Expression of the Cartilage Derived Anti-Angiogenic Factor Chondromodulin-I Decreases in the Early Stage of Experimental Osteoarthritis

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ABSTRACT. ***Objective.*** Chondromodulin-I (ChM-I), a cartilage derived anti-angiogenic factor, has been shown to regulate the vascular invasion during endochondral bone formation. We evaluated the expression and localization of ChM-I in articular cartilage during the progression of osteoarthritis (OA) in the rat, and correlated ChM-I expression with the increase in vascular invasion into OA articular cartilage.

Methods. Expression of ChM-I, type II collagen, basic fibroblast growth factor, vascular endothelial growth factor (VEGF), and matrix metalloproteinases MMP-9 and MMP-13 were examined in articular cartilage of intact growing and adult rats and in the surgically induced OA model using *in situ* hybridization, Western blot analysis, and immunohistochemistry. Co-immunostaining for ChM-I and CD-31 was performed to localize ChM-I and neovascularization in articular cartilage at advanced stage of OA.

Results. Abundant expression of ChM-I protein was detected in avascular regions of the developing and adult healthy articular cartilage. In early OA, ChM-I expression decreased in the superficial zone of articular cartilage, while levels of proteoglycan and type II collagen were comparable to control. In advanced OA, ChM-I expression was reduced in all zones of articular cartilage, and the number of VEGF-expressing cells was increased. Immunohistochemical studies showed that vascular invasion occurred in proximity to chondrocytes with high expression of pro-angiogenic markers, and decreased expression of ChM-I.

Conclusion. High expression of ChM-I was detected in articular cartilage of growing and normal adult joints, implicating its role in the maintenance of avascularity of intact articular cartilage. Expression of ChM-I decreased, while expression of VEGF and other pro-angiogenic factors increased, in OA cartilage. These findings suggest the loss of ChM-I from articular cartilage might be responsible in part for promoting blood vessel invasion into the cartilage during progression of OA. (J Rheumatol 2003;30:2207–17)

Key Indexing Terms:
CHONDROMODULIN-I ARTICULAR CARTILAGE OSTEOARTHRITIS
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Articular cartilage is unique among the tissues of mesenchymal origin in that it is extraordinarily resistant to vascular invasion[1-3]. Normal articular cartilage remains avascular throughout life, while epiphyseal cartilages are replaced by osseous tissue through endochondral ossification during skeletal development[4]. The mechanism underlying preservation of avascularity in articular cartilage is unclear; however, it has been suggested that hyaline cartilage contains high concentrations of endogenous inhibitors of angiogenesis[3].

In osteoarthritis (OA) articular cartilage, blood vessels have been observed to penetrate into the calcified zone of cartilage[5,6]. Vascular invasion was shown to play a key role in the progression of cartilage degradation, leading to the reinitiation of endochondral bone formation and subsequent increase in subchondral bone density and cartilage thinning[5,6].

On the other hand, vascular endothelial growth factor (VEGF) expressed by hypertrophic chondrocytes was found to act as a potent angiogenic inducer during the process of endochondral bone formation during skeletal development[7]. Recently, concentrations of VEGF were found to be

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elevated in the cartilage and synovial fluids derived from patients with rheumatoid arthritis (RA) and osteoarthritis (OA)[8-11]. This supports the hypothesis that surface damage of articular cartilage together with increased subchondral bone formation cooperatively enhance mechanical forces in the uncalcified cartilage that lead to further destruction of OA cartilage[12]. To understand the pathogenesis of OA, it is thus important to examine the mechanism by which vascular invasion occurs in articular cartilage during the progression of OA.

Cartilage derived chondromodulin-I (ChM-I) was reported to stimulate chondrocyte proliferation and proteoglycan synthesis *in vitro*[13,14]. Moreover, ChM-I was shown to inhibit proliferation of vascular endothelial cells *in vitro*[14,15] and *in vivo*[16]. In embryonic epiphyseal cartilage, both mRNA and protein of ChM-I localize to the avascular zones, and were significantly reduced in the deeper hypertrophic and calcified zones, where angiogenesis was observed[14]. These findings suggest that the loss of ChM-I protein preceded vascular invasion, leading to endochondral bone formation in embryonic epiphyseal cartilage.

We report that ChM-I is highly expressed in articular cartilage of growing and adult rats, supporting the role of ChM-I as an anti-angiogenic factor in articular cartilage. Further, we show that ChM-I expression decreases in the hyaline cartilage matrix in the early stage of OA, which occurs concomitantly with an increase in VEGF and matrix metalloproteinase (MMP)-9 and MMP-13 expression, leading to vascular invasion in OA cartilage.

MATERIALS AND METHODS

Rat OA model. All procedures were carried out according to the Institutional Animal Care and Use Committee Guide of Niigata University School of Medicine. Fifty-five intact male Sprague-Dawley rats (Charles River Breeding Labs, Japan) were used in this study (n = 5 per group). Normal articular cartilage from growing (4 and 10-week-old) and adult (36-week-old) rats was histologically examined.

OA was surgically induced in 4-week-old rat knee joints with some modification of described procedures[17]. Briefly, the right knee joint was exposed via medial parapatellar approach. The anterior cruciate ligament and medial collateral ligament were severed, and medial meniscus was resected. After surgery, the joint surface was washed with sterile saline solution, and both capsule and skin were sutured using monofilament 4-0 nylon. Rats were then allowed to move freely in the cages. The animals were sacrificed on Day 5 and 2, 6, 8, and 10 weeks after surgery with sodium pentobarbital.

Tissue preparation. Resected knee joints were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffered saline (PBS) at 4°C for 24 h. Tissues were decalcified in 0.5 M EDTA solution (pH 7.6), then embedded in paraffin. Normal and OA cartilage specimens were subjected to histological analyses, *in situ* hybridization, and immunohistochemistry. Prior to protein extraction as described below, articular cartilages of distal femur and proximal tibia of both OA (right) and control (left) knee joints were carefully shaved to exclude subchondral bone and marrow tissues.

