Chondromodulin-I expression and correlation with angiogenesis in human osteoarthritic cartilage

BING DENG[1,2], CHENG CHEN[1], XIAOYUAN GONG[1], LIN GUO[1], HAO CHEN[1], LI YIN[1], LIU YANG[1] and FUYOU WANG[1]

[1]Center for Joint Surgery, Southwest Hospital, The Third Military Medical University, Chongqing 400038; [2]Department of Orthopedics, General Hospital of Chengdu Military Region, Chengdu, Sichuan 610083, P.R. China

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Abstract. The present study aimed to evaluate the expression and localization of chondromodulin-I (ChM-I) in human osteoarthritic cartilage and its correlation with vascular invasion during osteoarthritis (OA) progression. Osteochondral specimens were collected from patients with OA, as well as from young and aged donors without joint diseases. The grade and the number of vascular channels terminating in non-calcified cartilage of these collected specimens were assessed by Safranin-O/Fast green staining. ChM-I expression in articular cartilage was examined by immunohistochemistry, western blotting and reverse transcription-quantitative polymerase chain reaction analyses. ChM-I protein and mRNA levels in articular cartilage appeared to be consistent between the normal young and aged groups ($P>0.05$). In mildly degenerated cartilage, ChM-I expression decreased in the extracellular matrix (ECM) of the superficial zone and in the cytoplasm of the superficial and middle zone compared with normal cartilage ($P<0.05$). In moderately degenerated cartilage, ChM-I protein expression was reduced in the ECM of all zones of articular cartilage, but the immunostaining intensity in the cytoplasm was increased. In severely degenerated cartilage, ChM-I expression was detected primarily in the cytoplasm of the cluster-forming chondrocytes. The density of vascular channels was correlated with the ChM-I expression levels in cartilage ECM. ChM-I expression was reduced in OA cartilage matrix, compared with normal cartilage (both young and aged), and correlated with angiogenesis, indicating that loss of ChM-I may promote angiogenesis in OA cartilage. Expression of ChM-I protein in the cytoplasm was decreased in mildly degenerated cartilage, whereas ChM-I expression increased in moderately degenerated cartilage accompanied by chondrocyte proliferation. These findings suggested that attenuation of ChM-I in the cartilage ECM may be due to decreased expression of ChM-I in cytoplasm of early stage OA and increased depletion of ChM-I in the ECM of advanced stage OA.

Correspondence to: Professor Liu Yang or Professor Fuyou Wang, Center for Joint Surgery, Southwest Hospital, Third Military Medical University, 30 Gaotanyan Road, Chongqing 400038, P.R. China
E-mail: yangliujoint@126.com
E-mail: wfyjoint@126.com

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Introduction

Osteoarthritis (OA) is a leading cause of pain and disability in the aging population. A primary characteristic of OA is angiogenesis, characterized by the formation and invasion of new blood vessels into the hyaline cartilage (1). It has previously been demonstrated that angiogenesis is important in the progression of cartilage degradation, which results in the re-initiation of endochondral bone formation and the subsequent increase in subchondral bone density and cartilage thinning (2). Angiogenesis results in the innervation of articular cartilage, therefore providing a potential source of pain in OA (3). Healthy cartilage is avascular and aneural, and the mechanisms underlying blood vessel initiation and invasion into cartilage during OA remain unknown.

The endogenous, antiangiogenic factor chondromodulin-I (ChM-I) is specifically expressed in cartilage. Previous studies have demonstrated that ChM-I may stimulate DNA synthesis and growth of chondrocytes in culture (4,5), but may inhibit DNA synthesis and growth of endothelial cells (5-7). Furthermore, ChM-I may inhibit vascular endothelial growth factor-A-stimulated chemotactic migration of endothelial cells (8) and tube morphogenesis of endothelial cells (5). The expression of ChM-I is specific to the avascular zone of cartilage in the developing bones of cattle (5), mice (9) and humans (10). These results suggested that ChM-I may be involved in the antiangiogenic properties of cartilage, and the absence of ChM-I expression may create a permissive microenvironment for vascular invasion of cartilage under physiological conditions.

