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Research Article

NITEGE-Expression in Meniscal Matrix. A New, Immunohistochemical Marker of Meniscal-Tissue Degeneration

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Abstract *Background.* No marker reflecting the pathobiology of meniscal degeneration (MD) is established so far. NITEGE is a hexapeptide fragments produced when aggrecan is cleaved. *Methods.* In vitro IL-1 exposition of bovine menisci ($n = 4$) caused extracellular NITEGE deposits detected by immunofluorescence. A retrospective immunohistochemical analysis of ($n = 60$) patients after meniscectomy for NITEGE deposits was performed. MD was graded as follows: no/little (grade $0/1$), medium (grade 2) or severe (grade 3). *Results.* NITEGE deposits in areas of degeneration were demonstrated in 55% of the patients with grade 2 or 3 MD (PPV & specificity 100%), while no extracellular NITEGE deposits in the menisci with grade 0 or 1 MD could be detected. Age correlated with NITEGE deposits ($r = 0.46$) and grade of MD ($r = 0.48$). NITEGE-positive cell density and size were significantly higher close to a tear ($p < 0,001$). *Interpretation.* Extracellular NITEGE deposits may be regarded as markers of medium and severe MD and might be used in diagnostic histopathology.

Keywords NITEGE; aggrecan; meniscal degeneration; immunohistochemistry; histopathology

1 Introduction

The major meniscal functions are to distribute stress across the knee during weight bearing, provide shock absorption, serve as secondary joint stabilizers, facilitate joint gliding, and protect the joint margins (Brindle et al. [6]).

Meniscal degeneration (MD) goes along with a loss of the meniscal function described above contributing to a higher incidence of tears. It is a common disorder that has been morphologically described long ago; however studies concerning the pathogenesis are scarce. All we know today about MD is almost exclusively derived from conventional HE-, Alcian-Blue or vG-staining, while only few immunohistochemical markers for MD are available (Dankof et al. [8]).

Histomorphologically three different variants of MD have been described: calcification, acellular hyaline degeneration and mucoid or myxoid degeneration (Ferrer-Roca et al. [9,10]). Several reasons for developing MD have been considered: trauma (Romanini et al. [21], Smillie [24]) malnutrition, vascular disturbance (Burri [7], Krenn et al. [16], McDevitt et al. [20]). Some studies demonstrated MD to be a physiological state of age (Könn [15], Slany [23]).

All conflicting concepts can be consolidated by considering the basic distinction between primary (Slany [23]) and secondary MD. Primary MD is defined as all morphological alterations in cellular and fibrillar meniscal components that are stronger than usual age-related changes. Secondary MD is seen in degenerative, inflammatory, posttraumatic, and metabolic joint diseases (Andreesen [3], Aufdermaur [4]).

The microstructural characteristics of the menisci dictate their mechanical properties. The menisci are composed of 70% water and 30% organic matter. Collagen (Type I) constitutes 75% of the organic matter, while roughly 8% to 13% of the remaining dry matter consists of proteoglycans (Adams et al. [1], Brindle et al. [6]). They make up only 1% of the wet weight of the meniscus but contribute significantly to its material properties (Fithian et al. [12]).

Degradation of one of the components of the extracellular meniscal matrix either collagens or proteoglycans is resulting in degeneration of the meniscus.

Aggrecan is the main proteoglycan of the extracellular meniscal matrix (Hoberg et al. [14], Verdonk et al. [27]), and NITEGE is one of the G1-fragments produced when the proteoglycan aggrecan is cleaved by aggrecanase activity. NITEGE is a hexapeptide consisting of Asn-Ile-Thr-Glu-Gly-Glu, hence the name. It has been suspected to be a marker for MD, however there has been no study so far that investigated the NITEGE distribution in degenerated human meniscal tissue.

We therefore studied by in vitro analysis of bovine meniscal fragments whether NITEGE may be expressed after stressing IL-1 exposition. Secondly, we carried out immunohistochemical analysis of human menisci for NITEGE expression and correlated NITEGE patterns with different grades of MD to see if NITEGE is of use as a pathogenetic marker for MD.