Immunohistochemistry. Tissue sections were deparaffinized in xylene, hydrated in graded ethanol, then treated with 500 U/ml testicular hyaluronidase (Sigma, St. Louis, MO, USA) at 37°C for 60 min. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 5 min. Tissue sections were then incubated with primary antibodies for 60 min at 25°C, using either anti-rat ChM-I polyclonal antibodies (Pab), anti-rat type II collagen (LSL, Tokyo, Japan) Pab, anti-rat CD31 monoclonal antibody (Mab; Endogen Inc., Woburn, MA, USA), anti-VEGF Mab (Oncogene Research Products, San Diego, CA, USA), anti-MMP-9 (Cell Sciences, Norwood, MA, USA), or anti-MMP-13 antibodies[18]. All antibodies were prepared with a diluent solution (Dako, Carpinteria, CA, USA). After rinsing in PBS, they were incubated with horseradish peroxidase (HRP) conjugated or alkaline phosphatase (AP) conjugated anti-mouse or anti-rabbit antibody (Envision Plus System, Dako) for 30 min as described[18,19]. These sections were rinsed again with PBS, and developed in 0.5 mg/ml 3,3'-DAB (Vector, Burlingame, CA, USA), 50 mM Tris HCl (pH 7.6), 0.01% hydrogen peroxide, or fast red substrate chromogen system (Dako) for 2 min.

Double-labeled immunohistochemical studies with anti-rat CD31 and ChM-I antibodies were as described with modifications[20]. Briefly, tissue sections were incubated with anti-rat CD31 Mab, followed by AP conjugated anti-mouse antibody, and developed red using Vector Red I® (Vector). They were washed twice with glycine/HCl buffer for 1 h, incubated with anti-ChM-I Pab, then with HRP-anti-rabbit antibody, and developed black with 0.5 mg/ml 3,3'-DAB and $NiCl_2$ (Vector). As a negative control, the same procedures were carried out either without primary antibody or with preimmune sera.

In situ hybridization. Plasmids (in pGEMeasy) encoding for rat ChM-I cDNA sequence were prepared as described[15,21]. Plasmid was linearized by appropriate restriction enzyme and transcribed to generate antisense/sense digoxigenin-11-UTP labeled single-strand RNA probes using a digoxigenin RNA labeling kit (Boehringer Mannheim, Germany). *In situ* hybridization was as described[21-23]. Each section was digested in 5 µg/ml of proteinase K (Boehringer Mannheim) at 37°C for 20 min, and hybridized with antisense probes at 50°C for 16 h. Sense probes were used as negative control. Consecutive sections were stained by hematoxylin-eosin (H&E) for histological characterization. Toluidine-blue-O stained slides were used to estimate the proteoglycan content in the cartilage[24].

Western blot analysis. Western blot was performed as reported[25], using either anti-rat ChM-I, anti-VEGF, anti-type II collagen (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-basic fibroblast growth factor (bFGF; Advanced Targeting Systems, San Diego, CA, USA), or anti-α-tubulin (Sigma). Surgically induced OA in 5 rats was as described above. One week after the surgery, proteins were extracted from both medial and lateral compartments of OA (right) and normal (left) knee joint articular cartilages, respectively. In brief, proteins were extracted with 8 M urea, 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA. After overnight incubation, the samples were centrifuged for 20 min at 14,000 g to remove debris. Protein concentrations were determined by BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL, USA) according to the manufacturer's instructions. Samples (25 µg each) were separated on 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted onto Immobilon-P membrane. After blocking with 100 mM NaCl, 10 mM Tris, 0.1% Tween-20, and 2% bovine serum albumin, the membrane was incubated with primary antibodies, followed by HRP conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) and detected with the ECL chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). To confirm equal protein loading, the gel was stained with Coomassie-blue. Protein bands in the blots were quantitated using an imaging densitometer (model GS-700, BioRad, Hercules, CA, USA). Recombinant mouse VEGF-120 and –165 (R&D Systems, Minneapolis, MN, USA) and recombinant human bFGF were used as positive controls.

RESULTS

High expression of ChM-I in normal articular cartilage in early development. Developmental changes of the lateral

tibial plateaus were examined by histological and immunohistochemical analysis. Normal articular cartilage in growing rats (4-week-old) exhibited smooth surfaces with intact layers of flattened cells in the superficial zone and hypertrophic-like chondrocytes in the deep zone (Figure 1A). With immunostaining, ChM-I expression was detectable in the superficial, and intense in the middle and upper deep zones of the cartilage. No ChM-I expression was detected in the lower deep zone or in the calcified cartilage. Interestingly, ChM-I was detected in the cytoplasm as well as in surrounding extracellular matrix (ECM) of the articular chondrocytes (Figure 1B). Immunostaining for type II collagen showed a diffused pattern of expression in all zones of articular cartilage (Figure 1C).

In 10-week-old rat knee joints, thinning of articular cartilage was apparent, possibly due to increased endochondral ossification associated with expansion of the secondary ossification centers. In addition, the 10-week-old articular cartilage appeared to be hypocellular compared to that in 4-week-old rats. However, high ChM-I protein expression was detected in chondrocytes and the surrounding ECM of the middle and deep zones of articular cartilage, and to a lesser extent in cells of the superficial zone. Type II collagen immunostaining was detected in all zones of the articular cartilage (Figures 1D, 1E, 1F).

In 36-week-old knee joints, tidemarks separating the calcified zone of articular cartilage and the thin layer of uncalcified articular cartilage were clearly observed to be associated with an intensely Toluidine-blue-positive metachromasia region (Figure 1G). We could not detect any significant signal for ChM-I mRNA by *in situ* hybridization (data not shown), although its protein products were weakly

