Cartilage—The experimental procedure was as described above using the following PCR primers for Ddr2, 5′-CCGCAAAGGATCGGATGCG (forward) and 5′-CTCGGCTCCTTGCTGAAA-3′ (reverse), at the concentration of 200 nM/200 nM.

Chondrocyte Cultures—Primary costochondral chondrocytes were isolated from 2-week-old C57BL mice and cultured as described in a previous study (39). The immortalized human chondrocyte cell line, C-28/I2, was cultured in modified Eagle’s medium (DMEM)/Ham’s F-12 (1/1, v/v; Invitrogen) containing 10% fetal calf serum for 12 or 24 h. For all experiments, culture plates coated with the various collagen preparations, native (commercial), denatured, and renatured type II collagens, was prepared and purified for transfection into chondrocytes using the EndoFree plasmid maxi-kit (Qiagen).

Transient transfection experiments were carried out in human C-28/I2 cells using Lipofectamine PLUS™ Reagent (Invitrogen). Cells were seeded 24 h prior to transfection in 6-well plates at 3.5 × 10^5 cells/well in DMEM/F-12 containing 10% fetal calf serum. For each well, 200 ng of plasmid construct, 6 μl of PLUS reagent, and 92 μl of serum-free DMEM/F-12 were mixed and incubated for 15 min at room temperature. Lipofectamine PLUS reagent (4 μl) in 100 μl of serum-free medium was then added to each reaction mixture, and incubation was continued for an additional 30 min 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. After incubation for 4 h at 37°C, the transfection mix was diluted with an equal volume of DMEM/F-12 containing 20% fetal calf serum, and incubation was continued for 24 h to permit expression of the wild-type and truncated DDR2 cDNAs. The cells were then scraped and transferred to 6-well plates without or with coating with type II collagen and incubations were continued for 24 h. The levels of MMP-1, MMP-3, and MMP-13 mRNA were examined by real-time PCR, and the PCR conditions were the same as those described above. GAPDH was used as internal control.

Treatment of Chondrocyte Cultures with Protein Kinase Inhibitors—The C-28/I2 cells were cultured in type II collagen-coated 10-cm plates for 4 h allowing the cells to adhere. Protein kinase inhibitors were added as follows: 1) MEK inhibitor, PD98059 at 10 μM (from CALBIOCHEM); 2) phosphodiesterase, 5′-kinase/Akt inhibitor, Wortmannin at 25 μM (from Calbiochem); and 3) JAK2 inhibitor, AG490 at 10 μM (from Calbiochem). No protein kinase inhibitor was added to control cultures. Incubations were carried out for 24 h, and the cells were washed twice with cold PBS and harvested for RNA extraction.

RESULTS

Age-related Increase in Matrix Metalloproteinase Activity in Articular Cartilage of Knee Joints in chol+ Mice—To determine whether the age-related cartilage degradation that we previously observed in chol+ mice was associated with increased activities of cartilage-degrading proteinases, we analyzed metalloproteinase activity in extracts of articular cartilage from knee joints. Using a fluorescent substrate to detect combined collagenase/gelatinase activity, we found that metalloproteinase activity was increased about 2-fold (left panel) and 6-fold (right panel) in chol+ mice at the ages of 3 and 6 months, respectively, compared with wild-type littermates.

EndoFree plasmid maxi-kit (Qiagen).

To determine more specifically which matrix-degrading enzymes are up-regulated in the articular cartilage of knee joints in chol+ mice, we examined the levels of Mmp-3, Mmp-8, Mmp-13, Adams-4, and Adams-5 mRNAs.
were set at 1.0.

Values representing levels in wild-type mice (Mmp-3) but not at 3 months (the left panel) and 6 months (the right panel). Mmp-8 was unchanged in 3 months and undetectable in 6 months. Values representing levels in wild-type mice were set at 1.0.

using real-time PCR. We also examined interleukin-1 (Il-1) mRNA, since Il-1 may be associated with increased cartilage degradation in this model. Real-time PCR analysis of cartilage extracts showed that the mRNA level of Mmp-13 was increased 3-fold in cho+/+ mice at the age of 6 months; no difference between mutant and wild-type mice was seen at 3 months (Fig. 2). In contrast, the mRNA levels of the other proteinases tested were either not increased (Mmp-3) or undetectable (Mmp-8) in articular cartilage from cho+/− mice compared with wild-type littermates at the age of 6 months. These results suggest that Mmp-13 may be a major factor contributing to the age-related articular cartilage degradation in cho+/− mice.

