Topographic and zonal distribution of tenascin in human articular cartilage from femoral heads: normal versus mild and severe osteoarthritis

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Summary

Objective: The extracellular matrix glycoprotein tenascin (TN) is upregulated in articular cartilage with severe osteoarthritis (OA). This study gives a detailed description of TN expression in areas of articular cartilage from femoral heads with mild OA showing structural lesions and in structurally normal areas of the same femoral heads compared with normal cartilage and cartilage with severe OA.

Methods: Immunohistochemical evaluation was performed on cryosections stained with antibodies against TN. Sections were selected as follows: from each macroscopically normal femoral head (n=6) a normal central and peripheral biopsy; from each femoral head with macroscopically mild OA (n=8) a central biopsy that showed structural lesions and a peripheral normal biopsy; from each femoral head with severe OA (n=9) a central and a peripheral biopsy with structural lesions. Central biopsies represent load bearing areas, whereas peripheral biopsies are non-load bearing.

Results: Central cartilage with mild OA contains significantly higher levels of TN in the superficial zone than structurally normal, peripheral cartilage from the same femoral heads. Normal cartilage and cartilage with severe OA do not display this topographic variation. Central cartilage with mild OA shows significantly higher levels of TN than normal, central cartilage. Peripheral, normal cartilage with mild OA shows significantly less TN than peripheral cartilage with severe OA.

Conclusions: In femoral heads with mild OA, TN is accumulated in areas displaying structural damage. This proposes mild OA to be a localized disorder. Extreme caution is necessary for sampling of articular cartilage, especially from joints with mild OA.

Key words: Tenascin, Osteoarthritis, Grading, Immunohistochemistry.

Abbreviations: CD, cluster of differentiation, Fn, fibronectin, IHC, immunohistochemistry, IL, interleukin, MMP, matrix metalloproteinase, OA, osteoarthritis, SFH, safranin-O, Fast Green, haematoxylin, TBS, Tris-buffered saline, TN, tenascin.

Introduction

In joints with severe osteoarthritis (OA) the entire articulating surface is damaged, manifested by disorganization and erosion of articular cartilage. In cases of less severe OA, the lesions include only localized areas of cartilage damage, at least at the macroscopical level. The extracellular matrix glycoprotein tenascin (TN) is involved in embryogenesis and wound healing, presumably through modification of the cellular adhesion to fibronectin (Fn). TN is highly expressed in human articular cartilage in relation to structural damage, as fibrillated and fissured areas of articular cartilage with severe OA are known to accumulate TN.

Several studies have documented elevated levels of TN in cartilage and synovial fluid from individuals suffering from severe OA. Recently, TN was shown to be the most promising molecular marker of cartilage degradation in synovial fluid from patients suffering from severe OA of the knee, whereas the initial stages of OA did not present increased levels of TN. TN is also increased in serum from patients suffering from severe OA, hence TN has been proposed as a marker of OA. In a rabbit model of OA, TN has been shown to be increased in the superficial zone of femoral condylar cartilage with moderate and severe OA. However, the distribution of TN has not been detailed in human articular cartilage with mild OA or in macro- and microscopically normal areas of cartilage from femoral heads with mild OA.

The objective of this study was to detail the expression of TN in load bearing areas of articular cartilage from femoral heads with mild OA showing structural lesions and in structurally normal non-load bearing areas of the same femoral heads compared with normal cartilage and cartilage with severe OA.

Methods

SAMPLING OF ARTICULAR CARTILAGE

Normal cartilage and cartilage with mild OA were obtained from the femoral head at autopsy. Biopsies
representing severe OA were obtained from patients undergoing hip replacement surgery due to OA. None of the patients had a history of a systemic inflammatory disease. The femoral heads were graded macroscopically according to Collins/McElligot\(^8\), and the location and nature of any pathological alterations were noted. Grade 0 femoral heads correspond to normal cartilage, whereas grades 1 and 2 correspond to mild OA. Grades 3 and 4 with areas of denuded bone correspond to severe OA (Table I).

Sampling of biopsies covering all aspects of the femoral head was performed in a standardized way, as described by Ostergaard and Salter\(^9\). In brief, biopsies anterior, posterior, medial, and lateral to fovea capitis femoris (perifoveal), next to the insertion of the articular capsule (peripheral), and in between (intermediate) (12 biopsies in total) were sampled from each femoral head and stored at \(-80°C\). In the following, perifoveal and intermediate biopsies together are referred to as central biopsies.