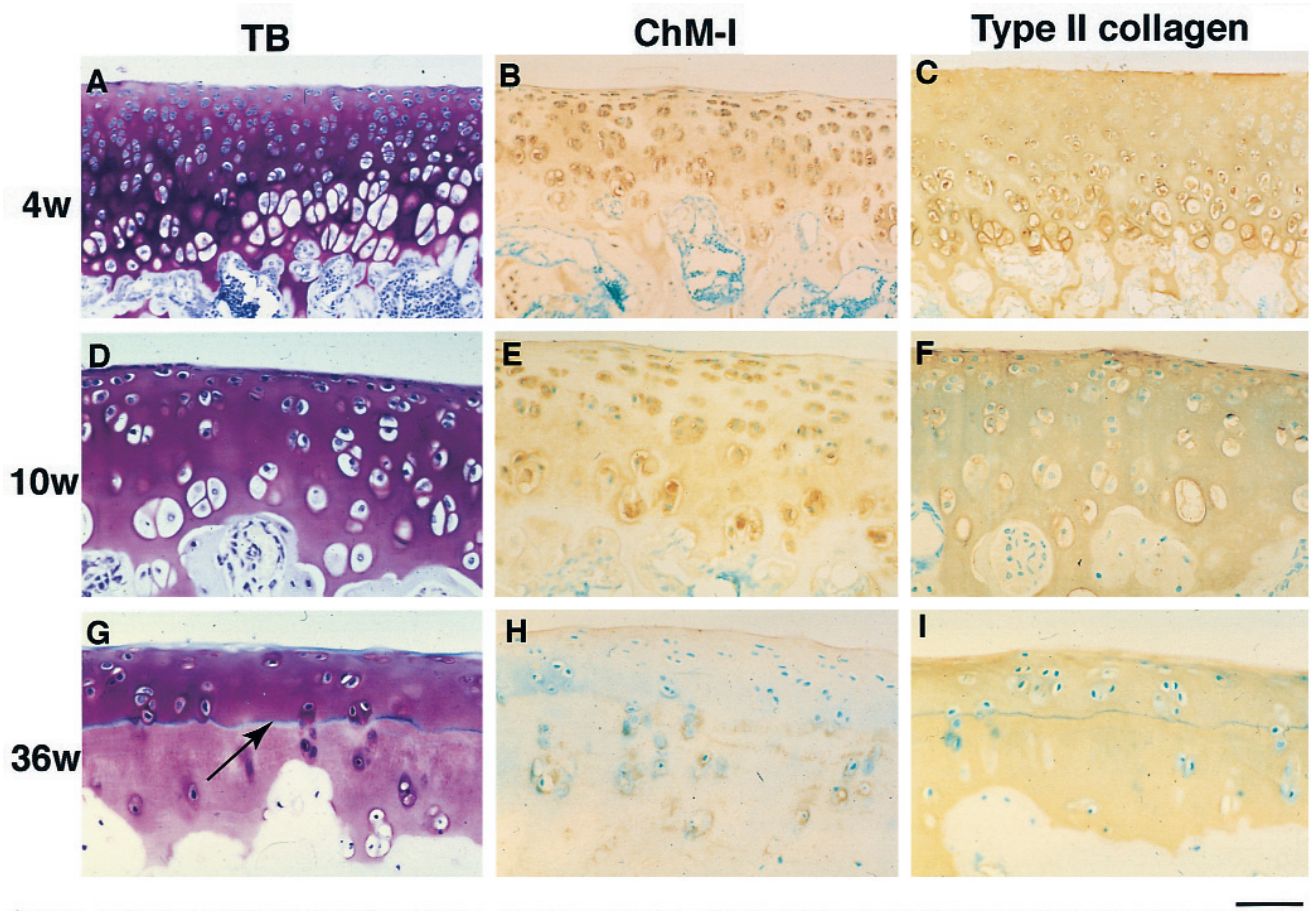

Figure 1. Immunohistochemical localization of ChM-I and type II collagen in growing and adult rat articular cartilage. Frontal regions of the lateral tibial plateaus were serial sectioned and stained with toluidine-blue (A, D, G) and immunostained for ChM-I (B, E, H) and type II collagen (C, F, I). In 4-week-old rats (A–C), ChM-I immunostaining was detected in the superficial, middle, and upper deep zones of the cartilage, and within chondrocytes and their surrounding ECM. Expression of type II collagen was observed in all zones of articular cartilage, while ChM-I immunostaining was significantly reduced in the lower deep zone. In 10-week-old rats (D–F), ChM-I immunostaining was detected from the middle to the upper deep zone of articular cartilage. Type II collagen immunostaining was detected in all zones of articular cartilage. In 36-week-old rats (G–I), there was an obvious tidemark (arrow) and significant decrease in the number of chondrocytes in articular cartilage. ChM-I protein was detected in the middle and deep zones, while type II collagen was detected strongly in all zones. (A–C: original magnification ×50, bar = 100 µm. D–I: original magnification ×100, bar = 50 µm)

positive in chondrocytes embedded in the middle and upper deep zones of the cartilage (Figure 1H). On the other hand, a high protein expression of type II collagen was detected in both uncalcified and calcified zones (Figure 1I), suggesting that type II collagen remains intact in normal articular cartilage of aged animals. By omitting primary antibody or using preimmune rabbit serum, the negative controls showed no staining (data not shown).

Accelerated loss of ChM-I expression in articular cartilage of rats with surgically induced OA. After transection of the anterior cruciate ligament and removal of the menisci, rats recovered within 3 days and their walking patterns were similar to the non-operated animals. No wound healing or inflammation problems were observed. We used articular cartilage of contralateral knee joints as controls, since they showed normal histological appearance compared with the sham-operated animals.

At 5 days after surgery, most articular cartilage of the tibial plateaus showed no difference from controls (Figure 2A). Occasionally, small cracks were observed in the cartilage surface of the medial tibial plateau (Figure 2D). Both of the medial and lateral tibial plateaus had normal proteoglycan contents. By 2 weeks after surgery, medial tibial plateau cartilage was focally and severely degenerated in the weight-bearing regions (Figures 2B, 2E). Meanwhile, articular cartilage of the lateral tibial plateau showed normal surface without loss of proteoglycan content in the matrix (Figure 3E). Although articular cartilage thickness was not quantitated in this study by histomorphometry, the deep zone associated with calcified cartilage and the increasing number of hypertrophic chondrocytes appeared to be extended during the progression of OA (Figure 3E). However, the rats used in this study were still growing, and we thus could not rule out the effects of growth on articular cartilage thickness. On the other hand, we could observe cell proliferation and cloning in both compartments. By 6 weeks after surgery, extensive cartilage thinning associated with loss of toluidine-blue staining was observed in the medial tibial plateau (Figures 2C, 2F), while the lateral tibial plateau showed high cellularity without significant cartilage