2 Materials and methods

In vitro study

Meniscal explant disks were isolated from bovine menisci (16-24 months-old cattle), procured from a local abattoir with authorization from the relevant meat inspectors. Full thickness tissue cylinders (10 mm in diameter) were punched perpendicular to the meniscus bottom surface. Tissue disks of 1 mm thickness were sliced including the original meniscal surface using a sterile scalpel blade, and four to five smaller explant disks (3 mm in diameter $\times$ 1 mm thick) were isolated using a biopsy punch (HEBUmedical, Tuttlingen, Germany) and cultured in Dulbecco's Modified Eagles medium (DMEM supplemented with 100 U/mL penicillin G, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B; Sigma-Aldrich, St. Louis, MO, USA) in a 37 °C, 5% CO_2 environment. Three explants/mL medium/well were cultured for 6 days in the absence or presence of 10 ng/mL interleukin-1 alpha (R & D Systems, Minneapolis, MN, USA) in a 24-well plate. Medium was exchanged after 3 days. Then, the explants were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Serial sections (7 μm) were cut sagittally through the entire thickness of the explant disks, immobilized on glass slides, and deparaffinised. After incubation for 2.5 min in a digester at 100 °C (in 0.01 M citric acid, pH 6.0) they were incubated overnight at 4 °C with the primary antibody [anti-NITEGE (1:50 dilution in 1% BSA, ABR Affinity BioReagents, Golden, CO, USA)], rinsed in Tris-NaCl three times for 5 min and incubated with the secondary antibody AlexaFluor 488 goat anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. After further washing, the sections were labeled for nuclear staining with bisbenzimide (Sigma, St. Louis, MO, USA), mounted with fluorescence mounting medium (Dako, Glostrup, Denmark) and visualized using the Apotome (ZEISS, Jena, Germany) fluorescence microscope.

Study population

Meniscal tissue, obtained from 60 patients ($n = 60$, 15 ♀ and 45 ♂, ages 18–79, Median age 51 years) who underwent meniscectomy because of meniscal tears or knee endoprosthesis implantation was selected for this study.

Histochemistry

After fixation in formalin (4%), the human meniscal tissue was embedded in paraffin. The microtomed sections of 1–3 μm width were stained with haematoxylin and eosin.

Immunohistochemistry

After the tissue sections were deparaffinized, epitopes were demasked (pressure cooker, citrate buffer). Staining was performed using commercially available polyclonal rabbit antibodies, which were appropriately diluted (1:200; Aggrecan Neo [Affinity BioReagents, Rockford, USA]), to detect NITEGE. Antibody-binding was detected by using the labeled streptavidin-biotin-method (LSAB-Kit+, DAKO, Denmark): Peroxidase-linked streptavidin binds to biotin, which again is linked to the secondary antibody. DAB (3, 3 diaminobenzidine; DAKO, Denmark) served as a chromogen for the reaction with peroxidase. Endogenous peroxidase was blocked by H_2O_2. In a final step, nuclear counter-staining with haematoxylin according to Mayer was performed. Negative controls were obtained by omitting the primary antibody.

Evaluation of histological stainings and graduation of degeneration

The panel of histochemically stained paraffin sections (HE) was evaluated by two pathologists who used the criteria for meniscus degeneration according to Krenn et al. [16]. This scoring system which is partly based on the criteria of Copenhaver (Boya et al. [5]) focuses on the cellularity and matrix alterations of bradytrophic tissues leading to grades of low, moderate and severe degeneration (Figure 1).

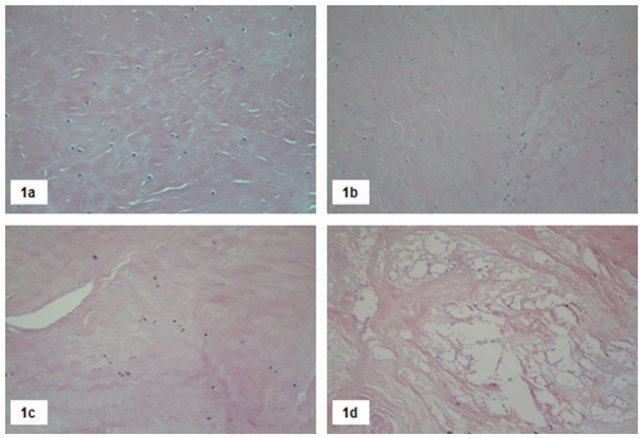

Figure 1: Histopathological characteristics of MD. The degeneration of (a) normal meniscal tissue, (b) sparse/grade 1, (c) medium/grade 2 and (d) severe/grade 3 (HE-stain, $\times$200). Pictures were formerly used in a German Rheumatologic journal in context different from this article.

