Original Article
Evaluation of correlation of articular cartilage staining for DDR2 and proteoglycans with histological tissue damage and the results of radiographic assessment in patients with early stages of knee osteoarthritis

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Abstract: Objective: To determine, if staining of articular cartilage for proteoglycans (natural element of healthy and functioning cartilage) and discoidin domain receptor 2 (DDR2) (a protein associated with articular cartilage degradation) is correlated with histological tissue damage or radiographic assessment score in patients with early stages of knee osteoarthritis (OA).

Method: 40 patients, with early stage OA were enrolled, from whom the biopsies for histological and immunohistochemical studies were obtained from edge of the femoral condyle during the arthroscopy. Semi-quantitative computer based analysis was used to evaluate the proportion of staining in histological sections. Results: No correlation was shown between the proportion of tissue stained for DDR2 and histological score or the results of radiographic assessment of tibiofemoral (TF) joint. There was a negative correlation between the proportion of tissue stained for DDR2 and radiographic grade of patellofemoral (PF) OA (Spearman r=-0.34; 95% CI -0.60 to -0.02; \( P = 0.03 \)). No correlation was shown between the proportion of tissue stained for proteoglycans and histological score or the results of radiographic assessment of TF and PF joints. A negative correlation was found between proportion of tissue stained for DDR2 and proteoglycans. Spearman r=-0.43; 95% CI=-0.66 to -0.12; \( P = 0.006 \). Conclusion: Production of DDR2 in articular cartilage could be related to early stages of OA, as it is significantly correlated to decrease of staining for cartilage proteoglycans. The role of production of DDR2 in cartilage may be decreased in stages, where higher grades of OA are detected on the radiographs.

Keywords: Osteoarthritis, DDR2, proteoglycans, tissue damage

Introduction

For some time now molecular mechanisms have been considered to play an important role in development of osteoarthritis (OA) and it is no longer considered to be a simple “wear and tear” condition. Although a lot of studies have been done to unveil the molecular mechanisms behind the OA pathogenesis, the specifics are still not clear. Determining the key molecules in the pathogenesis of OA would give an opportunity to perform early diagnosis and preventive treatment that would allow avoiding or postponing the joint replacement, which is unfortunately the only effective treatment in the advanced stages of the disease.

Early stages of OA are characterized by minor histological changes in the articular cartilage and are often not detected radiographically. However, certain molecular changes occur before structural changes in the cartilage and it would therefore be reasonable to direct the therapy towards the molecules participating in the early phases of the disease [1].

Along with the cellular changes in the cartilage, there are several extracellular matrix molecules degraded during OA, such as collagen II, which is the main collagen in the cartilage, but also other types of collagens, aggregans and proteoglycans [3, 16]. Collagen II and other structural molecules are degraded by matrix metalloproteinases (MMPs), produced by hypertrophic chondrocytes, which are found more in OA affected cartilage than in normal cartilage [17]. The most representative of MMPs responsible for cartilage erosion during OA is matrix metal-
loproteinase 13 (MMP-13 or collagenase 3), which preferentially degrades type II collagen [2, 3, 17]. However the use of MMP-13 as a therapeutic target is complicated due to side effects and therefore targets upstream of MMP-13 should be sought [1]. One of such targets is discoidin domain receptor 2 (DDR2). DDR2 is a cell surface receptor tyrosine kinase that predominantly interacts with type II collagen and amplifies the MMP-13 production in chondrocytes [4]. Therefore DDR2 could have a potential role in early stages of OA. It is hypothesized that DDR2 can interact with collagen II in the articular cartilage only after the significant proteoglycan loss. It has been shown, that DDR2 knockout mice are resistant to surgically induced OA [4]. On the other hand another mouse model of overexpression of DDR2 in cartilage demonstrated, that increased DDR2 did not induce MMP-13 expression and additional activation was needed in the mature articular cartilage to induce OA [5].

It is to be determined which local condition actually induces the expression of DDR-2 in the cartilage and in what stage of cartilage damage this expression could be detected.