After macroscopical grading, histopathological evaluation was performed to choose the desired biopsies—from each grade 0 femoral head a central and a peripheral biopsy that showed no structural changes; from each grade 1 and 2 femoral head, a central sampling site showing pathological features of OA at the macroscopic level, a biopsy that showed fibrillation or fissures in the superficial zone (located lateral intermediate or posterior perifoveal), and a peripheral biopsy that did not show any structural change at either the macroscopical or the histological level; from each grade 3 and 4 femoral head a central and a peripheral biopsy displaying severe structural changes (i.e. fibrillation and clefts). In five cases of severe OA no peripheral biopsy could be obtained. In total 49 biopsies were selected. Two central biopsies from normal femoral heads and one central biopsy from a femoral head with severe OA could not be evaluated due to either floating off or extensive folding of the section. Forty-six sections were suitable for immunohistochemical evaluation.

### Table I

<table>
<thead>
<tr>
<th>Macroscopical grading</th>
<th>No. of cases (sex)</th>
<th>Median age (range)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8 (6M, 2F)</td>
<td>59.5 (32–77)</td>
<td>Autopsy</td>
</tr>
<tr>
<td>Mild OA</td>
<td>10 (5M, 5F)</td>
<td>68 (59–89)</td>
<td>Autopsy</td>
</tr>
<tr>
<td>Severe OA</td>
<td>9 (6M, 3F)</td>
<td>77 (39–85)</td>
<td>Total hip arthroplasty</td>
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</table>

### Table II

<table>
<thead>
<tr>
<th>Score</th>
<th>Cell score (0–4)</th>
<th>Matrix score (0–2)</th>
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<tbody>
<tr>
<td>0</td>
<td>All cells negative</td>
<td>No staining</td>
</tr>
<tr>
<td>1</td>
<td>Less cells negative</td>
<td>Weak, localized staining</td>
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<tr>
<td>2</td>
<td>Equal number of negative and positive cells</td>
<td>Extensive staining</td>
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<tr>
<td>3</td>
<td>More cells positive</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>All cells positive</td>
<td></td>
</tr>
</tbody>
</table>

### HISTO- AND IMMUNOHISTOCHEMICAL PROCESSING

Five micrometer cryosections were mounted on SuperFrost/Plus glass slides (Eire Scientific, Portsmouth, NH, USA). Sections for histochemistry were fixed in formalin for 24 h, while sections for immunohistochemistry (IHC) were fixed in acetone for 15 min at room temperature as described in reference 10. All stainings were performed using a Shandon Sequenza.

Stainings were performed with Weigert’s acid iron chloride haematoxylin for 6 min, Fast Green for 3 min, and safranin-O for 5 min (SFH stainings). The first wash was with tap water, the second with 1% acetic acid, and the two washes following safranin-O were with 96 and 100% alcohol, respectively.

The IHC procedure has been described previously\(^11\). In brief, 100 µl of the primary antibody was applied diluted to optimal concentration in Tris-buffered saline (TBS). The concentration of the monoclonal anti-TN antibody (clone TN2, DAKO, Glostrup, Denmark) was 0.654 µg/ml. The antibody recognizes all TN isoforms. The primary antibody was incubated for 24 h at 4°C in a humidified chamber. The following incubations were performed at room temperature and 100 µl of each solution was added (all supplied by DAKO). The specimens were washed twice with 2 ml of TBS, and incubated for 25 min with a biotinylated rabbit anti-mouse secondary antibody. The washing procedure was repeated and endogenous peroxidase activity was blocked by incubation for 8 min with 3% H\(_2\)O\(_2\). Horseradish

### Table III

<table>
<thead>
<tr>
<th>Structure</th>
<th>Normal</th>
<th>Fibrillation</th>
<th>Sup. fiss.</th>
<th>Middle fissure</th>
<th>Deep fissure</th>
<th>Cells</th>
<th>Normal</th>
<th>Clones</th>
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<td>Peripheral (8)</td>
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<td>1</td>
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<td>2</td>
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<td>2</td>
<td>7</td>
<td></td>
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<tr>
<td>Peripheral (4)</td>
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<td></td>
<td></td>
<td>1</td>
<td>3</td>
<td></td>
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Sup. fiss., superficial fissure. Numbers refer to number of cases displaying each feature.
Fig. 1. Histology of normal articular cartilage and articular cartilage with mild and severe OA. SFH stainings. (A) Normal articular cartilage (magnification approximately ×40). (B) Articular cartilage with mild OA showing mild fibrillation at the entire surface and loss of safranin-O staining (magnification approximately ×20). (C) Articular cartilage with severe OA showing more advanced fibrillation, a fissure through the superficial zone, but no loss of safranin-O staining intensity (magnification approximately ×20).
peroxidase-conjugated streptavidin was added and incubated for 25 min. After washing, antibody binding was visualized by adding a buffered solution of \( \text{H}_2\text{O}_2 \) and 3-amino-9-ethyl-carbazole for 15 min, yielding a crispy red end-product at the sites of the antigen. Specimens were then washed with tap water and counterstained with Maier's haematoxylin.

