Chondrocyte-mediated collagenolysis correlates with cartilage destruction grades in osteoarthritis

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Abstract

Objective

Osteoarthritis (OA) is associated with destruction of type II collagen-rich hyaline articular cartilage. We hypothesized that classical interstitial collagenases cleave collagen type II, leading to the increased expression of the 3/4 native type II collagen fragment (COL2-3/4C) and the corresponding denatured type II collagen fragment (COL2-3/4M), which could correlate with different cartilage destruction grades. In addition, we assessed whether these fragments could be measured in joint fluid and serve as diagnostic markers.

Methods

Cartilage specimens were obtained from the femoral heads of hip joints from total hip replacement operations. Articular gliding surfaces of the cartilage were categorized into normal (G0), fibrillated (G1), superficially fissured (G2) and deeply fissured (fissures that reach to the subchondral bone) (G3). A histological scoring of the cartilage was also used. COL2-3/4C and COL2-3/4M were detected by immunohistochemical staining. Dot blotting was used to detect these fragments in joint fluid.

Results

COL2-3/4C and COL2-3/4M were found in the perichondrocyte matrix around lacunae. Such COL2-3/4C (p < 0.05) and COL2-3/4M (p < 0.05) immunoreactivity was significantly increased in G3 and G2 compared to G0 and G1. A positive correlation (n = 35, Spearman rank correlation) was observed between the histological score and the percentage of COL2-3/4C positive lacunae (r = 0.43, p = 0.01) and COL2-3/4M positive lacunae (r = 0.53, p = 0.001). All 7/7 joint fluid samples contained COL2-3/4C in dot blots whereas only 4/7 contained COL2-3/4M.

Conclusion

Collagenase-cleaved collagen – both native and denatured – increases as the severity of OA increases, assessed using a macroscopic clinical and microscopic histological grading system. Collagen degradation was always most apparent around chondrocytes. Furthermore, the native COL2-3/4C fragment has potential as a joint fluid marker for OA.

Key words

Collagenase, collagen type II, osteoarthritis, pannus.

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Introduction

Osteoarthritis can be a painful disease, which may lead to crippling and disability (1). Degradation of hyaline articular cartilage is its main pathological feature (2). Collagen type II is a main structural component of the cartilage matrix. Collagen type II is composed of three \( \alpha_2 \)-chains which form triple helical fibrils organized to stable collagen fibers and fibrillar networks. Collagen type II is mainly responsible for the mechanical strength of the cartilage and controls the swelling pressure of the hydrophilic proteoglycan-rich cartilage matrix. The counterpressure exerted by the collagen network prevents swelling and the composite of collagen and proteoglycans provides elasticity (3). The concentrations of other minor collagens, such as IX, XI and X, are much lower.

Cartilage is able to withstand high physical load and assures the congruence of joint during movement. Once it is damaged, cartilage has a very limited repair potential, in part probably due to low cellularity, lack of blood vessels, mechanical strain and a highly organized supramolecular structure which is difficult to restore (4). Normally, the collagen type II fibril network is resistant to most proteolytic enzymes (5). Only interstitial collagenases (collagenase-1, -2, and -3) catalyze the initial and specific cleavage of collagen at the intrahelical \( \text{Gly-775-Ile} \) bond (6). This generates the characteristic three-quarter (3/4) and one-quarter (1/4) collagen fragments. These fragments are different from those produced by cathepsin K, which cleaves at multiple sites at the end stage of osteoarthritis (7, 8). These fragments produced by mammalian collagenases are spontaneously denatured at body temperature and then rapidly degraded by collagenases and gelatinases (9, 10). Collagenase-1 (fibroblast collagenase or MMP-1) is expressed by fibroblast but also by chondrocytes (11). Collagenase-2 can be expressed by chondrocytes (12). Collagenase-2 binds to the collagen II cleavage site, as has been demonstrated using atomic force microscopy (13). Also collagenase-3 can be expressed by chondrocytes and synoviocytes, and it is the most effective collagenase in collagen II degradation (14,15). Collagenase activity in joint fluid correlates with cartilage damage (16). However, chondrocytes are also able to produce tissue inhibitors of metalloproteinases (TIMPs), which have been detected in joint fluid (17). An imbalance between collagenases and their endogenous inhibitors has been suggested to result in cartilage collagenolysis (18). Collagenases have been considered as the rate-limiting enzymes in collagen degradation. Collagenase-1, -2 and -3 are all synthesized by chondrocytes (19,20,21). Collagenase levels in the synovial fluid and serum correlate with cartilage destruction in OA (16). Some evidence has been presented to prove that collagenases are activated, overcome their endogenous antiproteinase inhibitor shield and cleave cartilage collagen fibrils (22,23). Recently developed neo-epitope-specific antibodies make it possible to demonstrate collagenase-cleaved hyaline articular type II collagen (23).