GRADE 0: normal histological morphology: the matrix of an intact fibrocartilaginous meniscus consists of chondrocytes distributed homogenously in a homogenous-eosinophilic-stained, ground substance (see Figure 1(a)).

GRADE 1: sparse degeneration: low reduction of cellularity (small areas with reduction of cellularity), matrix with variable staining intensity, small clefts in the matrix (see Figure 1(b)).

GRADE 2: moderate degeneration: moderate reduction of cellularity (large areas with reduction of cellularity), variability in size and shape of chondrocytes, moderate clefts in the matrix (see Figure 1(c)).

GRADE 3: severe degeneration: strong reduction of cellularity, reticular and basophilic matrix staining, large areas with complete loss of chondrocytes, matrix with large/pseudocystic clefts and tears (see Figure 1(d)).

Signs of reparation, regeneration and calciumpyrophosphate-depositions (CPPA) were additionally documented. The study population was divided into three groups showing no or low (grade 0/1), moderate (grade 2) or high MD (grade 3).

Evaluation of immunohistochemical stainings

The immunohistochemical stainings were evaluated for extracellular NITEGE depositions, cell size and cell density of NITEGE positive cells in meniscal tissue close to a tear and the meniscal tissue showing the least degenerative changes in the section, which was defined as being distant of the tear.

The evaluation of cell size and density was carried out semi-quantitatively. The cell size was estimated by using a scoring system with 1 indicating normal, 2 double, 3 triple or 4 quadruple cell size. The cell density was estimated by using a scoring system with 1 indicating normal, 2 little, 3 medium or 4 massive increase in cell density.

Statistics

Statistical procedures were performed with Microsoft Excel and with Statpoint LLC STATLETS version 2.01.

3 Results

In vitro study

Bovine meniscal explants were isolated and cultured for 6 days under common culture conditions. Untreated control tissue showed little to no staining of NITEGE in the extracellular matrix, but some staining is associated with the meniscal cells (Figure 2). Incubation of the explants with 10 ng/mL IL-1 induced a clear immunohistochemical signal in the matrix, indicating cleavage of aggrecan at the NITEGE site. This staining was found in all fibrocartilaginous areas of the explants.

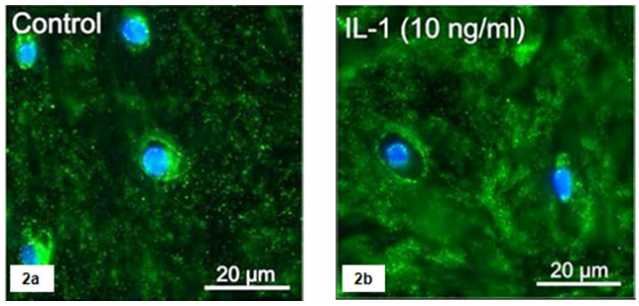

Figure 2: Immunofluorescent staining (green) of the aggrecan fragment NITEGE in bovine meniscal explants from 2-year-old cattle cultured for 6 days with (b) or without (a) 10 ng/mL interleukin-1 (IL-1). There is a weak staining in the extracellular matrix of control tissue but a strong signal in IL-1-treated explants. Both experimental groups show a cell-associated staining of NITEGE. Nuclei of the cells are labeled with bisbenzimide (blue).

Study-population

The study population was divided into three groups showing no or little (grade 0/1), medium (grade 2) or massive MD (grade 3). Ten patients had no signs of reparation or regeneration.

Group 0/1. Group 0/1 included 16 patients (3 ♀, 13 ♂) with no or little degenerative changes in meniscal tissue. The median age was 36 with a range between 18 and 61. No patient suffered from CPPA. Nine patients had regenerative or reparative changes in their meniscal tissue. Six patients had a history of trauma. Clinically MD was not known.