**PROCEDURE CONTROLS**

Both omission of the primary antibody and application of non-immune mouse immunoglobulins in the same protein concentration were used as negative controls. To test for demasking of TN antigens, the specimens were either pretreated with 0.1% trypsin for 30 s at 37°C or a combination of hyaluronidase (2 mg/ml) and collagenase (10 mg/ml) for 5 min at 37°C before the standard immunostaining, but no demasking effect was observed (data not shown).

**EVALUATION OF HISTO- AND IMMUNOHISTOCHEMICAL STAININGS**

Grading of both histo- and immunohistochemical stainings was performed by two blinded observers in companion, and the scores were agreed upon.

SFH stainings evaluated the structure of cartilage and the cells. For structure, five classes were employed: normal, fibrillation, fissure involving the superficial zone only, fissure into the middle zone, and fissure into the deep zone. Cells were evaluated for the presence of clones. A clone was defined as a clustering of at least three cells located in the same rounded lacuna.

IHC stained preparations were evaluated as described by Ostergaard and Salter. In brief, cartilage sections were divided into the superficial (upper 1/4), deep (1/4 of section above tidemark), and middle (1/2 in between) zones. Each zone received a cellular score from 0 to 4 and a matrix score in the peri- and intercellular compartments from 0 to 2 as shown in Table II. Cellular staining represents intracellular as well as membrane associated staining, as these cannot be definitely distinguished from the methods used. The grading system used does not directly reflect the actual concentration of TN, but rather the extension of staining. Quantification of non-fluorescent immunohistochemical stainings is not sufficiently reproducible.

**STATISTICS**

Comparison of the scores of central and peripheral biopsies from the same subject was performed by Wilcoxon matched-pairs signed rank test in normal cartilage and cartilage with mild and severe OA. Comparison of scores of normal cartilage and cartilage with mild and severe OA at both the central and peripheral sampling sites was performed by Mann-Whitney rank sum test. \( P \) values less than 0.05 were considered significant.

**ETHICS**

The study was approved by a regional ethics committee as part of a larger project involving articular cartilage and bone.

**Results**

**HISTOPATHOLOGICAL FEATURES**

Macroscopically normal cartilage (grade 0) showed only occasional structural defects. Cartilage sampled from the lesonal central sampling site from femoral heads with mild OA (grades 1 and 2) showed histological defects in all cases ranging from fibrillation to fissures into the middle zone. All cartilage biopsies from femoral heads with severe OA (grades 3 and 4) showed structural defects ranging from fibrillation to fissures into the deep zone. In 65% of all sections clones were present. There was a tendency for the clones to be increased in femoral heads with mild or severe OA (Table III and Fig. 1).
Fig. 2 (A) and (B)
Fig. 2 (C), (D) and (E)
TENASCIN EXPRESSION IN CENTRAL VERSUS PERIPHERAL ARTICULAR CARTILAGE

In macroscopically normal cartilage, the highest level of expression of TN was located in the peripheral biopsies. Only half of the central biopsies showed cellular staining and only one biopsy showed matrix staining. Only one central biopsy showed TN staining in the middle or deep zone. Almost all the peripheral biopsies showed TN staining in the cellular compartment and half of the biopsies showed matrix staining in the superficial zone. In the middle and deep zones almost half the peripheral biopsies showed TN staining in all compartments; these differences did not achieve statistical significance (Table IV).

In mild OA, the superficial zone of lesional, central cartilage shows a significantly increased TN expression compared with the peripheral biopsies. In the majority of the central biopsies the cellular TN staining spread into the middle zone, whereas in the peripheral biopsies this was only occasionally the case. Matrix staining was rarely present in the middle or deep zones (Table IV).

Cartilage from femoral heads with severe OA showed very high levels of TN in the superficial zone of central as well as peripheral biopsies. Staining was present in all compartments in the middle zone of almost all biopsies and in the deep zone of half of the biopsies of both central and peripheral cartilage. Regarding cartilage with severe OA no difference between central and peripheral biopsies is present (Table IV and Fig. 2).