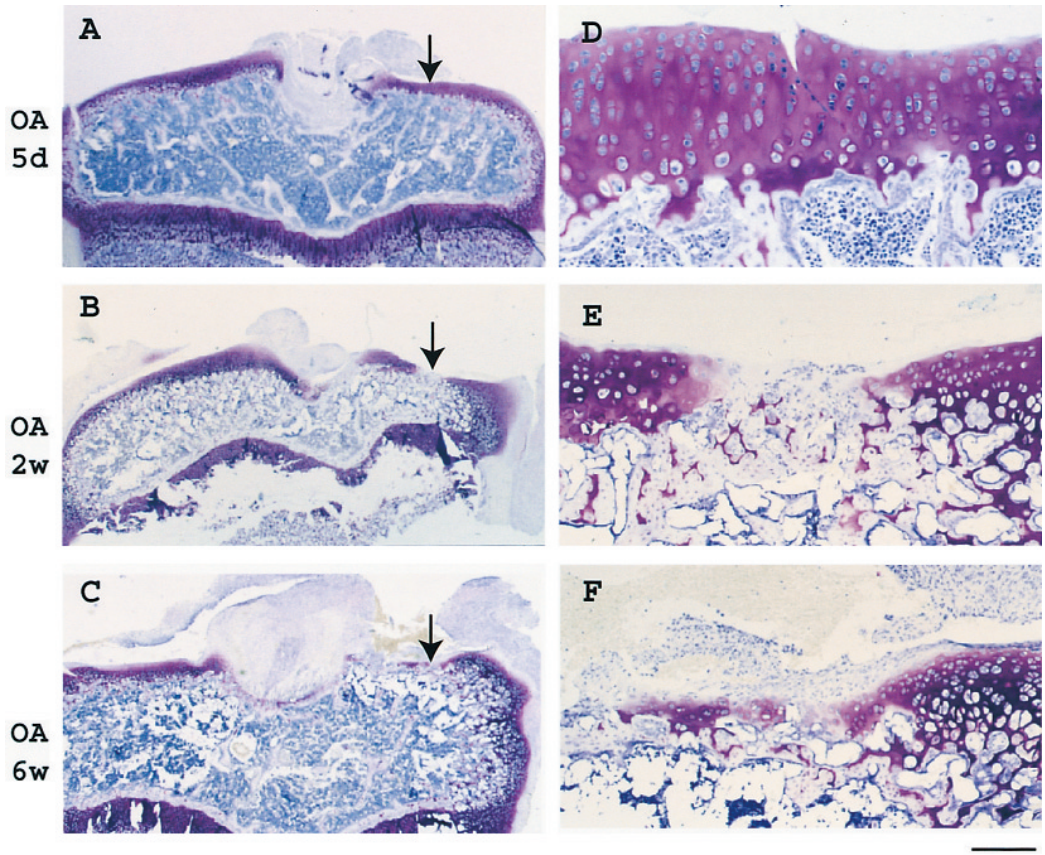

Figure 2. Progressive loss of articular surface and proteoglycans in cartilage of surgically induced rat OA. Proteoglycan contents were examined in the tibial plateaus of right knee joints at 5 days (A, D), 2 weeks (B, E), and 6 weeks (C, F) after surgery. The right panels show higher magnifications of medial tibial plateaus that are indicated by arrows in the corresponding left panels. Sections were stained with toluidine-blue-O. (A–C: original magnification ×4, bar = 1000 µm. D–F: original magnification ×20, bar = 50 µm)

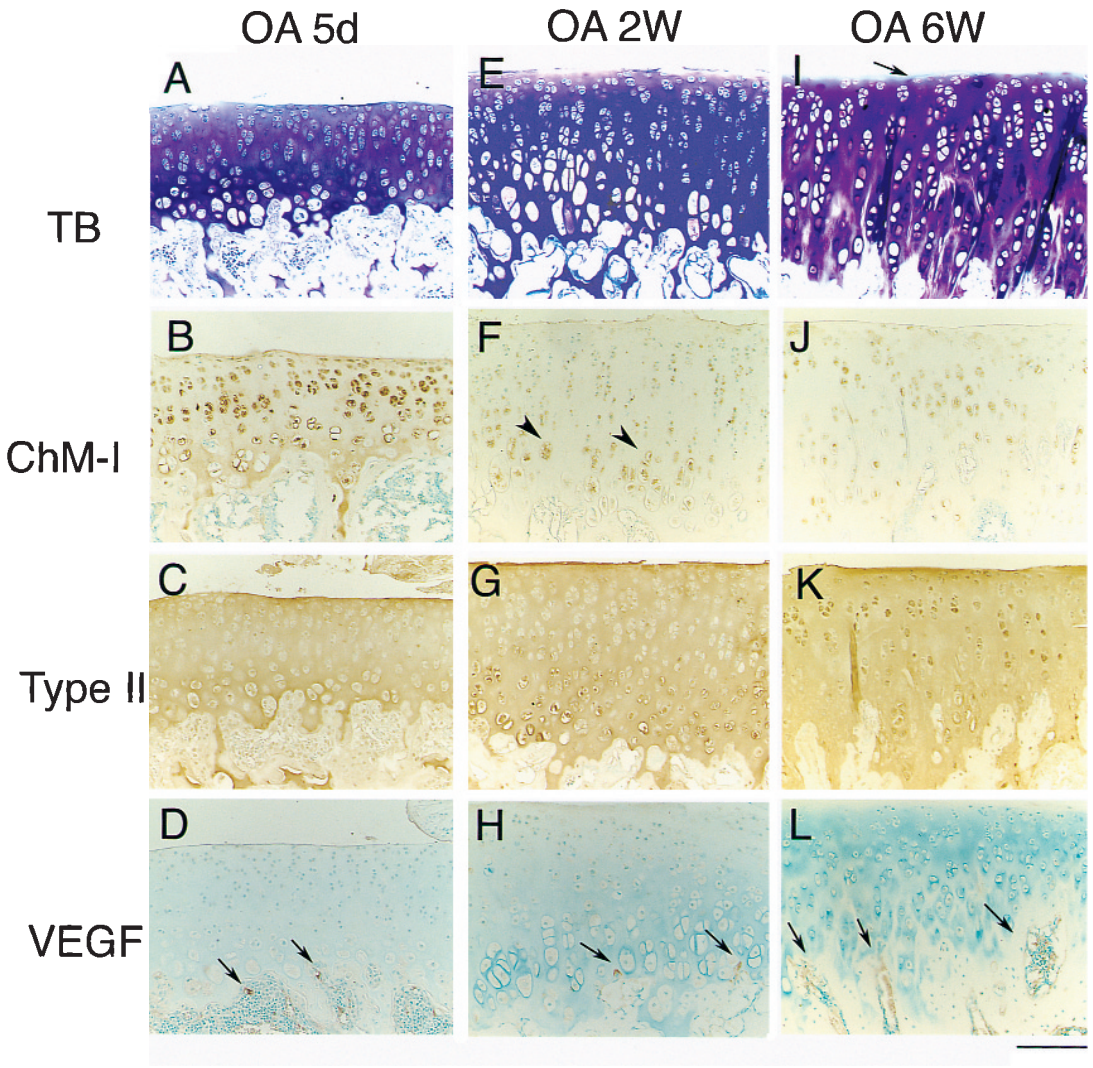

Figure 3. Immunostaining of ChM-I and type II collagen in OA cartilage at 5 days, 2 weeks, and 6 weeks after surgery. Frontal regions of lateral tibial plateaus were serial sectioned and stained with either toluidine-blue (A, E, I) or antibodies to ChM-I (B, F, J), type II collagen (C, G, K), or VEGF (D, H, L, arrows). Five days after surgery (A–D), the lateral tibial plateau showed normal appearance and expression of proteoglycan, ChM-I, and type II collagen in the ECM. VEGF was detected only at the border of cartilage and bone. Two weeks after surgery (E–H), the superficial zone of cartilage showed fraying. ChM-I protein was detected only in chondrocytes in the middle and deep zones (F, arrowheads) and was significantly decreased in the superficial zone of the ECM. Intense immunostainings of type II collagen and proteoglycan were observed in all zones. VEGF immunostaining was observed at the border of cartilage and bone. Six weeks after surgery (I–L), there were no longer flat cells even in the superficial zone (I, arrow). Clusters of chondrocytes were detected in the superficial zone. High concentrations of proteoglycan and type II collagen were detected, while ChM-I was significantly reduced in the ECM in all zones of OA cartilage. (A–D: original magnification ×40, bar = 125 µm. E–L: original magnification ×50, bar = 100 µm)