Increased Degradation of Type II Collagen in Articular Cartilage of cho+/− Mice—To understand whether the high-level expression of Mmp-13 in articular cartilage of cho+/− mice results in the increased activity of the enzyme, we examined the degraded type II collagen in the articular cartilage by immunostaining using an antibody that recognizes the carboxyl terminus of the three-quarter fragment produced by collagenase activity. There was no difference in degraded type II collagen at the age of 3 months in cho+/− mice compared with wild-type mice (data not shown). However, more degraded type II collagen molecules (brown color staining) were observed in cho+/− mice at the age of 6 months (Fig. 3). We noticed that more degraded type II collagen appeared in the pericellular matrix. These data suggest that the collagenase activity of Mmp-13 is increased in cho+/− mice.

Altered Ultrastructure of Extracellular Matrix and Expression of a Collagen Receptor in Articular Cartilage of cho+/− Mice—Altered contact between type II collagen fibrils and a collagen receptor on chondrocytes may play a role in initial and/or progression in OA pathogenesis, at least in the case of hereditary OA caused by type IX and type XI collagen mutations. In the articular cartilage of wild-type mouse knee joints, we observed some type II collagen fibrils around the chondrocytes, but more fibrils are located in the interterritorial region (Fig. 4A, bottom panel). In contrast, the increased number of thick type II collagen fibrils were concentrated in the pericellular region in cho+/− mice (Fig. 4A, top panel). These results suggest the possibility that the exposure of chondrocytes to type II collagen fibrils in the pericellular matrix is enhanced in cho+/− mice.

To determine whether the expression of collagen receptors on chondrocytes is affected by the cho+/− mutation, we examined the protein expression of three different types of collagen receptors, Ddr1 and Ddr2, β1 integrins, and annexin V, in the articular cartilage of knee joints at the ages of 3 and 6 months.

As shown in Fig. 4B (top panels), increased numbers of Ddr2-positive cells were observed in the articular cartilage of cho+/− mice at the age of 6 months compared with wild-type mice, whereas no difference could be detected at 3 months (data not shown). There was no difference observed in the staining intensity of annexin V when cho+/− and wild-type mice were compared at either age (Fig. 4B, lower panels). Ddr1 and β1 integrins were barely detected by immunohistochemistry at both ages, and no difference could be detected between cho+/− and wild type mice (data not shown).

To determine whether the increased protein expression of Ddr2 was due to increased gene expression, we examined the level of Ddr2 mRNA in mice at the ages of 3 and 6 months by real-time PCR. As shown in Fig. 4C, the level of Ddr2 mRNA in cho+/− knee joints was not increased at the age of 3 months, but it was increased 3-fold compared with wild-type mice at the age of 6 months. These data indicate that the expression of Ddr2 in the articular cartilage of cho+/− knee joints at 6 months of age is increased at both the mRNA and protein levels.

Increased Ddr2 and Mmp-13 mRNA Levels in Chondrocytes Cultured on Type II Collagen-coated Plates—Since fibrillar collagens are known to activate Ddr2 and thereby induce expression of a metalloproteinase (MMP-1) (28, 29), we performed in vitro experiments to determine the effects of culturing chondrocytes on type II collagen-coated plates on the expression of matrix-degrading enzymes. Mouse cells with phenotypic characteristics of chondrocytes, including polygonal morphology and extracellular matrix staining by Alcian blue, were obtained from costal cartilage. Col2a1 mRNA was detected in cultured mouse chondrocytes by RT-PCR, whereas Col10a1 mRNA, a marker for hypertrophic chondrocytes (34), was absent, indicating that the cells in the cultures were proliferating, nonhypertrophic chondrocytes. The immortalized human chondrocyte cell line, C-28/I2, was also used as a model to further experiments to study mechanisms. We prepared culture plates coated with native type II collagen and used non-coated plates as controls.