COMPARING TENASCIN EXPRESSION IN CENTRAL CARTILAGE FROM NORMAL JOINTS AND FROM JOINTS WITH MILD AND SEVERE OA

In cartilage with mild or severe OA, the staining of TN in the superficial zone was significantly increased in all compartments (Fig 4). Cartilage with severe OA showed the highest level of staining, but this was not significantly different from the level in mild OA (Table IV).

In the middle zone, the expression of TN was significantly increased in the cellular and pericellular compartments in cartilage with severe OA when compared with normal cartilage (P<0.05). Cartilage with mild and severe OA only differed significantly in this zone in the pericellular compartment, whereas no significant difference was present between normal cartilage and cartilage with mild OA (Table IV).

In the deep zone, the expression of TN was highest in cartilage with severe OA, where half the sections showed cellular staining. Staining was only occasionally present in the deep zone of normal cartilage or cartilage with mild OA. This difference did not achieve statistical significance (Table IV).

COMPARING TENASCIN EXPRESSION IN PERIPHERAL CARTILAGE FROM NORMAL JOINTS AND FROM JOINTS WITH MILD AND SEVERE OA

In the superficial zone, there was a significantly increased expression of TN in cartilage with severe OA in all compartments when compared with mild OA (P<0.05) (Fig. 5). There was no significant difference between normal cartilage and cartilage with mild OA, but significant difference was observed only in the intercellular compartment when comparing normal cartilage and cartilage with severe OA (P=0.008).

In the middle and deep zones no statistically significant difference between any of the three groups was present; however, the biopsies with severe OA showed a tendency towards increased expression of TN (Table IV).

Discussion

This study shows that the expression of TN is focally increased in human articular cartilage with mild OA, whereas cartilage with severe OA shows increased expression through all zones and compartments at both the central and the peripheral sampling sites. Consideration of TN as a molecular marker of OA supports the idea of mild OA being a focal condition regarding both depth and width, whereas severe OA extends to all zones and areas of the femoral head. To our knowledge, this is the first study to describe the topographic differences in the expression of a potential molecular marker of OA in human cartilage with mild OA compared with normal cartilage and with cartilage with severe OA.

The individuals with femoral heads with mild OA reported no symptoms of joint pain in their case books. One could then speculate that the cartilage degradation could be attributed to age-related degenerative changes. But the central sampling sites used in this study correspond to load bearing areas of the femoral head and are thus very likely to represent functionally important and clinically significant areas, even though this cannot be documented.

The increasing presence of TN with increasing severity of OA in our study corresponds to what was found in a rabbit model of moderate and severe OA. In the rabbit model, TN staining appears to spread from the middle and deep zones to the superficial zone with increasing severity of OA. In our and in other studies on humans the strongest staining is reported pericellular in the superficial zone in normal cartilage as well as in cartilage with OA. With increasing severity of OA, TN staining appears to extend to the deeper zones. This is in accordance with a study of the progression of OA, wherein the extent of denaturation of collagen type II fibrils in human femoral condylar cartilage.

Fig. 2. TN stainings of normal articular cartilage and articular cartilage with mild and severe OA. (A) Normal articular cartilage from the central sampling site. Only occasional positive chondrocytes are seen in the superficial zone but a discrete line of TN staining is present in the uppermost part of the superficial zone indicating that the source of TN is primarily the synovial fluid (magnification approximately ×10). (B) Normal articular cartilage containing a cluster of chondrocytes showing the cellular localization of TN in one chondrocyte (magnification approximately ×40). (C) Central articular cartilage with mild OA showing extensive staining of the fibrillated areas. Underneath are positive chondrocytes with a discrete zone of TN in the pericellular compartment superficial to the chondrocytes (magnification approximately ×10). (D) Peripheral articular cartilage from a femoral head with mild OA. Only a few chondrocytes are positive (magnification approximately ×10). (E) Articular cartilage with severe OA showing diffuse staining in the peri- and intercellular compartments and positive chondrocytes in the middle and deep zones (magnification approximately ×20).
Fig. 3. Negative control stainings with non-immune mouse immunoglobulins. (A) Normal articular cartilage. (B) Articular cartilage with mild OA. (C) Articular cartilage with severe OA. No staining is present. Magnification approximately ×10.
was studied\textsuperscript{12}. This study therefore proposed that OA originates around chondrocytes in the superficial zone and propagates to the deeper zones in later stages of the disease. The rabbit model\textsuperscript{7} describes secondary OA in the knee joint due to meniscectomy, whereas our study engages primary OA in the hip joint. This could also explain the differences in zonal distribution of TN between our study and the rabbit model.

In accordance with our findings, Chevalier \textit{et al.}\textsuperscript{3} reported increased expression of TN around clefts and...
fibrillation in a study of human femoral head cartilage with severe OA, suggesting a role for TN in the repair of cartilage.