Various studies have demonstrated that the loss of ChM-I expression in articular cartilage may be partly responsible for promoting the invasion of blood vessels into cartilage during OA progression (11-13). However, the pattern of ChM-I expression varies in different cartilage degeneration models. For example, in a surgically induced rat knee OA model, ChM-I expression was at first upregulated in the extracellular

matrix (ECM) and cytoplasm of chondrocytes and then the expression decreased (11). In a study on immobilized ankle joints of rats, the percentage of ChM-I-positive cartilage was significantly decreased compared with normal ankle joints (12). In a rat temporomandibular joint OA model, the expression of ChM-I in the cytoplasm was reported to first decrease, and then increase (13).

The expression of ChM-I and its correlation with angiogenesis in human cartilage remains to be elucidated. The present study evaluated the mRNA and protein expression of ChM-I the in articular cartilage of patients with OA, followed by an examination of the association between ChM-I and angiogenesis in non-calcified cartilage. In conclusion, the expression of ChM-I in the cytoplasm initially decreased and was followed by an increase, in line with cartilage degeneration. However, the ChM-I in the ECM decreased gradually, and was correlated with angiogenesis. These results suggest that maintaining ChM-I levels in the ECM may improve the ability to resist vascular ingrowth of cartilage, especially in mild osteoarthritis.

Table I. Clinical parameters of donors.

Grade	n	Sex (M/F)	Sides (L/R)	Age (years)	BMI (kg/m^2)
G0 (Y)	6	2/4	2/4	26.2±3.4	22.6±3.6
G0 (A)	7	2/5	2/5	69.8±2.2	26.3±1.8
G1	7	0/7	3/4	57.1±1.2	25.0±2.4
G2	10	2/8	6/4	56.8±1.1	25.2±3.0
G3	10	1/9	6/4	57.0±1.6	25.5±3.1
F/χ^2		3.554[a]	2.802[a]	48.243[b]	7.965[b]
P-value		0.470	0.591	0.000	0.093

[a]Pearson's χ^2 test statistic. [b]Analysis of variance statistic. G0(Y), normal young donors; G0(A), normal aged donors; G1, mild OA donors; G2, moderate OA donors; G3, severe OA donors; BMI, body mass index; F, female; M, male; L, left; R, right; n, number of tissue samples.

Materials and methods

Patients and samples. The present study was approved by the ethics committee of The Southwest Hospital of The Third Military Medical University (Chongqing, China), and each participant provided written informed consent, according to the Declaration of Helsinki. In the OA group, osteochondral samples (1.5x0.5x1.0 cm) were collected from the weight-bearing area of the lateral femur condyle of 27 patients (3 males, 24 females, aged 55-60 years) with OA who were undergoing total knee arthroplasty (Fig. 1A). In the young group, a total of 6 normal cartilage samples were obtained from the weight-bearing area of the medial and lateral femur condyle of 3 young patients (1 males, 2 females, aged 18-30 years) that had previously undergone amputative procedures (Fig. 1B). In the aged group, 7 additional normal cartilage samples were obtained from the weight-bearing area of the femur head of 7 aged patients (2 males, 5 females, aged 65-72 years) that had previously undergone total hip arthroplasty for femoral neck fracture (Fig. 1C). In the OA group, any patients with lower extremity trauma or other joint diseases were excluded. In the young group and the aged group, any patients with arthropathy were excluded. Each cartilage sample was subdivided into three parts for subsequent immunohistology, western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Histology. Tissue blocks were decalcified in 10% EDTA for 14 days at 4˚C and then embedded in paraffin. Sections (5 μm) were cut from each paraffin block using an automatic microtome. Following deparaffinization, sections were stained with hematoxylin for 3 min and differentiated in 1% acid alcohol for 15s. Sections were subsequently stained in 0.02% aqueous Fast Green for 3 min, washed in 1% acetic acid for 15 s to remove remnant stain, and counterstained in 0.1% Safranin-O for 3 min. Sections were dehydrated through serial dilutions of ethanol, cleared in xylene, and mounted using neutral gum. Sections were scored according to the Osteoarthritis Research Society International grading system (14) by two different observers (blinded to the study). The cartilage samples were classified as normal (G0), mild OA (G1), moderate OA (G2) or severe OA (G3). According to the method described by Fransès *et al* (15), osteochondral vascular density was determined as the number of vascular channels that terminate in the non-calcified cartilage divided by the section length. A DP26 colored CCD camera (Olympus Corporation, Tokyo, Japan) mounted onto an Olympus BX51-PMS binocular light microscope (Olympus Corporation) and the cellSens Life Science Imaging Software (Olympus Corporation) were used for digital image evaluation. The results of the evaluation were consistent between the two observers (r>0.9). The mean values of the two measurements were used for statistical analysis.