erosion (Figure 3I). By 8 weeks after surgery, cartilage in both medial and lateral tibial plateaus was significantly eroded, associated with severe loss of proteoglycan content in the medial compartment (Figure 6A).

Expression of ChM-I was also evaluated biochemically in both lateral and medial compartments of the OA cartilage at one week after surgery, compared with the contralateral cartilage (Figure 5). Collected cartilage samples from 5 joints were pooled and Western blot analyses for ChM-I expression were repeated 3 times, with reproducible results. Figure 5 shows representative data. Both the mature secretory form of ChM-I (~25 kDa) and the precursor transmembrane form of the molecule (~37 kDa)[15] were detected in both control and OA cartilages (Figure 5). While concentrations of ChM-I precursors were the same, expression of the mature form of ChM-I was significantly decreased (by 60%) in both medial and lateral tibial plateaus of OA cartilage compared with the control. In the same tissues, we found that whereas expression of VEGF-121 remained unchanged in OA and normal cartilage, expression of VEGF-165 was significantly upregulated in the medial (2.8-fold) compared with the lateral compartment of OA cartilage or with the control cartilage (Figure 5).

We also examined the expression pattern of bFGF in the

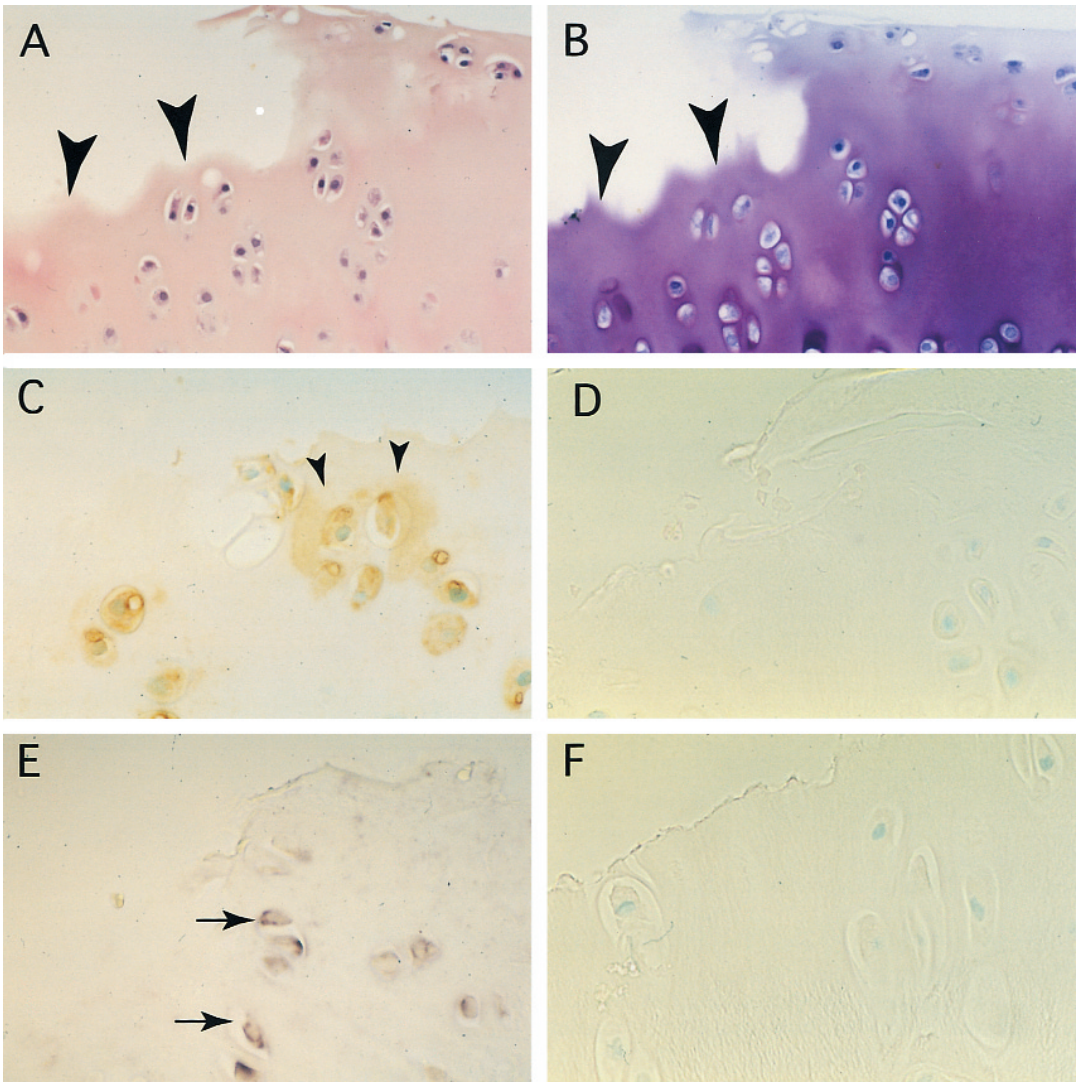

Figure 4. Localization of ChM-I in OA cartilage 10 weeks after surgery. Expression of ChM-I was examined in the frontal section of medial tibial plateaus. In advanced changes, the articular surface was found to be severely eroded upon H&E staining (A, arrowheads) and toluidine-blue staining (B, arrowheads) associated with decreased proteoglycan content. ChM-I protein (C) was only detected within the cytoplasm of clustered chondrocytes and their surrounding ECM (arrowheads). These cells also expressed ChM-I mRNA, detected by *in situ* hybridization (E, arrows). Negative controls for ChM-I immunostaining (D) and mRNA (F) are shown. (A, B: original magnification ×100, bar = 50 µm; C–F: original magnification ×200, bar = 25 µm)

same OA and control cartilage by Western blot. Interestingly, the concentrations of bFGF were consistently higher in the medial than in the lateral compartments, although there was no difference between OA and control cartilage. Expression of type II collagen and α-tubulin was not significantly changed in these tissues relative to the total proteins, as shown by Coomassie-blue stained gel.