Proteoglycans have a significant role in maintaining the structural integrity of extracellular matrix of the cartilage as they provide osmotic resistance necessary for cartilage to resist compressive loads [6]. It is noted, that one consistent pathogenic feature of OA is the loss of matrix macromolecules from the cartilage, especially aggrecan, which is a predominant proteoglycan in the cartilage [7]. However, it has been shown in experimental arthritis models, that cartilage can be heavily depleted of proteoglycans for weeks by sustained local exposure to inflammatory stimuli, without the development of cartilage erosions and the proteoglycan content is re-established, when the inflammatory effectors are removed [1]. Since proteoglycan depletion is a change from which the cartilage is able to recover, it could be related to the early steps of the OA from where some regeneration of the tissue is possible. It would be of interest to determine the relation between MMP-13 predecessor, DDR2, production and the loss of cartilage proteoglycans and how is it related to the articular cartilage damage in early stages of OA.

The first objective of this study was to assess the correlation between cartilage staining for proteoglycan or DDR2 and values of histological tissue damage. The second objective was to evaluate the correlation between cartilage staining for proteoglycan or DDR2 and scores of radiographic assessments of the knee joint.

Materials and methods

Experimental subjects

In order to focus on the early stages of OA, the cartilage samples were obtained during knee arthroscopy from 40 patients (25 male and 15 female) aged 33-59 years, whose modified Mankin score was evaluated to be between 1 and 4.5. Enrollment was voluntary and all patients have been given informed consent. This consent allowed us to use patients’ medical records, in order to get information about the radiological grade of the damage, age and gender. This study was approved by the Research Ethics Committee of the University of Tartu.

Histology

Cartilage tissue samples were harvested from the edge of the condyle during arthroscopy, fixed in 4% formalin solution, dehydrated and embedded into paraffin. Samples were sectioned and hematoxyline-eosin staining was performed. The modified Mankin scores were evaluated by a pathologist in blinded manner. We used the modified Mankin score ranging from 0 (normal cartilage architecture) to 6 (severe damage), that has previously been described by Pritzker et al [8].

Radiographs

The tibiofemoral (TF) and patellofemoral (PF) joints of both knees in each subject were radiographed and assessed separately as described earlier by Kumm et al [9]. Joint space narrowing (JSN) and osteophyte size were classified on 4-point scales (grades 0-3) according to the system of Nagaosa et al [10] in medial as well as in lateral compartments. This way JSN was assessed in four and osteophyte size - in eight distinct regions of interest (ROIs) in each joint. Subject’s TFOA and PFOA diagnosis was based on as the highest grade documented in any ROI. All radiographs were interpreted by an experienced radiologist who was blinded to clinical and laboratory data.
Immunohistochemistry and toluidine blue staining

Deparaffinized histological sections were treated with 1% H₂O₂ to inactivate endogenous peroxidase and then with Dako REAL Antibody Diluent (S2022) (Dako Denmark A/S, Glostrup, Denmark) to block non-specific binding. After blocking, sections were incubated with mouse monoclonal antibody to DDR2 (ab63337; produced by Abcam Ltd., Cambridge, UK) overnight at 4°C. Visualization of the primary antibody was performed using commercial kit “Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse” (K5007) (Dako Denmark A/S, Glostrup, Denmark). The washing steps in-between were done in phosphate buffered saline (PBS) which contained 0.07% of Tween 20 as a detergent. No immunohistochemical staining was noted in negative controls where the primary antibody was omitted. Toluidine blue (Applichem, Darmstadt, Germany) was used to stain the cartilage for background proteoglycans [11].

Estimation of the DDR2 and proteoglycan content in cartilage samples

Overview pictures of the tissue sections were taken after staining at standard conditions using 20× objective microscope with necessary equipment and software (newCAST software, Visiopharm A/S, Hoersholm, Denmark). The analysis of the proportion of staining in the cartilage sections was performed using software Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA) as described by Lehr et al [12]. The procedure of determination of the proportion of tissue stained for DDR2 and proportion of tissue stained with toluidine blue included the following steps. The background (i.e. part of the picture that did not include the tissue section) was removed, so only the image of the tissue section remained as the analyzable area on the white background. Using the Magic Wand tool in the Select menu all the pixels of the tissue section were counted. After that the Magic Wand tool was used to select a typical stained area with the tolerance level set on 15 units. Applying the Similar command in the Select menu, all the stained areas were automatically selected. Histogram tool was used to obtain the pixel count of the selected area. The amounts of staining were calculated as proportions relative to the whole section area.

Statistics

Nonparametric correlation (Spearman r) test (GraphPad InStat software) was used to assess the correlation between proportion of cartilage tissue stained for DDR2 and proteoglycans and modified Mankin score and radiographic degree of damage.