Immunohistochemistry. Immunohistochemical staining was performed on adjacent sections using the SABC-POD Immunohistochemistry Staining kit (Boster Systems, Inc., Pleasanton, CA, USA), according to the manufacturer's protocol. Briefly, sections were deparaffinized in xylene and rehydrated in graded ethanol and water, and incubated with 3% H_2O_2 at room temperature for 10 min. The slides were washed several times with PBS, pre-incubated with 5% BSA (Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China) at room temperature for 20 min, followed by incubation with rabbit anti-ChM-I antibody (sc-33563; 1:100; Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 4˚C overnight. Following washes with PBS, the sections were incubated with the secondary goat anti-rabbit immunoglobulin G HRP antibody (SPN-9001; 1:300; ZSGB-BIO, Beijing, China) at 37˚C for 30 min. The color reaction was developed with 3,3'-diaminodenzidine and counterstained with hematoxylin. Normal rabbit serum served as a negative control in place of the anti-ChM-I antibody. Cytoplasmic immunostaining was graded on a scale of 0-3, where 0=no staining; 1=weak staining; 2=moderate staining; 3=strong staining. Quantitative analysis of ChM-I immunostaining intensity in ECM was performed by analysis of the computer gray scan, using Image-Pro Plus 5.0 (Media Cybernetics, Inc., Rockville, MD, USA).

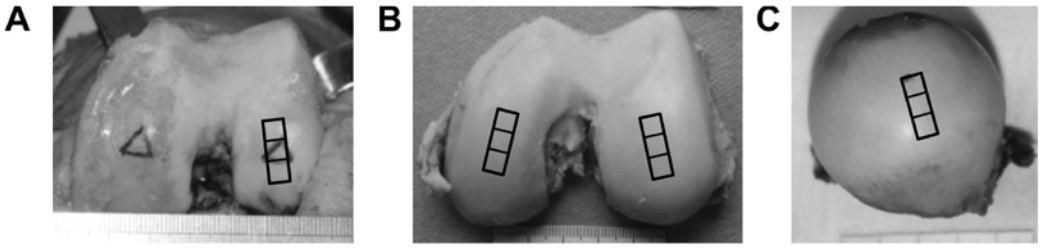

Figure 1. Sites of femoral cartilage sampling. (A) Lateral femur condyle of patients undergoing total knee arthroplasty. (B) Normal cartilage samples from young patients that previously underwent amputative procedures. (C) Femur head of aged patients that previously underwent total hip arthroplasty for femoral neck fracture. Three cartilage samples were obtained from each femur; black frames indicate sites of sampling.