Real-time PCR analysis demonstrated that the level of Mmp-13 mRNA was elevated about 5-fold in human chondrocytes cultured on type II collagen for 24 h (Fig. 5A). However, the level of MMP-1 mRNA was not increased above the low constitutive level. The mRNAs for Mmp-3, Mmp-8, ADAMTS-4, ADAMTS-5, and IL-1 were not detectable. Surprisingly, we found that the level of Ddr2 mRNA in human chondrocytes was also increased (about 6-fold) after incubation on collagen for 24 h. In mouse chondrocytes, Mmp-13 and Ddr2 mRNA levels were increased about 7- and 8-fold, respectively, in cells cultured in type II collagen-coated wells for 24 h compared with cells cultured in non-coated wells (Fig. 5B). The level of Mmp-3 mRNA was not increased. The mRNAs for Mmp-8, Adams-4, Adams-5, and II-1 were not detectable in cultured mouse chondrocytes. These results indicate that chondrocytes exposed to native type II collagen for 24 h are induced to express increased...
levels of DDR2 and MMP-13 mRNA. The absence of induction at 12 h suggests that the response requires early cellular events such as activation of gene expression and protein synthesis.

We also examined the responses of the human chondrocytes to denatured (gelatin) and renatured type II collagen. Culture on denatured collagen had no effect on levels of expression of MMP13 and DDR2 mRNA, whereas culture on renatured type II collagen increased the levels of DDR2 and MMP-13 mRNAs by 4- and 5-fold, respectively (Fig. 5C). These results indicate
that chondrocytes respond in a specific manner to triple-helical type II collagen.

**Increased MMP-13 mRNA Level in Human Chondrocytes in Response to Transient Overexpression of Full-length DDR2 cDNA**—To better understand the cause-effect relationship between DDR2 and MMP-13, we transfected full-length DDR2 cDNA (Fig. 6A) into human chondrocytes and cultured the cells on type II collagen-coated plates for 24 h. As shown in Fig. 6B, left panel, the level of DDR2 mRNA was increased about 4-fold, and MMP-13 mRNA was increased about 8-fold. This result indicates that overexpression of DDR2 and its activation by type II collagen results in induction of MMP-13 mRNA in chondrocytes.

To further establish that the MMP-13 response is linked to activation of DDR2, we transfected a truncated DDR2 cDNA (lacking the cytoplasmic domain) (Fig. 6A) into human chondrocytes. After the transfected cells were cultured on type II collagen-coated plates for 24 h, the levels of endogenous DDR2 and MMP-13 mRNA were reduced by about 60 and 65%, respectively (Fig. 6B, right panel). In addition, we also found that there was no change in the level of MMP-13 mRNA in human chondrocytes transfected with the full-length or truncated DDR2 and cultured on non-coated plates for 24 h (Fig. 6C). These results

**FIG. 5.** Induction of DDR2 and MMP-13 mRNA in chondrocytes cultured on type II collagen. A, analysis of mRNA levels in human chondrocytes. The mRNA levels of DDR2, MMP1, and MMP13 were detected but not increased in C-28/I2 cells cultured in collagen-coated plates for 12 h compared with uncoated plates. However, DDR2 and MMP-13, but not MMP-1, mRNA levels were increased about 6- and 5-fold, respectively, in chondrocytes cultured in collagen-coated plates for 24 h. B, analysis of mRNA levels in mouse chondrocytes. Drs2 and Mmp13 mRNA levels were increased in chondrocytes cultured for 24 h in collagen-coated plates compared with uncoated control. Mmp3 mRNA was detected and but showed no increase. C, analysis of mRNA levels in human chondrocytes cultured in plates with denatured (gelatin) or renatured type II collagen. There was no difference in the mRNA levels of DDR2 and MMP-13 in C-28/I2 cells cultured on gelatin. However, the DDR2 and MMP-13 mRNA levels were elevated about 4- and 5-fold, respectively, in cells cultured on renatured type II collagen. Values representing levels in uncoated plates were set at 1.0.