This study was conducted to test the hypothesis that the collagenase-cleaved native collagen II 3/4 fragment (COL2-3/4C) and its denatured counterpart (COL2-3/4M) correlate with cartilage destruction grades. The eventual presence of COL2-3/4C or COL2-3/4M in synovial fluid was also studied.

Methods

Patients and samples

Collection of tissue samples was approved by the Ethics Committee of Helsinki University Central Hospital. The guidelines of the Declaration of Helsinki were followed. Cartilage samples were from patients undergoing total hip replacement. All subjects gave their informed consent. Ten patients with a mean age of 76 ± 7 years (range 67-86) participated in this study. Patients fulfilled the clinical and radiological criteria of the American College of Rheumatology for osteoarthritis of the hip (24). In the case of joint replacement surgery, even one single femoral head may contain very different types of cartilage – from almost intact cartilage through all the grades of damaged cartilage. The degree of cartilage destruction of that part of the tissue...
which was used for analysis, was grad-
ed by the surgeon from 0 to 3 as fol-

dows (25). Cartilage specimens were
obtained from the femoral heads of hip
joints from total hip replacement oper-

tions. Cylindrical samples were snap-
frozen in liquid nitrogen, embedded in
OCT and stored at 80°C. Although more
than one sample might be collected
from patients, there was no repetition
since at most one sample of each grade
(G0, G1, G2, G3) was taken from a sin-
gle patient. In no case were two sam-
ples of the same grade (e.g. G0) taken
from the same patient. As the joint sur-
face wears differently in differently
loaded joint surface areas, it was possi-
ble to collect all different grades of car-
tilage (G0, G1, G2 and G3) in 7 pa-
tients, three grades in 2 patients (G1, G2
and G3) was taken from a sin-
gle patient. In no case were two sam-
ples of the same grade (e.g. G0) taken
from the same patient. The joint sur-
face wears differently in differently
loaded joint surface areas, it was possi-
ble to collect all different grades of car-
tilage (G0, G1, G2 and G3) and one grade (G3) in 1 pa-
tient.

Histological grading was done using a
modification of a method described
earlier (25). Briefly, cartilage sections
were fixed in freshly prepared 4% for-
maldehyde for 5 minutes followed by a
10 minute rinse in distilled water and
counterstaining of nuclei in iron haem-
atoxylin and of cell cytoplasm with
0.2% light green (Allied Chemical Cor-
poration, New York, USA) in dH2O for
5 minutes. Slides were washed in run-
ing tap water for 10 minutes and rin-
sed in 1% acetic acid, pH 4.6. Cartilage
matrix was stained as follows: 4% for-
maldehyde-fixed sections were coun-
terstained in iron haematoxylin and
0.2% light green, followed by 0.1% Sa-
franin O (Merck, Darmstadt, Germany)
solution in 0.1% acetic acid for 15 min-
utes before rinsing in 1% acetic acid and
running tap water for 10 minutes, dehydroxylation, clearing and mounting as
described before (26). Histological gra-
ding was based on the following char-
acteristics. For the cells: normal cellu-
laritv = 0, diffuse hypercellarity = 1, clo-
ing = 2, and hypocellularity = 3. For Safranin-O staining of matrix: normal
= 0, slight reduction = 1, moderate re-
duction = 2, severe reduction = 3, no
staining at all = 4.