Results

Histology and radiographs

The modified Mankin scores ranging from 0 (normal cartilage architecture) to 6 (severe damage), that has previously been described by Pritzker et al [8], were evaluated by a pathologist in blinded manner. The modified Mankin scores remained in the range of 1 to 4.5 (average 2.5). The radiographs were interpreted by an experienced radiologist in a blinded manner. The radiographic score of OA was in the range of 0 to 3 (average degree of radiographic TFOA score was about 1.0 and PFOA score about 0.8). See details in Table 1.

Immunohistochemistry and toluidine blue staining

DDR2 expression was found in all but one sample. Most of the DDR2 staining was noted intracellularly, but also extracellularly in few samples. The staining was predominantly noted in the superficial layers of articular cartilage as described by Sunk et al [13]. Toluidine blue staining was noted in the extracellular matrix of most of the cartilage samples. See Figure 1A-D.

The proportion of cartilage tissue stained for DDR2 and proteoglycans and their correlation with histological tissue damage

There was no significant correlation between the proportion of tissue stained for DDR2 and modified Mankin score, which was used for histological assessment of tissue damage (Spearman r=-0.14; 95% CI -0.19 to 0.44; Two tailed p-value =0.40).

There was also no correlation between the proportion of tissue stained for proteoglycans and modified Mankin score (Spearman r=-0.07; 95% CI -0.38 to 0.25; Two tailed p-value =0.65).
Correlation between the proportion of cartilage tissue stained for DDR2 and proportion of cartilage stained for proteoglycans

A significant negative correlation was found between proportion of tissue stained for DDR2 and proportion of tissue stained for proteoglycans. Spearman r=-0.43; 95% CI=-0.66 to -0.12; Two tailed P=0.006. This means that increase in staining for DDR2 was significantly correlated to decrease of staining for proteoglycans. Surprisingly this correlation remained significant only in men, when the patients were grouped based on gender. Men: Spearman
The proportion of cartilage tissue stained for DDR2 and proteoglycans and their correlation with the results of radiographic assessment of TF and PF joints

There was no significant correlation between the proportion of tissue stained for DDR2 and radiographic grade of TFOA (Spearman $r=-0.07$; 95% CI -0.38 to 0.26; Two tailed $P=0.66$).

There was a significant negative correlation between the proportion of tissue stained for DDR2 and radiographic grade of PFOA (Spearman $r=-0.34$; 95% CI -0.60 to -0.02; Two tailed $P=0.03$). This means that DDR2 was detected less in the samples obtained from the patients with higher PFOA grading. This correlation remained significant only in men, when the patients were grouped based on gender. Men: Spearman $r=-0.46$; 95% CI=-0.73 to -0.06; Two tailed $P=0.02$. Women: Spearman $r=-0.03$; 95% CI=-0.56 to 0.52; Two tailed $P=0.92$.

There was no significant correlation between the proportion of tissue stained for toluidine blue and radiographic grade of TFOA (Spearman $r=0.07$; 95% CI -0.25 to 0.38; Two tailed $P=0.66$).

There was no significant correlation between the proportion of tissue stained for toluidine blue and radiographic grade of PFOA (Spearman $r=0.19$; 95% CI -0.15 to 0.48; Two tailed $P=0.25$) (see Table 1).

Correlation between histological cartilage damage and results of radiographic assessment of TF and PF joints

There was no significant correlation between the modified Mankin score and radiographic...
grade of TFOA (Spearman r=0.26; 95% CI -0.07 to 0.53; Two tailed P=0.11).

There was however a significant correlation between the modified Mankin score and radiographic grade of TFOA (Spearman r=0.37; 95% CI -0.05 to 0.62; Two tailed P=0.02). This correlation remained significant only in women, when the patients were grouped based on gender. Women: Spearman r=-0.70; 95% CI= 0.25 to 0.90; Two tailed P=0.006. Men: Spearman r=0.19; 95% CI=-0.23 to 0.56; Two tailed P=0.36 (see Table 1).

It has to be mentioned here that lack of correlation between the histological scores and results of radiographic assessments has been noted before when radiographic findings were compared to histomorphometry of the cartilage of non-weight-bearing compartment [14].