Group 2. Group 2 included 14 patients (4 ♀, 10 ♂) with medium degenerative changes in meniscal tissue. The median age was 40 with a range between 20 and 79. No patient suffered from CPPA. All patients had regenerative or reparative changes in their meniscal tissue. One patient had a history of trauma. Clinically MD was known in three patients.

Group 3. Group 3 included 30 patients (8 ♀, 22 ♂) with massive degenerative changes in meniscal tissue. The median age was 56 with a range between 36 and 76. Two patients suffered from CPPA. And 27 patients had regenerative or reparative changes in their meniscal tissue. Two patients had a history of trauma. Clinically MD was known in 13 patients.

There was positive correlation between age and grade of degeneration ($r = 0.48$).

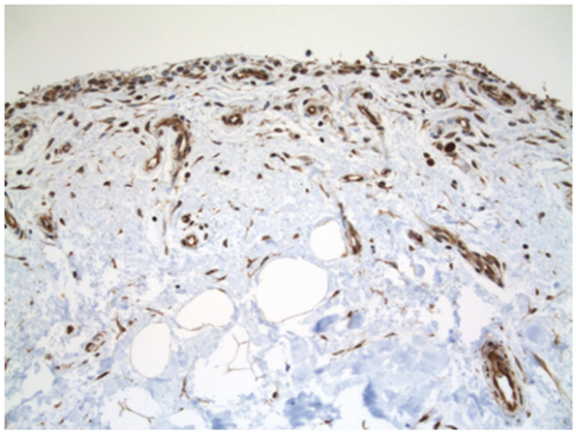

Figure 3: NITEGE positive staining in synovial lining layer cells and endothelial cells sparing the interstitial tissue (NITEGE immunohistochemistry, $\times 400$).

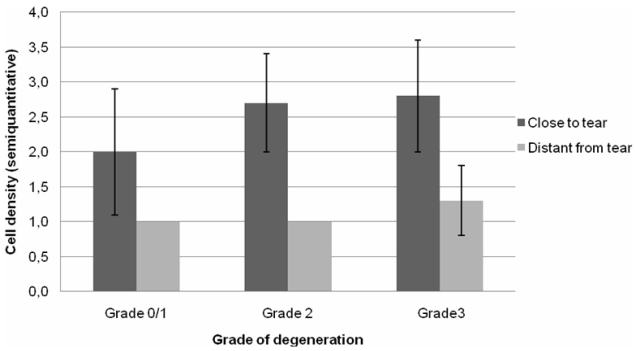

Figure 4: Comparison of cell density close and distant from a tear showing higher cell density in tissue close to a tear.

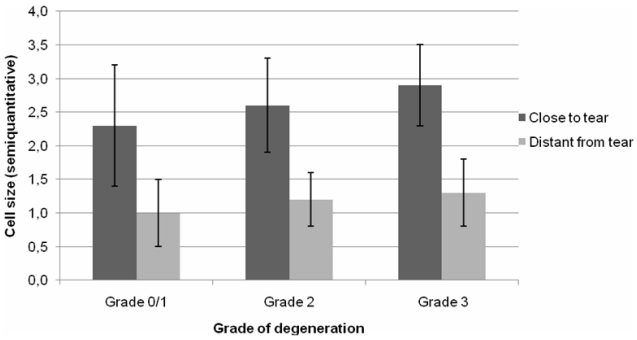

Figure 5: Comparison of cell size close and distant from a tear showing increasing cell size close to a tear.

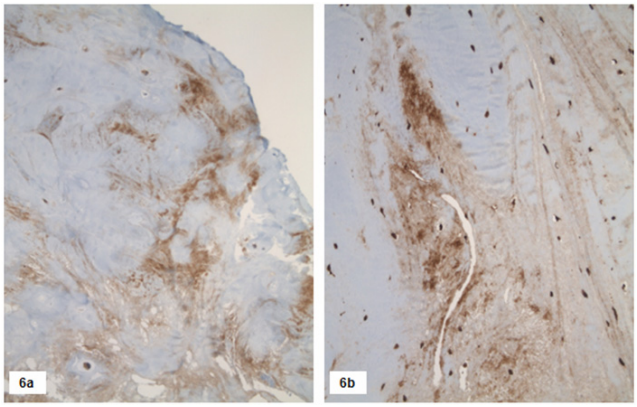

Figure 6: Extracellular NITEGE depositions in moderate to massive degenerated meniscal tissue (grade 2 and 3) showed a fine granular, extracellular confluating pattern (NITEGE immunohistochemistry, $\times 100$).