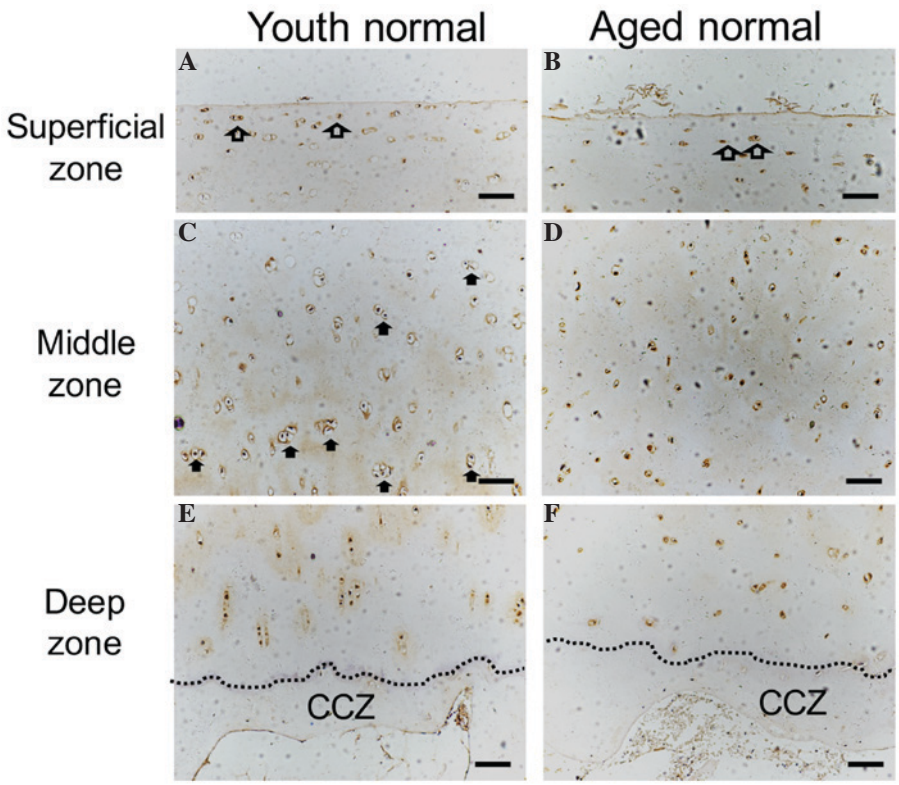

Figure 2. ChM-I protein localization in normal human cartilage. Chondrocyte cytoplasm with ChM-I-positive immunostaining (brown) were observed in the superficial zone of (A) young and (B) aged donors. ChM-I protein expression was detected in the cytoplasm and ECM in the middle zone of (C) young and (D) aged donors. Solid arrows indicate dividing cells, hollow arrows indicate flattened cells. In the deep zone and the CCZ of (E) young and (F) aged donors, ChM-I expression was decreased in the ECM, however, there was no change in cytoplasmic levels. Dashed line denotes the tidemark (separation of calcified and non-calcified). Scale bar, 200 μm. CCZ, calcified cartilage zone; ChM-I, chondromodulin-I; ECM, extracellular matrix.

Western blotting. In western blot analysis, total protein from OA cartilage and normal cartilage specimens were extracted using a Total Protein Extraction kit (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China). Protein concentrations were determined with the Pierce BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total proteins (40 μg) were separated by 8% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% skimmed milk at room temperature for 2 h, and incubated with rabbit polyclonal anti-ChM-I antibody (1:1,000; sc-33563; Santa Cruz Biotechnology Inc, Dallas, TX, USA) at 4˚C overnight. The membrane was washed with TBS + 0.1% Tween (TBST) three times for 10 min each, and incubated with the secondary goat anti-rabbit-IgG-horseradish peroxidase antibody (1:5,000; ZDR-5306; ZSGB-BIO) at room temperature for 90 min. The membrane was subsequently washed with TBST four times for 20 min each. Blots were stripped and reprobed with mouse monoclonal anti-GAPDH antibody (1:1,000; G8795; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to confirm equivalence in loading. Analysis of absorbance was performed using the Quantity One v4.6.7 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RT-qPCR. Total RNA was extracted with Cartilage RNAout kit (Beijing Tiandz Gene Technology Co., Ltd., Beijing, China) and reverse transcribed into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan). Target gene primers were designed as follows: ChM-I, forward 5'-GAAGGCTCGTATTCCTGA GG-3', reverse 5'-GGC ATG ATCTTGCCTTCCAG-3'; and GAPDH (used as an endogenous