Discussion

This study confirms that DDR2 expression can be detected in OA patients in early stages of the disease. Sunk et al have shown positive correlation between increased DDR2 staining in articular cartilage and the degree of tissue damage [13]. They also detected reduced proteoglycan staining in samples with higher grade of OA.

In this study we were able to show the correlation between reduced proteoglycan staining and increased DDR2 staining. This suggests, that higher expression of DDR2 protein could be related with the depletion of cartilage form proteoglycans. However we could not show correlation between cartilage stained for DDR2 and proteoglycans and histological tissue damage and furthermore the proportional staining of cartilage for DDR2 was negatively correlated to radiographic grade of PFOA. This suggests that production of DDR2 in cartilage may be indeed related to very early stages of OA and DDR2 would be decreased in stages, where higher grades of OA are detected on the radiographs. Since it has been shown that DDR2 knockout mice are resistant to surgically induced OA [4], it could be that DDR2 production is involved in the molecular processes that initiate the cartilage destruction and in the more advanced stages of OA the other molecular pathways leading to cartilage destruction are more dominant [17]. Considering the DDR2 to be of one of the possible key molecules in initiation of OA in articular cartilage, it is reasonable to expect some other signs of disorders in the cartilage would appear as DDR2 production increases. Depletion of cartilage from proteoglycans could be considered as one of such signs. The fact that we could not demonstrate the correlation between DDR2 or proteoglycan staining and histological tissue damage, could be because depletion of proteoglycans in cartilage does not itself cause erosions and cartilage is even able to recover from such depletion [1]. Therefore it could be that such correlations are not so obvious in very early stages of molecular processes that finally lead to OA. However, it is strange that in some samples with extensive proteoglycan staining and lower staining for DDR2, the Mankin scores were pretty high (Figure 1C). According to the data from this study it is not possible to connect the degree of cartilage damage and expression of DDR2 and proteoglycan content of the articular cartilage in patients with early OA. We cannot also tell whether increased proportion of DDR2 staining in articular cartilage is caused by the proteoglycan depletion or vice versa. We could only hypothesize that DDR2 expression could be related to cartilage proteoglycan content in some kind of tissue response mechanism in early OA, assuming that appropriate proteoglycan content is essential for normal function of the cartilage [15].

We could further hypothesize that proteoglycan depletion-related DDR2 expression sets the stage for cartilage degradation, since DDR2 is the protein upstream from the major enzyme involved in OA cartilage erosion (MMP-13 or collagenase 3) [1]. Since it has been described in mice that DDR2 would act on cartilage specific collagen only after proteoglycan loss [4], it is possible that increased DDR2 expression is the response to the proteoglycan depletion and it introduces the production of erosive MMP-13 in the tissue [1]. Unfortunately the results from this study cannot be used to confirm such hypotheses.

The methods used in this study for semi-quantitative assessment of immunohistochemistry results are relatively rough and able only to detect obvious differences in the quantity, since the DDR2 and proteoglycan measurements are done proportionally based on the
staining of the histological tissue section. On the other hand this could be an advantage, because the differences shown with this method are clearly visible and widespread throughout the tissue. The usability of Adobe Photoshop for semi-quantitative assessment of immunohistochemistry results has been described by Lehr et al [12].

The material for histological and immunohistochemical studies was taken during arthroscopy, which sets certain limitation to the size of the samples and the samples could not be taken through all the layers of the articular cartilage up to the bone. It has to be stated, that the samples of better quality were used in the study (considering the necessary amount of cartilage to perform histological studies on) and the poor samples were excluded from the study - the 40-patient group represents the patients with good samples. The effect of the non-standardized samples was reduced by the fact that the major finding of the study was determined by comparing two stains on the same slide.

In conclusion, this study does not show correlation between the proportion of tissue stained for DDR2 and proteoglycans and modified Mankin score. Instead we found a correlation between increased radiographic grade of PFOA and decreased proportion of cartilage stained for DDR2. We also showed correlation between increased proportion of tissue stained for DDR2 and decreased proportion of tissue stained for proteoglycans. It could be assumed that staining for a protein related to degradation of cartilage (DDR2) is increased when cartilage is depleted of proteoglycans. Further studies have to be done to describe the role of DDR2 in early processes that lead to development of OA and to assess, if the role of DDR2 production is less significant in advanced stages of OA, where other factors could be more involved in degrading the articular cartilage.

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Disclosure of conflict of interest

None.

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