NITEGE distribution in human meniscal tissue

All meniscal cells, vessels and the synovial-membrane were stained positive with NITEGE (Figure 3). The cell size and density of NITEGE positive meniscal cells increased close to a tear and was associated with reparative and regenerative changes in all three study groups.

The mean for cell size and density in meniscal cells distant from a tear was between 1.0 in group 1 and 1.3 in group 3 indicating normal or slightly increased cell size and density in tissue with no or minor degenerative changes.

In group 0/1 average cell size and density doubled in the meniscal tissue close to a tear. This difference was even higher in the groups 2 and 3. The average enlargement of the cell size had a value of 2.6 in group 2 with a standard deviation of 0.6 and a value of 2.9 in group 3 with a standard deviation of 0.5. The average cell density was nearly threefold in groups 2 and 3 with a value of 2.8 and 2.9 with standard deviations of 0.7 and 0.8 respectively. In the whole study population, the mean cell density and cell size were 2.7 in the meniscal tissue close to tear with a standard deviation of 0.8 and 0.7, respectively. Cell size and density were nearly the same in all groups when the meniscal tissue distant from a tear was evaluated. The difference of cell size and density when comparing meniscal tissues close and distant from a tear was significant ($p < 0.0001$) concerning the whole study population (Figures 4 and 5).

Extracellular NITEGE depositions

The morphology of the extracellular NITEGE depositions showed a fine granular confluating pattern (Figure 6). Tissue changes mimicking NITEGE depositions with a diffuse non-granular pattern resulted from diathermic artifacts and calcium-pyrophosphate crystals. They were easily distinguishable from the real depositions.

In group 1 no extracellular NITEGE depositions were detected. The groups 2 and 3 showed such depositions in 50% (7/14), 57% (17/30) and 55% (24/44), respectively when combined (Figures 7 and 8). Specimens with very

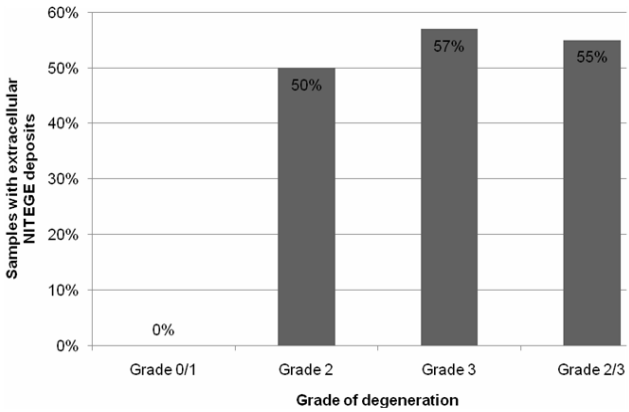

Figure 7: Extracellular NITEGE depositions are associated with moderate (grade 2) and massive (grade 3) meniscal degeneration.

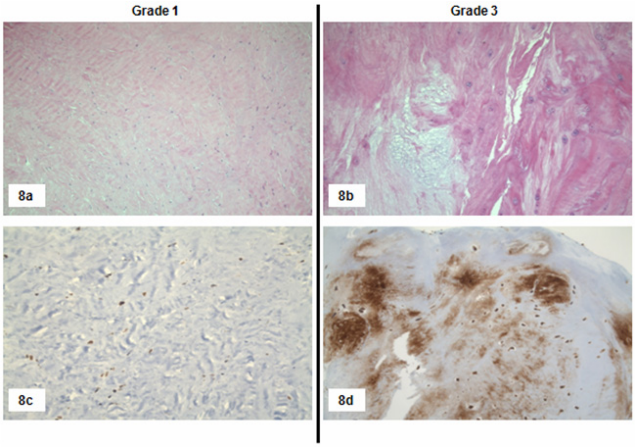

Figure 8: Comparison of meniscal tissue with low, Grade 1 (a, c) and massive MD, Grade 3 (b, d). In degenerated meniscal tissue the meniscocytes, which stain positive with NITEGE, are enlarged and show a higher cell density indicating an upregulation of ADAMTS in degenerated meniscal tissue with reparative changes (HE $\times 100$, NITEGE immunohistochemistry $\times 200$).