**FIG. 6.** Response of human chondrocytes to transient overexpression of full-length or truncated DDR2 cDNA. A, construct for expressing full-length and truncated DDR2 cDNAs. The full-length DDR2 cDNA contained 2568 nucleotides including extracellular domain, 1–1200, transmembrane domain, 1201–1263, and intracellular domain, 1264–2568. Within the cytoplasmic domain, the tyrosine kinase catalytic unit corresponds to the region 1687–2547 nucleotides. The truncated DDR2 cDNA construct does not contain the tyrosine kinase catalytic unit. B, overexpression of full-length DDR2 or truncated DDR2 cDNA in human chondrocytes cultured on type II collagen. Levels of DDR2 and MMP-13 mRNA were increased in the C-28/I2 cells transfected with full-length DDR2 cDNA compared with cells transfected with the vector alone, whereas there was no change in the level of MMP-1 mRNA (left panel). When the chondrocytes were transfected with the truncated DDR2 cDNA and cultured on type II collagen for 24 h, the levels of endogenous DDR2 and MMP-13 mRNA were reduced by about 60 and 65%, respectively (right panel) (note: the difference in scale between the left and right panels). C, overexpression of full-length or truncated DDR2 cDNA in C-28/I2 cells cultured in non-coated plates. There was no change in the MMP-13 mRNA level when cells transfected either with the full-length or truncated DDR2 were compared with cells transfected with vector alone. Values representing levels in cells transfected with vector alone were set at 1.0.
FIG. 7. Effects of protein kinase inhibitors on the levels of DDR2 and MMP-13 mRNA in human chondrocytes cultured on type II collagen. The C-28/I2 cells were plated on type II collagen and treated with different protein kinase inhibitors for 24 h, and the levels of DDR2 (left panel) and MMP-13 mRNA (right panel) were analyzed by real-time PCR.

demonstrate that activation of DDR2 induces MMP-13 mRNA levels and that the interaction between chondrocytes and type II collagen is required for the elevation of MMP-13 and DDR2 mRNA levels in chondrocytes.

The MEK1 Inhibitor, PD98059, Suppresses the Induction of MMP-13 mRNA Levels in Human Chondrocytes Cultured on Type II Collagen-coated Plates—To examine the potential signaling pathways involved in the up-regulation of MMP-13 by type II collagen-dependent DDR2 activation, we examined the effects of pretreatment with three protein kinase inhibitors, AG490 (JAK-2 inhibitor), wortmannin (phosphatidylinositol 3-kinase inhibitor), and PD98059 (MEK1 inhibitor). As shown in Fig. 7, the DDR2 mRNA levels in all three inhibitor-treated cells were increased, but the level of MMP-13 mRNA in chondrocytes cultured on type II collagen-coated plates with the inhibitor, PD98059, was reduced to 25% of the level in cells cultured on type II collagen-coated plates in the absence of inhibitor (as control). In chondrocytes treated with the other two inhibitors, AG490 and wortmannin, the MMP-13 mRNA levels were the same as in the control cells. These results indicate that the MEK1 inhibitor does not interfere with the pathways involved in up-regulation of DDR2 mRNA but inhibits signaling downstream of DDR2, resulting in decreased MMP13 expression.

DISCUSSION

In this study, we found that metalloproteinase activity was slightly increased in articular cartilage of knee joints of chal+/ mice at the age of 3 months and further increased with aging. This is consistent with our previous observation that the initial sign of articular cartilage degeneration in knee and temporomandibular joints of chal+/ mice is detected at the age of 3 months. By real-time PCR we found that mRNA levels of several proteinases including Mmp3, Mmp8, Mmp13, Adamts-4, and Adamts-5 were not elevated in the articular cartilage of knee joints in chal+/ mice at the age of 3 months. It is possible that the increased enzyme activity at 3 months is due to accumulation of protein synthesized over time and to the post-translational modification and activation of the previously synthesized enzymes. In our previous study, we found that the protein expression of Mmp-3 is increased in chal+/ mice at the age of 3 months. However, the increased protein expression is not reflected in elevated Mmp-3 mRNA level in chal+/ mice in analysis performed by real-time PCR at a single time point. One possibility is that the pooling of the cartilages from chal+/ mice for real-time PCR may dilute the regional increase of Mmp-3 mRNA. Furthermore, it is unlikely that the expression and activity of Mmp-13 in chal+/ mice at the age of 3 months are elevated because we do not observe the difference in Mmp-13 immunostaining (14) or in the degradation of type II collagen between wild-type and chal+/ mice at the age of 3 months. However, the further increase in metalloproteinase activity with age in chal+/ mice at the age of 6 months is associated with the easily detectable increase in Mmp-13 mRNA and protein levels. In addition, more degraded type II collagen molecules appear in chal+/ articular cartilages as the consequence of a high level activity of Mmp-13. Levels of mRNAs encoding other enzymes with collagen- and proteoglycan-degrading activities either were not increased or were undetectable in the articular cartilage of knee joints of chal+/ mice. These results suggest that level and activity of Mmp-13, an enzyme that can degrade aggrecan as well as collagen (35), may be a critical factor in the progression of OA in knee joints of chal+/ mice. This finding is consistent with numerous reports that MMP-13 is up-regulated in the articular cartilage of human OA joints (16, 21, 22).