In a study of human synovial fluid from knees of 65 patients with pain and joint impairment, TN proved to be the most promising molecular marker of cartilage degradation compared with cartilage oligomeric matrix proteins, YKL-40 and MMP-1, -3, and -13. TN levels were only correlated to the most severe arthroscopic grades of OA, though. As shown in our study, the expression of TN in cartilage with mild OA is only increased localized to lesional areas while peripheral, non-lesional cartilage displays levels of TN similar to or even less than macroscopical and histological normal cartilage. This might account for the lack of correlation between levels of TN and the mild grades of OA found at arthroscopy. Thus, more sensitive TN assays might be able to detect elevated TN levels in synovium from joints with mild OA.

The Arg-Gly-Asp-sequence in TN binds Fn and integrin αβ3. Other TN ligands are syndecan-15, integrin αβ14, and annexin II13. The integrin subunits αβ and ββ are present in normal human articular cartilage and in cartilage with severe OA, the α2 subunit has been localized in cartilage with severe OA only, whereas the β3 subunit is localized neither in the normal cartilage nor in cartilage with severe OA.

High levels of Fn (especially Fn fragments) induce a catabolic response through release of proinflammatory cytokines, catabolic enzymes and proteoglycan depletion, whereas low levels induce enhanced rates of proteoglycan synthesis17. Enhanced levels of TN might counteract the chondrolytic effects of the high levels of Fn found in OA4. One would then suspect a co-localization of Fn and TN in central human articular cartilage with severe OA. Increased staining of Fn as well as TN is indeed found in areas of fibrillated rabbit cartilage17. In central human articular cartilage with severe OA, staining of Fn is increased in the superficial layer and extends into the middle zone (unpublished observation), like the expression of TN in our study.

The source of TN in articular cartilage is still unclear. In this study, cellular staining was present in combination with strong pericellular staining. Occasionally, single chondrocytes contained staining in what resembles the intracellular compartment only [Fig. 2(B)]. This cellular staining of TN is not likely to be unspécific due to dying cells, as this was not found in the negative controls [Fig. 3(A–C)]. In most sections of fibrillated cartilage from femoral heads with mild OA, an area of cartilage superficial to the chondrocytes was strongly positive, suggesting that chondrocyte-derived TN is secreted and directed towards the articulating surface [Fig. 2(C)]. IL-1β upregulates the expression of TN in the pericellular compartment18 and bovine articular chondrocytes synthesize TN19. On the other hand, occasional staining of the extracellular matrix was present in the uppermost part of the superficial zone with only few chondrocytes positive for TN [Fig. 2(A)]. This suggests that TN may be derived from both the chondrocytes and the synovium.

In normal cartilage and cartilage with severe OA no topographical variation was observed in this study, whereas central and peripheral cartilage with mild OA differed significantly regarding the expression of TN. This underlines the importance of including topography and macroscopical description of the sampling site, as random sampling from a femoral head with mild OA could result in false conclusions. This is supported by the knowledge that several molecules are known to be expressed with topographic (e.g. YKL-4020 and collagen type VI21) or zonal variation (e.g. integrin α5 and CD4411).

The monoclonal antibody used in this study recognizes all variants of TN so that the differential expression of the variants cannot be determined. As the TN variants possess different functions (i.e. regarding the binding of Fn)13 caution is needed when deducing the consequences of our results for cartilage metabolism.

As quantitative evaluation of IHC stainings is not adequately reproducible22, a rather crude evaluation was used in this study. Minor differences may be missed, but using this method of evaluation the results are expected to be more reproducible.

The small sample size of this study (n=4) is very likely the reason why the obviously increased expression of TN in peripheral biopsies from femoral heads with severe OA did not achieve statistical significance in more than one compartment.

In conclusion, we have detailed the increased expression of TN in load bearing areas of human cartilage with mild OA to be accumulated in areas showing structural damage and to be primarily localized in the superficial zone. This suggests that TN is involved in the early events of OA. Cartilage with severe OA expresses increased levels of TN in all zones and compartments and the increased expression of TN includes central as well as peripheral areas. Since TN is considered to be a molecular marker for OA, this proposes mild OA to be a localized disorder, whereas severe OA is generalized. Extreme caution is necessary for sampling of articular cartilage, especially from joints with mild OA.

Future research should identify metabolic or biomechanical factors responsible for the enhanced expression of TN. In addition, a more accurate role for TN in articular cartilage should be determined, especially considering the functions of the various TN isoforms as antagonists for Fn in vivo.

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References