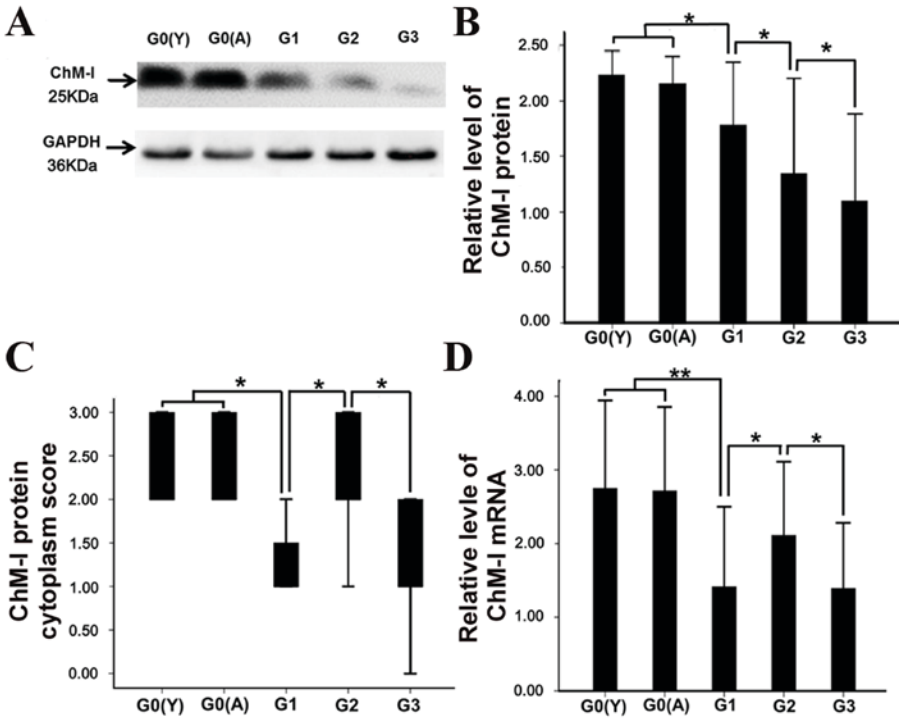

Figure 3. Comparison of ChM-I protein and mRNA levels between normal and osteoarthritic cartilage. (A) Representative western blotting image and (B) densitometric quantification of total protein levels in cartilage. (C) Protein levels in chondrocytes were determined and scored by immunostaining. (D) ChM-I mRNA expression levels detected via reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation; *P<0.05; **P<0.001. ChM-I, chondromodulin-I; G0(Y), normal young donors; G0(A), normal aged donors; G1, mild OA donors; G2, moderate OA donors. G3, severe OA donors.

control), forward 5'-GCACCGTCAAGGCTGAGA A-3', reverse 5'-TGGTGAAGACGCCAGTGG A-3'. qPCR was performed in a reaction volume of 25 μl with the QuantiTect SYBR-Green PCR kit (Qiagen, Inc., Valencia, CA, USA). The cycling program was performed under the following conditions: 5 min at 95˚C, followed by 40 cycles of 10 sec at 95˚C, 30 sec at 60˚C. Assays were performed in triplicate on the Applied Biosystems 7500 Real-Time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression of ChM-I was normalized to that of GAPDH using the $2^{-\Delta\Delta Cq}$ method (16).

Statistical analysis. Data were analyzed using the Statistical Package for Social Scientists (SPSS) version 14.0.1 (SPSS Inc., Chicago, IL, USA). The categorical variables were reported as absolute values and the continuous variables as the mean ± standard deviation. Pearson's χ^2 test was used to compare the categorical variables between the frequencies, corrected for continuity. The normality of the distribution for continuous variables was examined with the Kolmogorov-Smirnov test. One-way analysis of variance followed by the Fisher's least significant difference test was used to compare the different study groups for normally distributed continuous variables. The Kruskal-Wallis H test was performed, followed by the Mann-Whitney U-test and Bonferroni correction if data were not normally distributed. Correlation between two parameters was identified by Spearman rank correlation analysis. P<0.05 was considered to indicate a statistically significant difference. Each test was repeated six times.

Results

General condition. The present study evaluated age, sex, side and body mass index of 37 donors (Table I). No statistically significant differences were identified between these variables (P>0.05), except for the age of donors (P<0.001).