Immunohistochemistry
8 µm cryostat sections of cartilage were
cut to SuperFrost slides (Menzel Glä-
ser, Germany). The sections were fixed
in freshly prepared 4% (w/v%) parafor-
maldehyde in 0.1M phosphate-buffered
saline (pH 7.4; PBS) for 5 minutes at
+4°C. The sections were incubated in
1% (w/v) hyaluronidase (sheep testicu-
lar, type II, EC 3.2.1.35; Sigma, St Louis, MO) in PBS at room tempera-
ture for 30 minutes in order to increase
the permeability of the cartilage matrix.
Endogenous peroxidase activity was
blocked using 1% (v/v) H2O2 in meth-
anol for 30 minutes at +22°C. Non-spe-
cific antibody binding was blocked
with incubation in normal goat or rab-
bit serum (1:20) in 0.1% bovine serum
albumin in PBS for 30 minutes at +22°
C. Incubation with the primary polyl-
clonal rabbit anti-human COL2-3/4C an-
tibody (Ibex, Montral, Canada) (dilu-
tion: 1:800) (23) or monoclonal mouse
anti-human COL2-3/4M antibody (Ib-
ex, http://www.ibex.ca) (dilution 1:
500) was performed overnight at +4°C
(22). COL2-3/4C and COL2-3/4M,
which also recognize their human coun-
terpart (23, 25), were visualized using
the Vectastain® Elite ABC Kit (Vector,
Laboratories, Burlingame, CA). 8.3%
3,3’-diaminobenzidine tetrahydrochlo-
ride (DAB, Sigma) in PBS with
0.006% H2O2 was used for colour de-
velopment for 3 minutes. Negative stain-
ing controls comprised omission of the
primary antibodies and use of irrele-
vant normal rabbit or mouse IgG at the
same dilution as and instead of the pri-
mary antibodies.

The peptide used to immunize rabbits
to produce antibodies able to recognize
denatured collagenase-produced colla-
gen neoepitopes was alpha1(II)-CB11-
B, which was conjugated to ovalbumin
through its amino-terminal cysteine.
Serum samples from immunized mice
were tested in an ELISA for reactivity,
followed by preparation of hybridomas
producing antibodies reacting with both
the immunogenic alpha1(II)-CB11B pepti-
and the heat-denatured type II
collagen (22, 28).

Quantitative assessment
Using a 20x objective magnification,
immunostained sections without coun-
terstaining were inspected with an Oly-
mpus Provis AX-70 microscope (Oly-
mpus Optical Co. GmbH, Hamburg,
Germany) coupled to a 12-bit CCD
camera (PCO SensiCam; PCO Compu-
ter Optics GmbH, Kelheim, Germany).
Images were analyzed using a semi-
automated Analysis Pro 3.2 image an-
alysis and processing system (Soft Ima-
ging System GmbH, Münster, Ger-
many). The number of COL2-3/4C or
COL2-3/4M immunoreactive (de-
monstrated by perilacunar staining) and
the number of total lacunae were analyzed
from the total area of the sections. At
least 200 lacunae were counted for
COL2-3/4C or COL2-3/4M positivity
using one section from each cartilage
sample. The results are expressed as a
percentage value (%) of immunoreac-
tive lacunae in comparison to the total
lacunae.

Dot blots
Hyp joint fluid samples were diluted in
ten-fold series (1:1 to 1:10,000) in 50
mM Tris-HCl buffer, 0.15M NaCl and
1mM CaCl2, pH 7.5. Synovial fluid
samples analyzed for collagen degrada-
tion fragments using dot blot were de-
derived from a different set of patients
and were not paired with those used for
the immunohistochemistry.

1.5 µL serial samples were dotted on
nitrocellulose membrane (Bio-Rad Lab-
oratories, Richmond, CA) and air dried
at +22°C for 30 minutes.
(1) The membrane was dipped into 50 mM Tris-HCl buffer, pH 7.5, 0.01% Tween-20 washing buffer for 5 minutes.
(2) Non-specific binding sites were blocked by soaking in 5% bovine serum albumin in 50 mM Tris-HCl, pH 7.5, 0.01% Tween-20 for 1 hour at room temperature.
(3) Membranes were incubated with COL2-3/4C (1:200) or COL2-3/4M (1:200) antibodies dissolved in 2% bovine serum albumin in 50 mM Tris-HCl, pH 7.5, 0.01% Tween-20 overnight at +22ºC, followed by 3 x 5 min washes in buffer alone.
(4) Alkaline phosphatase conjugated goat anti-rabbit or -mouse IgG antibody (1:3000, Bio-Rad) incubation was performed for 1 hour at +22ºC followed by washes in washing buffer and, finally, in 50 mM Tris-HCl, pH 7.5.
(5) Colour was developed in alkaline phosphatase buffer with 1/100 5-bromo-4-chloro-3-indolyl phosphate and 1/100 nitroblue tetrazolium (Conjugate Substrate Kit; Bio-Rad) for 10 minutes +22ºC after which the reaction was stopped by washing in dH2O for 15 min.