severe MD and combined loss of nearly all meniscal cells showed no extracellular NITEGE depositions. The sensitivity for detecting at least medium degenerative changes was 55% combined with a specificity of 100%. The negative predictive value was 44%, and the positive predictive value was 100%. There was positive correlation between age and appearance of extracellular NITEGE depositions ($r = 0.46$) concerning the whole study population. Patients with moderate MD (group 2) were not significantly older than patients with little or no MD ($p = 0.44$), though they are significantly younger than patients with severe MD (group 3) ($p = 0.045$). The mean

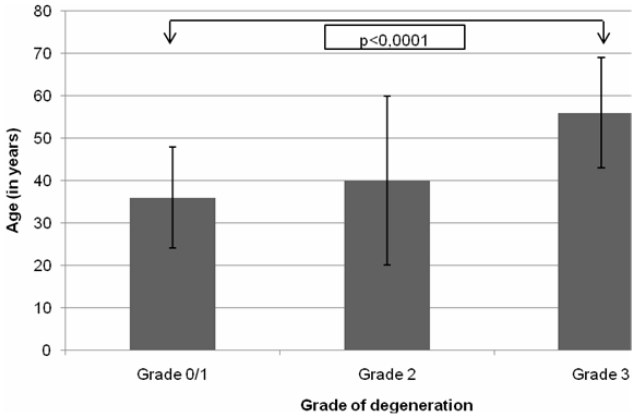

Figure 9: The mean age of patients with grade 0/1 degeneration (36 years) is significantly lower than the mean age of patients with grade 3 degeneration (56 years).

age of patients with grade 0/1 MD was high-significantly lower than the mean age of patients with grade 3 MD ($p < 0.0001$) (Figure 9).

4 Discussion

Although the frequent occurrence of MD is a well-known fact and its consequences include rupture and even loss of the meniscus, the pathogenetic factors are only insufficiently established. As up to now only histomorphological descriptions of degenerative meniscal changes, based on conventional staining methods like HE- and Alcian-Blue-staining, exist.

We provide with NITEGE an immunohistochemical substrate for degeneration of fibrous cartilage which might be used systematically in order to define the degree of MD. Aggrecan is cleaved by aggrecanases (like ADAMTS) and matrix metalloproteinases (MMPs) (Lark et al. [17], Valiyaveettil et al. [26]). The role of aggrecan and its main cleavage product NITEGE has not been studied systematically in human meniscal tissue so far.

A positive correlation between age and prevalence of extracellular NITEGE depositions could indeed be demonstrated. This is consistent with previous findings (McAlinden et al. [19]) that showed that the synthesis and turnover of aggrecan in the human meniscus is influenced by the age of the individual with increasing aggrecan mRNA expression with rising age.

Our in vitro results showed prominent extracellular NITEGE depositions in stress exposed bovine meniscal tissue after IL-1 exposition, while there was little to no staining in the matrix of control tissue. This suggests that aggrecan cleavage might be part of MD. In contrast, NITEGE signals were found in western blottings of healthy fetal, newborn, as well as adult bovine meniscal tissue and suggested that aggrecan cleavage at the NITEGE site

might be part of the normal meniscal aggrecan turnover (Sandy et al. [22]). Reasons for these different findings might be the fact that western blotting is not a sufficient method for quantification and samples analyzed by western blottings include matrix as well as cells, while in immunohistochemical stainings, cellular signals and stainings in the matrix were evaluated independently. There was a constant staining associated with the cells; this could be the signal that was found even in healthy tissue (Sandy et al. [22]). It is therefore suggested that NITEGE formation might occur as part of the normal turnover associated with the cells, while aggrecan cleavage at the NITEGE site in the matrix might be part of degenerative processes in the meniscus.