As a result of the type XI collagen mutation in cho mice, thick type II collagen fibers are present and the integrity of the type II collagen fibril network is altered (14). Although the amount of type II collagen in the articular cartilage of mutant knee joints may not be affected, the distribution of type II collagen in the articular cartilage is affected, the increased number of thick type II collagen fibers in close contact with chondrocytes. This altered distribution of type II collagen may enhance the interactions between chondrocytes and type II collagen resulting in the altered expression of a collagen receptor. Our data demonstrate that the protein and mRNA levels of Ddr2 are increased in the articular cartilage of chal+/ mice at the age of 6 months. We did not detect an altered expression of other collagen receptors, including β1 integrins, annexin V, and Ddr1 on chondrocytes. This suggests that the up-regulation of Ddr2 mRNA and protein may result from the receptor binding to its own ligand, type II collagen, in chal+/ mice.

The correspondence between the increased levels of Ddr2 and Mmp-13 protein in the articular cartilage of chal+/ mice prompted us to investigate a possible role of Ddr2 in regulating the expression and activities of other matrix-degrading proteinases in chondrocytes. Data from our in vitro experiments demonstrate that the mRNA level of MMP-13 is increased in human and mouse chondrocytes when the cells are cultured on type II collagen-coated plates for 24 h and that the mRNA levels of other matrix-degrading proteinases including Mmp-1, Mmp-3, Mmp-8, ADAMTS-4, and ADAMTS-5 are either not increased or undetectable. Our data also showed that the mRNA level of DDR2 is increased in cells cultured on type II collagen. Furthermore, we showed that transfection of a full-length DDR2 cDNA in human chondrocytes results in elevated levels of MMP-13 mRNA. In contrast, the level of MMP-13 mRNA is reduced after transfer of a truncated DDR2, lacking the cytoplasmic domain, into human chondrocytes cultured on type II collagen. On the basis of these data, we conclude that signaling through DDR2 results in up-regulation of MMP-13 in chondrocytes cultured on type II collagen-coated plates. In addition, we conclude that interaction between chondrocytes and type II collagen is required, since the MMP-13 mRNA level is not changed in chondrocytes, when they are cultured on plastic, after transfection with either full-length or truncated DDR2 cDNA.

The data indicate that the activation of DDR2 by type II collagen, not only increases expression of MMP-13, but also up-regulates the expression of DDR2 itself in chondrocytes. Since there is little information on the immediate downstream signaling pathways activated by DDR2, we examined three signaling pathways to understand if these pathways are implicated in the up-regulation of MMP-13 by DDR2 activation. Based on our results, we conclude that the Ras/Raf/MEK/ERK...
pathway is involved in the increased expression of MMP-13 by DDR2 activation but not in the up-regulation of DDR2 expression.

It has been reported that IL-1 can induce MMP-13 expression in chondrocytes and that signal transduction by the NF-κB, p38 MAPK, and JNK pathways are required for IL-1-induced transcription of MMP-13 (36, 37). To know whether IL-1 is an intermediate in the regulation of MMP-13 expression by activation of DDR2, we examined IL-1 mRNA levels in mouse and human chondrocytes cultured on type II collagen for 12 or 24 h. Since IL-1 mRNA was undetectable in the chondrocytes cultured on type II collagen-coated plates, it is unlikely that IL-1 plays a role in the regulation of MMP-13 expression by DDR2 activation in our chondrocyte culture system.

The activation of DDRs requires native collagens such as type I, II, and III collagens (28, 29). Interestingly, we found that the increased number of thick type II collagen fibers in pericellular region in chol/+ articular cartilage. We speculate that the altered structure and arrangement of type II collagen fibrils in the cartilages of chol/+ mice may elicit the interaction between chondrocytes and type II collagen fibrils. As a consequence, DDR2 is activated to trigger a series of cellular events leading to synthesis, release, and activation of matrix-degrading proteases such as MMP-13 resulting in OA.

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