Statistical analysis
Statistical calculations were made using the BMDP 02.00 software package (Los Angeles, CA). Shapiro and Wilk’s W test were used to test for normality. The percentage of positive COL2-3/4C and COL2-3/4M lacunae was correlated with different grades of cartilage destruction using the Spearman rank correlation test. The Kruskal-Wallis test in ANOVA was applied to test for differences of COL2-3/4C and COL2-3/4M between the groups. When the difference was significant at the level of $p < 0.05$, the Rank sum test was further used to localize the differences. The results are expressed as the mean ± the standard error of the mean.

Results
COL2-3/4C and COL2-3/4M immunoreactivity was localized to the perichondrocyte matrix surrounding the lacunae in the superficial and middle layers of the cartilage in the G0 and G1 groups. No enlarged lacunae or damaged chondrocytes were found in these areas. Almost no COL2-3/4C and COL2-3/4M positive lacunae were seen in the deep zone (Fig 1; Fig 2). Intensive COL2-3/4C and COL2-3/4M immunoreactivity was scattered in all cartilage layers in the G2 and G3 samples. In the G2 and G3 samples, some lacunae were large and contained damaged chondrocytes (Fig 1; Fig 2).

Quantitative analysis was first performed to assess an eventual relationship between collagen degradation fragments and macroscopic (clinical) grade of the cartilage. Briefly, there were significantly increased COL2-3/4C ($p < 0.05$) and COL2-3/4M ($p < 0.05$) positive lacunae in G3 (COL2-3/4C, n = 10, 22.78 ± 7.19%, Q1 = 0, Q3 = 50.53%; COL2-3/4M, n = 10, 29.76 ± 9.35%, Q1 =0.99, Q3 = 57.48) and G2 (COL2-3/4C, n = 9, 17.38 ± 6.13%, Q1 = 0, Q3 = 32.84%; COL2-3/4M, n = 9, 21.56 ± 7.15%, Q1 = 0, Q3 = 33.17) compared to G1 (COL-2-3/4C, n = 9, 4.77±3.48%, Q1=0, Q3=6.30; COL2-3/4M, n = 9, 7.19±7.13%, Q1=0, Q3=10.83).

Fig. 1. The COL2-3/4C (native) immunoreactivity patterns in different cartilage destruction grades: (A) G0: Only a few COL2-3/4C positive lacunae are seen in the superficial and middle layers. No COL2-3/4C positive lacunae are seen in the deep zone. (B) G1: COL2-3/4C immunoreactivity is shown in the superficial and middle layers. The number of COL2-3/4C surrounding lacunae is only slightly increased, compared to G0 (not significant statistically). (C) G2: COL2-3/4C positive lacunae are seen scattered in all layers of cartilage. Some of the positive lacunae surrounded by collagenase-cleaved collagen neopeptides are clearly larger than the normal size lacunae. (D) G3: Many COL2-3/4C immunoreactive lacunae are seen in all layers of the cartilage. Some fissures are also seen. In most cases the superficial layer has been worn off. Distorted and enlarged lacunae are frequent. All bars in A, B, C, D = 10 µm.
Quantitative analysis was also performed to assess an eventual relationship between collagen degradation fragments and histological grading of the cartilage. A positive correlation (n = 35, Spearman rank correlation, Fig. 3) was observed between a modified Mankin’s histological score and the percentage of COL2-3/4C positive lacunae (r = 0.43, p = 0.01) and COL2-3/4M positive lacunae (r=0.53, p= 0.001).

All synovial fluid samples used for dot blot analysis were from OA patients and a statistical comparison with controls was therefore not possible. Dot blot of synovial fluid was used to demonstrate that it is possible to apply this method to demonstrate both native and occasionally also denatured collagenase-cleaved collagen fragments in osteoarthritic synovial fluid. In dot blots, 7/7 OA joint fluid samples were COL2-3/4C positive. Two of these were positive even at a 1/10000 dilution. In contrast, only 4/7 cases contained COL2-3/4M immunoreactivity (Fig. 4).
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Discussion

Some evidence has been reported that collagenases in OA are activated, overcome their endogenous antiproteinase inhibitor shield and cleave cartilage collagen II fibrils. This eventually leads to irreversible cartilage destruction in human diseases and animal models (23, 28, 30, 31). This is confirmed by the present study showing increased immunoreactive COL2-3/4C and its denatured COL2-3/4M around lacunae upon increasing degeneration. Although the number of samples used was relatively low, the statistical methods adopted in the present study take into consideration such uncertainty, which is based on relatively low numbers of observations. It is therefore concluded that collagenase-cleaved collagen, native and denatured, increases as the severity of OA increases, based on an assessment using a macroscopic clinical and microscopic histological grading systems.