ChM-I expression in normal cartilage of young and aged donors. The surface of normal cartilage from both young and aged donors was observed to be smooth and intact. Flattened cells were observed in the superficial zone, and ChM-I immunostaining was positive (Fig. 2A and B). In the middle zone, mitotic activity appeared to be higher in the young human cartilage compared with the aged cartilage specimen. ChM-I protein expression was detected in the cytoplasm and in the surrounding ECM of the articular cartilage (Fig. 2C and D). Vascular channels were not observed in the deep zone of either of the two groups, and ChM-I expression was decreased in the ECM of the lower deep zone and in the calcified cartilage zone compared with the middle zone (Fig. 2E and F). ChM-I expression in the deep zone appeared to be lower in the aged samples compared with the youth samples, however, no statistical significance (P>0.05) was identified for ChM-I protein in the cytoplasm of whole cartilage (Fig. 3C). Furthermore, there was no significant difference between the young and aged normal cartilage samples in protein (Fig. 3A, B) and mRNA (Fig. 3D) expression levels.

ChM-I expression in OA cartilage. ChM-I expression in cartilage at different stages of OA was observed by immunostaining,

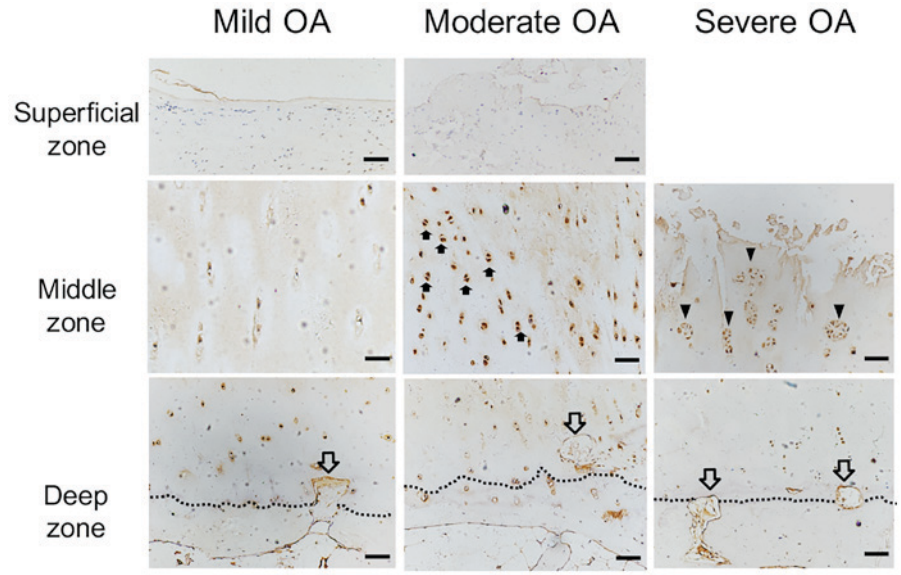

Figure 4. ChM-I protein expression localization in OA cartilage. ChM-I protein expression was detected by immunostaining (brown). ChM-I protein expression was markedly decreased in the cytoplasm and ECM in the superficial zone of mild OA cartilage compared with normal cartilage, and vascular channels (hollow arrow) invaded the non-calcified cartilage in the deep zone, where ChM-I expression was decreased. Compared with mild OA cartilage, the mitotically active cells with ChM-I protein expression (solid arrow) were significantly increased in the cytoplasm of the moderate OA cartilage middle zone, however there were no differences in the superficial and deep zones. Vascular channels were present in the deep zone. In severe OA, ChM-I expression was primarily observed in the ECM surrounding the cluster-forming chondrocytes (arrowheads), whereas ChM-I expression was reduced in the ECM in all other zones of OA cartilage. Scale bar, 200 μm. ChM-I, chondromodulin-I; ECM, extracellular matrix; OA, osteoarthritis.