Since matrix-associated NITEGE formation could be induced by IL-1, we decided to investigate samples from 60 patients regarding the deposition of NITEGE fragments. We observed extracellular NITEGE depositions in 55% of the specimens with grade 2 or 3 degenerative lesions. Extracellular NITEGE depositions were not associated with mainly secondary degenerative changes close to a tear in the group with no or little degenerative changes. These results are consistent with our in vitro data and a previous study performed in which NITEGE depositions were immunohistochemically more often seen in areas with extensive hyaline cartilage damage. Like in our study there were depositions surrounding the cells but also further removed in the interterritorial matrix (Lark et al. [17,18]). Furthermore we were able to show NITEGE-positive cells to be larger and having a higher density comparing meniscal tissue close to and distant from a tear. These results indicate a similar up-regulation of ADAMTS in meniscal tissue like in hyaline cartilage after trauma or in degeneration (Tortorella et al. [25]). In low-grade degeneration there must be a higher aggrecanase activity than in normal meniscal tissue but in contrary to meniscal tissue with moderate or severe degeneration there are enough meniscal cells that are capable of removing the aggrecan fragments by endocytosis (Fosang et al. [13]). The endocytosis may be one reason for the increasing size of the cells. The higher cell density of NITEGE positive meniscal cells is mainly caused by ongoing regenerative and reparative changes, which we were able to demonstrate in nearly all specimens. In later stages, MD goes along with meniscal cell depletion which seems to result in extracellular NITEGE depositions indicating moderate and severe MDs with a positive predictive value and a specificity of 100%.

But what happens to meniscal tissue distant from a tear when degeneration occurs? Morphologically, we were able to find areas of nearly unchanged meniscal tissue with small meniscal cells in nearly all specimens. All these cells stained positive for NITEGE but the small size and normal density may indicate that there is no upregulation of aggrecanase in tissue distant from tear when degenerative changes occur.

There remains the question why not all specimens with moderate or severe MD showed extracellular NITEGE depositions. A negative predictive value of 44% and a sensitivity of 55% seem to show a limited value of NITEGE in identifying MD but they can give a hint in which stages the MD proceeds. As long as you can find only small NITEGE positive cells there is little degeneration; when degeneration continues, meniscal cells grow bigger and when a severe loss of meniscal cells occurs it goes along with extracellular NITEGE depositions. At last when most of the meniscal cells are depleted there is strong myxoid degeneration. Aggrecan is completely depleted and/or no intact matrix containing aggrecanases is left with the result of highly degenerated meniscal tissue with no signs of NITEGE depositions. This interpretation comes very close to Aigner's description of hyaline cartilage degeneration quoted above (Aigner et al. [2]).

Another limitation to our study is that we only focused on immunohistochemistry and left out other molecular-biological testing on human meniscal tissue like MALDI-TOF mass spectometry for protein level and RT-PCR for DNA level.

The last question is whether NITEGE depositions can help to distinguish primary and secondary degenerative changes in everyday diagnostic with mainly arthroscopically obtained meniscal fragments which contain less informative value (Fisseler et al. [11]). In this study, extracellular NITEGE depositions were not associated with mainly secondary degenerative changes close to a tear in the group with no or little degenerative changes. In cases with moderate or severe MD, we were able to show extracellular NITEGE depositions. There was a positive correlation between extracellular NITEGE depositions and the grade of degeneration.

We interpret these results as a hint that up-regulation of aggrecan cleavage might be associated with primary degeneration and not with secondary degenerative changes close to a tear.

The detection of NITEGE in synovial fluid obtained from joint punctures as a marker of MD may be another clinical application as a joint puncture is much less invasive than arthroscopy in cases of unclear MD.

In any case, immunhistochemical expression of NITEGE could help to identify meniscal tissue with severe degeneration in arthroscopically obtained meniscus specimens contributing in this way to a pathophysiologically based and more objective diagnosis in meniscal histopathology.

NITEGE immunohistochemistry is currently used for grading of MD by a number of selected institutes for pathology (including world-class university affiliations) throughout Germany.

5 Conclusions

NITEGE is the G1-fragment of aggrecan after being cleaved by enzymes like aggrecanase. Aggrecan itself is the most common proteoglycan in meniscal tissue and provides crucial biological and mechanical features. In meniscal degeneration, aggrecan is lost and being cleaved by aggrecanases. In our study, we showed that extracellular NITEGE deposits can indicate moderate and severe meniscal degeneration and may therefore act as an immunohistochemical marker for histopathologists.

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