Collagenases produced by chondrocytes have the potential to mediate pericellular collagenolysis (29, 32, 33). Collagenases produced by fibroblasts in the pannus-cartilage tissue junction or in synovial tissue are other sources leading to irreversible cartilage lesions (34, 35). In the present study, COL2-3/4C and COL2-3/4M immunoreactivity around cartilage lacunae was seen in the superficial and the middle zones in G0 and G1 grade hyaline articular cartilage in OA. Based on the staining pattern, i.e. that both the native and denatured collagenase-cleaved collagen fragments were always found around chondrocyte lacunae, we conclude that chondrocytes may themselves be the cellular source of those mammalian collagenases, which are responsible for cartilage type II collagen cleavage. This suggests that the early stages of
cartilage destruction are characterized by peri-chondrocyte collagenolysis. Although our cartilage samples were obtained at joint replacement surgery performed due to severe osteoarthritis, we refer to early osteoarthritis due to the fact that different areas of the same joint can display apparently normal looking hyaline articular cartilage in non-weight bearing areas, whereas other areas subjected to weight bearing and/or pathological degeneration can display advanced macro- and/or microscopic changes of osteoarthritis. It is naturally not possible to exclude the possibility that the apparently healthy looking cartilage in an osteoarthritic joint is not totally healthy in spite of its normal looking macro- and microscopic appearance and low level of collagenase-cleaved collagen fragments. Perichondrocyte collagen matrix destruction was often so extensive that based on the visual assessment of the mean chondrocyte size and the size of the enlarged lacunae, it seemed that the destroyed lacunae were so large that they would provide space for a chondrocyte proliferative response, i.e. for at least two chondrocytes.

COL2-3/4 neoepitopes in articular cartilage and synovial fluid might only represent normal cartilage tissue remodeling. However, as the proportion of COL2-3/4 neoepitope containing perilacunar areas clearly increased as the degree (or grade) of degeneration increased, it can be concluded that at least this remodeling rate increases as the degeneration advances. Furthermore, it seems, based on the clinical and histological scores, that this enhanced remodeling is not able to keep up with the pace of cartilage tissue destruction. Increased COL2-3/4C and its denatured COL2-3/4M fragment were found in the early stages of OA before cartilage showed more prominent signs of destruction in the form of fibrillation or fissures. Many of the currently available imaging methods, such as computed tomography (CT), arthrography, ultrasonography, and magnetic resonance (MR) imaging, are not sensitive enough to detect the early stages of cartilage destruction (36). Joint arthroscopy can be used to check the articular surface, but does not reveal the underlying cartilage pathology (37). Because only small volumes of joint fluid are required for dot blotting, it has been suggested that it may be clinically useful in some other diseases.

In the present study, COL2-3/4C in OA joint fluid samples was more often positive than COL2-3/4M, even at high dilutions. This implies that COL2-3/4C might be a good marker for the destruction of articular cartilage. It could be helpful in the clinical diagnosis of osteoarthritis at an early stage of the disease. It is naturally possible that collagenase-cleaved native collagen fragments could also be found in normal joint fluid as a result of normal cartilage turnover. However, based on the morphometric measurements of collagenase-cleaved native collagen fragments in cartilage showing different degrees of osteoarthritic changes, we conclude that synovial fluid COL2-3/4C might be a good marker for ongoing collagen destruction, although this will require further studies. While the present work does not in any way prove that the COL2-3/4C fragment can be used as a marker for ongoing cartilage collagenolysis, the fact that the native collagen degradation fragment was found in all 7 synovial fluid samples studied indicates that this test merits further study to evaluate its potential as a biochemical marker for osteoarthritic joint tissue destruction. However, a good marker of cartilage degradation should be measurable from serum and urine, and not only from synovial fluid. Indeed, recent work in a canine model of arthritis demonstrates that COL2-3/4C can be measured in urine (38). In conclusion, enhanced COL2-3/4C and COL2-3/4M immunoreactivity is observed in OA and correlates with cartilage destruction grades in osteoarthritis. Collagenases produced by chondrocytes have been activated and overcome their endogenous tissue inhibitors, and are thus able to cleave across the triple helical collagen fibrils surrounding the cartilage lacunae. This also leads to the release of COL2-3/4C to the joint fluid, which has potential as a marker for diagnosis and disease follow-up.

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