Vascular invasion into OA cartilage. OA progression appeared to start in the medial compartment before the lateral tibial plateau in this rat OA model. Moreover, bone formation has been suggested to be induced in the weight-bearing medial plateau, and to lesser extent in the lateral cartilage compartment. In this study, reduction in ChM-I expression was observed in both compartments, while VEGF-165 expression was increased only in the medial cartilage compartment one week after surgery. These findings suggested that the reduction in ChM-I expression, which precedes the increase in VEGF expression, might be sufficient to drive angiogenesis. To test this hypothesis, we examined the recruitment of blood vessels and the changes in concentrations of angiogenic markers in the lateral cartilage compartment during progression of OA, as follows.

Early changes. Five days after surgery, the lateral tibial plateau appeared to be intact and rich in proteoglycan as

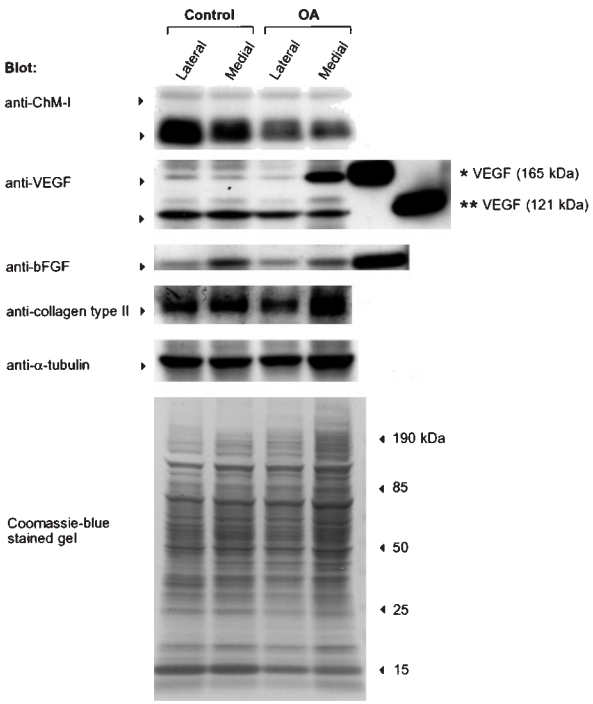

Figure 5. Western blot analysis of ChM-I in OA articular cartilage. One week after surgery (n = 5), proteins were extracted from the medial and lateral compartments of OA cartilage (right knee joint) and normal cartilage (left knee joint), and separated on SDS-PAGE. Expressions of ChM-I, VEGF isoforms, bFGF, type II collagen, and α-tubulin were examined by Western blot using respective antibodies. To confirm equal protein loading, the gel (25 µg) was stained with Coomassie-blue. Recombinant mouse VEGF121 (**), VEGF165 (*), and human bFGF (right lane) were loaded as positive controls. Western blots for ChM-I expression were repeated 3 times, with reproducible results. Result shown here indicates representative data.

detected by toluidine-blue staining (Figure 3A). Expression of ChM-I was strongly upregulated in both ECM and cytoplasm of chondrocytes (Figure 3B). Type II collagen was found in all zones of the articular cartilage except calcified cartilage (Figure 3C). Interestingly, VEGF was detected only at the border of cartilage and bone (Figure 3D). By 2 weeks after surgery, the cartilage surface appeared to be normal (Figure 3E). The concentrations of ChM-I protein were significantly decreased in the superficial zone, and were found only in the middle and deep zones (Figure 3F). In contrast, high levels of proteoglycan (Figure 3E) and type II collagen (Figure 3G) were detected in all zones, including the superficial zone. VEGF was weakly detected in the perivascular cells at the border of cartilage and bone (Figure 3H). Six weeks after surgery, the number of chondrocytes appeared to be higher in OA cartilage compared to control cartilage (Figures 1D, 3I). ChM-I immunostaining was significantly reduced in the ECM in all zones of the OA cartilage (Figure 3J). The expression of proteoglycan and type II collagen was maintained in all zones (Figures 3I, 3K). VEGF positive vascular pericytes or endothelial cells were clearly associated with blood vessel formation at the border of cartilage and subchondral bone (Figure 3L).

Advanced changes. Ten weeks after surgery, extensive degeneration of cartilage was detected in the medial tibial plateau. The cartilage surface was severely eroded and the number of chondrocytes decreased. Many cluster-forming chondrocytes could be detected just below the eroded surface (Figure 4A). Proteoglycan was significantly decreased toward the articular surface, by toluidine-blue staining (Figure 4B). ChM-I protein was found primarily in cytoplasm and at the surface of the cluster-forming chondrocytes, and in their surrounding ECM (Figure 4C). We also detected ChM-I mRNA expression in these chondrocytes (Figure 4E). Negative controls for ChM-I immunostaining (Figure 4D) and *in situ* hybridization (Figure 4F) showed no specific localization.

In advanced OA, vascular invasion from the subchondral space resulted in thinning of avascular cartilage, with diminished toluidine-blue staining (Figures 6A, 6B, 6C). Double immunostaining for ChM-I and CD31 showed that the cells expressing these markers were in proximity to one another (Figure 6D). CD31 positive cells associated with vascular formation penetrated the cartilage matrix, surrounding the cluster-forming chondrocytes expressing low cytoplasmic concentrations of ChM-I (Figure 6D). VEGF immunostaining was observed in the perivascular cells and hypertrophic chondrocyte-like cells (Figure 6E). Negative controls showed no specific staining (Figure 6F).

MMP-9 and MMP-13 immunolocalization in early stage OA. Metalloproteinases, particularly MMP-9 and MMP-13, have been reported to play an important role in vascular invasion and ECM degradation in cartilage during development[26,27]. We thus examined the immunolocalization of MMP-9 and MMP-13 in cartilage of the rat model at 2 weeks after surgery. The lateral tibial plateau in the intact left joint showed no detectable expression of MMP-9 or MMP-13 (Figures 7A, 7D). In OA joints, expression of MMP-9 and MMP-13 increased in proximity to the articular surface damage (Figures 7B, 7E). MMP-13 was strongly stained in hypertrophic chondrocytes in the deep zone and in flattened cells in the superficial zone (Figure 7B). Intense MMP-9 staining was observed at the border of cartilage and vasculature in bone marrow space. To confirm the specificity of the antibodies, we also evaluated localization of MMP-9 and MMP-13 in the growth plate, where MMP-13 was detected in hypertrophic chondrocytes (Figure 7C) and MMP-9 at the border of cartilage and bone marrow in controls (Figure 7D) and in OA joints (data not shown), as described[27].