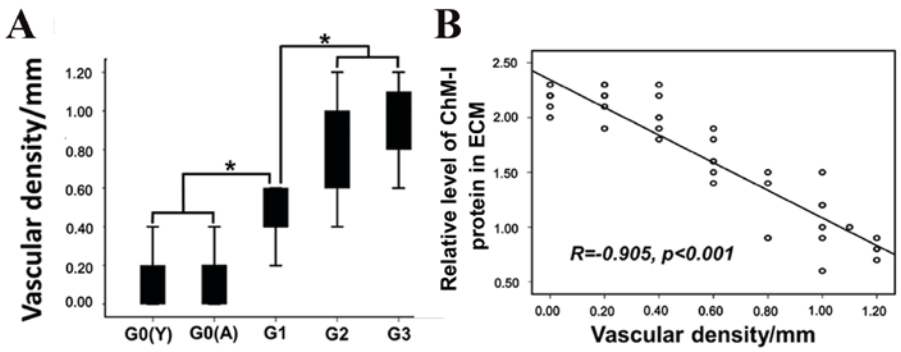

Figure 5. Vascular density in non-calcified cartilage and its correlation with ChM-I expression in human cartilage. (A) Vascular density was determined as the number of vascular channels that terminate in the non-calcified cartilage divided by the section length. Vascular density increased with cartilage degeneration and (B) was negatively associated with ChM-I expression in the ECM. Data are presented as the mean ± standard deviation; *P<0.05. ChM-I, chondromodulin-I; ECM, extracellular matrix. G0(Y), normal young donors. G0(A), normal aged donors; G1, mild OA donors; G2, moderate OA donors; G3, severe OA donors.

and the results are presented in Fig. 4. In mild OA cartilage, chondrocyte fibrosis was observed by Safranin-O/Fast green and ChM-I protein expression in the cytoplasm was significantly decreased in the superficial zone, compared with the superficial zone of normal cartilage (Fig. 4A). In the middle zone, there were no changes in cell morphology and ChM-I expression compared with the middle zone of normal cartilage (Fig. 4C). In the deep zone, vascular vessels were observed invading the non-calcified cartilage and ChM-I expression was decreased compared with the deep zone of normal cartilage (Fig. 4F). The mRNA levels of ChM-I were decreased in mild OA compared with normal cartilage (Fig. 3D; P<0.05).

In moderate OA cartilage, small cracks were observed in the cartilage surface and ChM-I expression was markedly decreased in the cytoplasm and ECM of the superficial zone (Fig. 4B). In the middle zone, the mitotic activity of chondrocytes appeared to be greater compared with the young and aged group. ChM-I immunostaining was stronger in the cytoplasm of moderate OA chondrocytes compared with the mild OA cartilage (Fig. 4D). In the deep zone, compared with the normal cartilage, a further decline in the number of chondrocytes and cytoplasmic ChM-I expression was observed (Fig. 4G). ChM-I mRNA expression levels in moderately degenerated cartilage were significantly higher compared with mildly degenerated cartilage (Fig. 3D; P<0.05).

In all severe OA cartilage specimens, the cartilage surface was severely damaged and the chondrocytes had been lost. Numerous cluster-forming chondrocytes were detected below the eroded surface (Fig. 4E). ChM-I protein expression was reduced in the ECM in all zones of the OA cartilage (Fig. 4E and H). ChM-I expression was detected primarily in the cytoplasm of the cluster-forming chondrocytes (Fig. 4E).

Angiogenesis at the osteochondral junction. The number of vascular channels terminating in the non-calcified cartilage was significantly greater in the OA cartilage samples compared with normal human cartilage (Fig. 5A; $P<0.05$). The density of vascular channels appeared to be correlated with the immunostaining intensity of ChM-I in ECM (Fig. 5B; $r=0.905$; $P<0.001$); however no correlations were identified between vascular channel density and cytoplasmic ChM-I protein expression or mRNA levels.

Discussion

It has previously been demonstrated that ChM-I is expressed in the proliferative and hypertrophic zones of rabbit condylar cartilage; however, is not expressed in subchondral bone (17). Increased expression levels of ChM-I have also been reported in the articular cartilage of growing and normal adult rat joints (11). The present study demonstrated that ChM-I was expressed in non-calcified zones of cartilage of young and aged donors and were without vasculature. These results indicated that there may be a regulatory role for ChM-I in vascular invasion during endochondral bone formation (5).