DISCUSSION

The role of ChM-I as a potent angiogenic inhibitor was previously reported[14,15]. Further, ChM-I was also reported to act as a growth factor for chondrocyte proliferation[13,14]. We

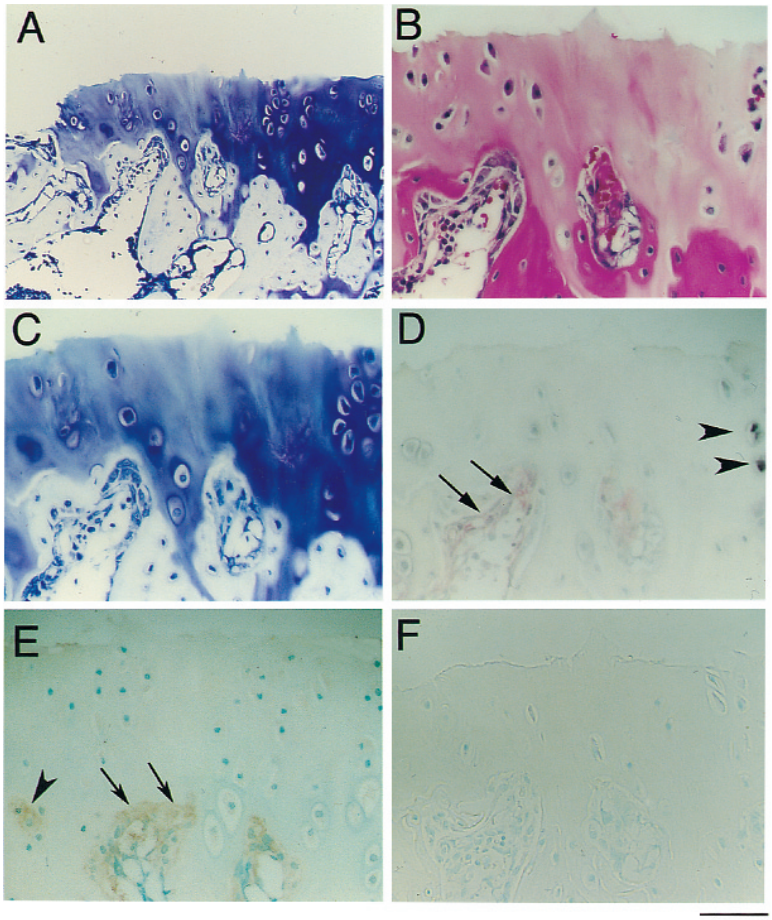

Figure 6. Localization of ChM-I in chondrocytes and CD31 in vascular endothelial cells in OA cartilage at 8 weeks after surgery. Serial sections of the frontal compartment of medial tibial plateaus were stained at the 8th week with toluidine-blue (A and C) and H&E (B). ChM-I immunostaining decreased in the matrix (D); strong expression was observed in the cluster-forming chondrocytes (D, arrowheads). CD31 positive cells with tubular vascular formation invaded the cartilage matrix (D, arrows). E: VEGF immunostaining was detected in perivascular cells (arrows) and hypertrophic-like chondrocytes (arrowhead). F: Negative control. (Original magnification ×100, bar = 50 µm)

found that ChM-I is highly expressed in the avascular zone of articular cartilage in both growing and adult rats. On the other hand, ChM-I mRNA levels were relatively lower in adult, compared with growing rats (data not shown). Therefore, our observation supports the notion that ChM-I may play a key role in the maintenance of articular cartilage as avascular tissue and promote the regenerative potential of articular cartilage in response to injury[28].

OA is a degenerative joint disease characterized by progressive deterioration of articular cartilage. The disease process arises from an imbalance between matrix synthesis and degradation[29,30]. The superficial zone of articular cartilage, which contains more densely packed collagen fibrils than other zones[29], could serve a function similar to that of protective dermis in skin, by limiting the access of extracartilage macromolecules and egress of large cartilage ECM molecules. Thus, disruption of the superficial zone in the early stage of OA has been suggested to promote leakage of cartilage constituents into the synovial space[30-32]. Our study demonstrates that ChM-I proteins are significantly decreased in the superficial zone prior to the loss of proteoglycan and type II collagen. We also found that decreased expression of the secreted form of ChM-I appeared to be an early event in cartilage degeneration. Upon deterioration of the superficial layer, hydrophilic mature ChM-I (25 kDa) was suggested to escape from the interterritorial matrix, and synthesis of the membranous precursor form of ChM-I (37 kDa) could be elevated from these chondrocytes to compensate for the loss of ChM-I from the matrix. As OA progressed, ChM-I as well as proteoglycan were depleted in cartilage extracellular matrices. Diminished expression of ChM-I expanded to all zones of the articular cartilage. This observation suggests that newly secreted ChM-I might not be retained in the degenerated matrices due to either its high

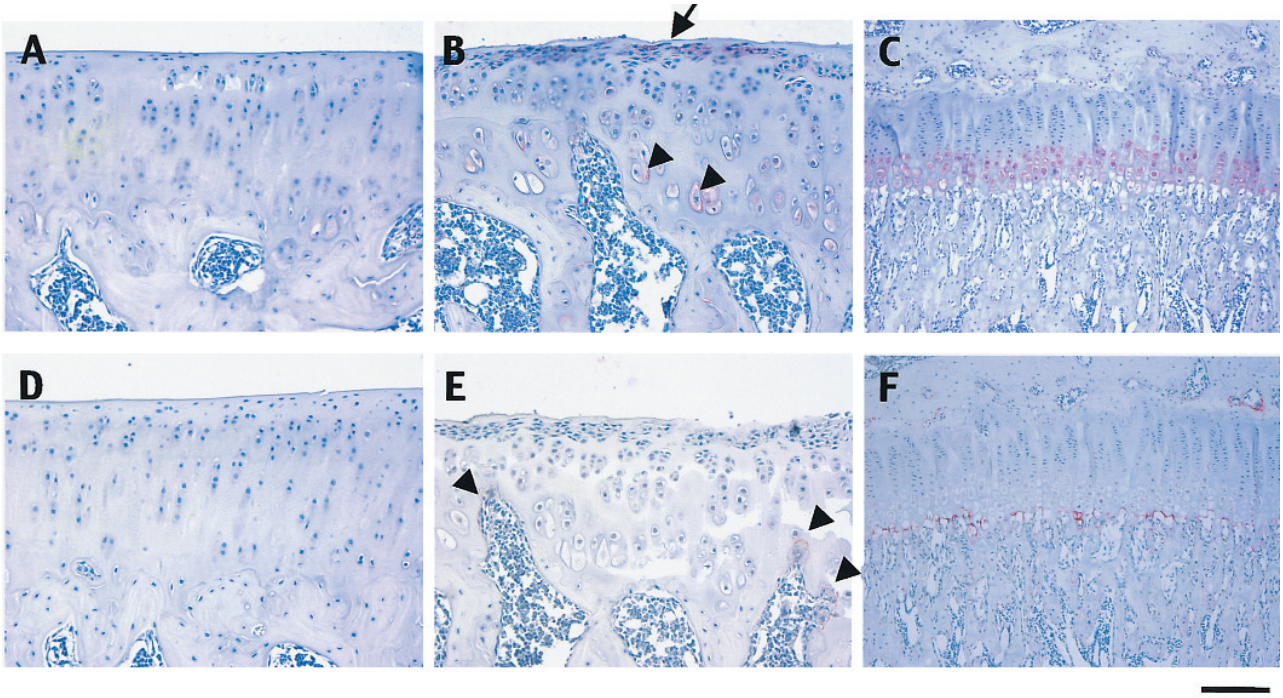

Figure 7. Immunostaining of MMP-9 and MMP-13 in OA cartilage at 2 weeks after surgery. Frontal regions of lateral tibial plateaus and growth plates were serial sectioned and stained with antibodies to MMP-13 (A, B, C) and MMP-9 (D, E, F). Control left joint showed normal appearance and undetectable MMP-13 (A) or MMP-9 (D) expression. In OA joints, MMP-13 (B) was found in hypertrophic chondrocytes in the deep zone (arrowheads) and small cells in the superficial zone (arrow), while strong staining for MMP-9 (E) was observed at the border of cartilage and blood vessels (arrowheads). In the growth plate, MMP-13 (C) was intensely stained in hypertrophic chondrocytes, while MMP-9 staining was observed at the border of cartilage and bone marrow (F). (A, B, D, E: original magnification ×200, bar = 200 µm. C and F: original magnification ×100, bar = 100 µm)

solubility in aqueous fluid or breakdown of an unknown anchoring molecule[16].

Elevated synthesis of ECM molecules in articular chondrocytes of OA cartilage has been reported[29-31]. In addition to the increase in ChM-I mRNA levels in OA chondrocytes (Figure 7), we observed a net loss of ChM-I protein from the cartilage matrix. This finding suggested that sufficiently high concentration of ChM-I in the cartilage is required to prevent vascular invasion into this tissue[14]. Further, substantial loss of ChM-I protein is coincident with onset of angiogenesis in the same region in early stage OA, suggesting this process might precede the subchondral bone changes in the late stage of this model (data not shown). We have shown that ChM-I mRNA was highly expressed in rabbit primary chondrocytes, and was markedly downregulated in response to growth and differentiation stimuli, including FGF-2, transforming growth factor-ß (TGF-ß), and parathyroid hormone related protein[33]. Regulation of ChM-I in chondrocyte cultures by inflammatory cytokines and mechanical stress will be subjects of future investigations.

It has been hypothesized that increased angiogenesis in tumors during tumor development is regulated by an imbalance of inducers and inhibitors of endothelial cell proliferation[34]. In the joints, normal articular cartilage is known to be impervious to vascular infiltration, and this property is suggested to be somehow lost in OA cartilage. The presence of angiogenic inducers such as VEGF or bFGF in cartilage matrix has been reported[7,35]. Recently, levels of VEGF were found to be elevated in chondrocytes and synovial fluid from patients with RA or OA[8-11].

In addition to angiogenic growth factors, neovascularization depends on ECM proteolysis by MMP, which are regulated by tissue inhibitors (TIMP)[36]. Proteolysis of the ECM allows cell migration and may also release stored growth factors, and MMP are thus implicated in these processes owing to their ability to cleave ECM components. While expression of specific TIMP subtypes during degeneration of articular cartilage has not been well defined, MMP-9 and MMP-13 are reported to play an important role in neovascularization and ECM degradation in cartilage[26,27]. Target disruption of MMP-9 in mice caused delayed endochondral bone formation, suggested to be due to decreased ECM degradation, leading to reduction of release of angiogenic factors, including VEGF, from the ECM[37]. Moreover, blocking VEGF function using its soluble receptor in mice was also shown to impair vascular invasion into the growth plate, and thus resulted in a bone phenotype resembling that observed in MMP-9 deficient mice[7,27]. On the other hand,

MMP-13 or collagenase 3 is highly expressed in the hypertrophic chondrocytes in growth plate and has been suggested to play a major role in cartilage degradation. More recently, MMP-9 and MMP-13 were reported to work cooperatively in degrading the unmineralized cartilage, and their expression was also known to be upregulated in OA cartilage and/or synovium[26,27]. Our observations on the increased expression of MMP-9 and MMP-13 in OA joints within 2 weeks after surgery thus support the potential role of these MMP in promoting vascular invasion as an early step in OA pathogenesis. Expression and regulation of MMP and their endogenous inhibitors, TIMP, during vascular invasion and cartilage degradation in this rat OA model will be subjects of future studies.

Recently, ChM-I null mice were generated with no obvious phenotype in cartilage and vasculogenesis[38]. Expression of other angiogenic factors such as VEGF, FGF-2, and TGF-ß was not upregulated in epiphyseal cartilage in these mice during growth. However, this lack of phenotype in the ChM-I deficient mice did not rule out an anti-angiogenic function for ChM-I in the maintenance of the avascular state of articular and epiphyseal cartilage in adult animals, nor did it rule out compensatory changes in other unknown angiogenic or antiangiogenic factors.

In conclusion, a role for ChM-I in maintenance of the avascular state in normal articular cartilage is suggested in this study. Moreover, the decrease in ChM-I and increase in VEGF expression, as well as other pro-angiogenic factors, occurred in the early stage of OA, suggesting that an imbalance of the pro- and anti-angiogenic factors plays an essential role in promoting vascular invasion into articular cartilage. Because the elevated subchondral bone remodeling and osteophytosis of OA may involve endochondral bone formation preceded by vascular invasion into hypertrophic cartilage[39], our observations suggest that angiogenesis in the articular cartilage is an early contributing factor to the progression of OA.

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