In addition, it was observed that vascular density in OA cartilage gradually increased with the level of cartilage degeneration, whereas ChM-I protein expression levels in the ECM decreased. This suggested that decreased ChM-I expression may lead to a decreased ability to inhibit angiogenesis during OA progression. Besides ChM-I, there are other factors that inhibit angiogenesis, such as thrombospondin-1, type XVIII-derived endostatin, secreted protein acidic and rich in cysteine and the type II collagen-derived N-terminal propeptide (18). However, the mechanism that results in ChM-I reduction in OA cartilage remains to be elucidated. The results of the present study indicated that the reasons for reduced ChM-I expression differed at various stages of OA progression. In early OA, the degeneration of cartilage is mild; a decrease of ChM-I gene expression in cytoplasm may be associated with cartilage fibrosis. Fibrillation on the surface of mildly degenerated cartilage has been observed in a rat OA model and in human OA samples (11,13), and ChM-I expression was significantly decreased in the superficial zone of mildly and moderately degenerated cartilage (19). The expression of certain factors that promote cartilage fibrosis, such as transforming growth factor-β and basic fibroblast growth factor, have been demonstrated to be increased in the superficial zone of OA cartilage and have been reported to inhibit ChM-I expression (20-24).

The present study also observed more chondrocyte clusters in moderately and severely degenerated cartilage compared with mildly degenerated cartilage. ChM-I protein expression was increased in the cytoplasm of chondrocytes in the middle zone of moderate OA cartilage. Similarly, ChM-I mRNA levels were increased in moderately degenerated cartilage compared with mildly degenerated cartilage. These results appear to be consistent with the theory that ChM-I promotes chondrocyte proliferation (25). It has been suggested that the increase in ChM-I protein expression in the cytoplasm may be associated with the anoxia of OA cartilage (26,27). Fibrosis of the cartilage surface disrupts oxygen diffusion from the synovial membrane to the cartilage, and cartilage hypoxia further promotes ChM-I expression via hypoxia inducible factor 2α (28). Therefore, chondrocyte proliferation and increased ChM-I protein expression in cytoplasm may be upregulated through a defense mechanism against the hypoxia during the degenerative processes of cartilage (29).

In the middle zone of moderately and severely degenerated cartilage, ChM-I protein expression in the cytoplasm was increased; however in the ECM of the middle zone and the deep zone, the overall level of ChM-I significantly decreased. Therefore, in the late stages of OA when the cartilage is more damaged, cartilage matrix loss is very serious, which leads to decreased ChM-I in the cartilage ECM. A previous study revealed that ChM-I precursor proteins are cleaved intracellularly and the mature glycopeptide is rapidly secreted (30). This observation suggested that newly secreted ChM-I may not be retained in the degenerated matrices owing to its high solubility in aqueous fluid. Alternatively, the decrease of ChM-I expression may indicate an increase of proteolytic enzymes in OA cartilage (30,31).

Notably, the alterations in ChM-I mRNA and protein expression levels in the rat OA model differ from that in human OA cartilage. This may be due to the fact that the rats used in the studies aforementioned were still growing (11). Chondrocytes are proliferative during the growing stages and may be able to rapidly respond to cartilage damage. However, OA primarily occurs in the aged, where the vitality of chondrocytes in OA cartilage is weak and cell response to cartilage damage is slow (32). Therefore, the use of adult or aged animal models to study OA may be able to mimic human OA more successfully.

Several limitations existed in the present study: First, due to traditional practices in China, few elderly patients are willing to undergo amputation, causing difficulty in obtaining knee specimens form elderly patients. The specimens of aged patients were obtained from the femoral heads, where the biomechanics are different from that of knee; however, the obtained specimens were from a weight-bearing area and are close to the mechanical environment. Second, vascular channels were not identified with a specific antibody, however the morphology was observed to be different from other tissues at the chondro-osseous junction (33).

In conclusion, with the degeneration of cartilage, the expression of ChM-I in the cytoplasm decreased in mild OA cartilage and then increased in the moderate OA cartilage. The ChM-I in ECM of cartilage decreased gradually that was correlated with the angiogenesis in cartilage. These results suggest that maintaining ChM-I levels in the ECM may help to improve the ability to resist vascular ingrowth to the cartilage, especially in mild osteoarthritis.

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