

Original Article

Immunohistochemical detection of cartilage intermediate layer protein 1 in the synovial membrane of patients with knee complaints

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Received April 27, 2016; Accepted June 22, 2016; Epub August 1, 2016; Published August 15, 2016

Abstract: Cartilage intermediate layer protein 1 (CILP-1) is a structural component of the cartilage extracellular matrix. As CILP-1 localization outside the articular cartilage is noted, we aimed to detect CILP-1 in the synovial membranes. Synovial membrane samples were collected from 30 patients, who formed a subset of an extensive osteoarthritis (OA) study and who had undergone knee arthroscopy due to knee complaints. Immunohistochemistry was used to detect CILP-1 in the synovial membranes. Double immunofluorescence staining was applied in order to confirm the presence of CILP-1 in synovial macrophages (CD68 positive cells). Immunohistochemical staining of CILP-1 was found in cells of synovial membrane as well as in blood vessels, particularly in medial layer. However, CILP-1 staining was not seen in all cells or blood vessels. No correlation was found between the semi-quantitative score of CILP-1 staining and histological score of synovitis. Conclusions: The CILP-1 was detected in the synovial membranes and was demonstrated to be localized in macrophages and in synovial blood vessels. However no correlation between semi-quantitative assessment of CILP-1 staining and histological score of synovitis could be shown.

Keywords: Cartilage intermediate layer protein, synovial membrane, immunohistochemistry

Introduction

The extracellular matrix of cartilage contains several specific proteins. The most dominant matrix molecule in the extracellular matrix of the articular cartilage is collagen type 2, but other minor collagens and noncollagenous glycoproteins are required to maintain and support the properties of the collagen fibrils [1]. One of such noncollagenous glycoproteins is cartilage intermediate layer protein (CILP). The CILP can be found in two isoforms (CILP-1 and CILP-2), which are considered to be components of extracellular matrix of cartilage and are described to be 50.6% identical [2]. The CILP-1 and CILP-2 are proteins, which are thought to play a role in cartilage scaffolding [3, 4]. It is suggested that changes in the cartilage content of CILP are involved in joint pathologies such as osteoarthritis (OA) [4, 5]. Although CILP is considered cartilage specific, its expression has been shown in other tissues such as murine skeletal muscle and heart [4]. In recent studies CILP was detected during proteomic

analysis of the human adipose tissue [6] and in a porcine myocardium after ischemia [7]. A porcine homologue of human nucleotide pyrophosphohydrolase (encoded by the gene associated to the one that encodes CILP) was detected in the synovial fluid [8] and in the case of calcium pyrophosphate dihydrate crystal deposition disease CILP staining has been noted in synovial lesions [9] suggesting that CILP could be expressed in the synovial membrane and be involved in its pathological conditions.

In this study we aimed to detect CILP-1 in the synovial membrane of patients with knee complaints, determine its specific localization in synovial structures and correlate the semi-quantitative score of CILP-1 immunohistochemical staining to the score of synovitis.

Materials and methods

Patients

The sample for this study consisted of 30 patients, who had formed a subset of an exten-

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sive OA study and who had undergone knee arthroscopy at Tartu University Hospital in 2007-2010 due to chronic knee pain and restriction of movement. Biopsies of the synovial tissue (weighing 10-50 mg) from the synovial capsule (suprapatellar recessus with a macroscopic lesion) were harvested using the biopsy forceps (Piranha Rongeur, Atlantech) for histological evaluation. The study was approved by the Ethical Committee of Human Research, University of Tartu, and informed consent was obtained from all patients before arthroscopy.

Immunohistochemical staining of CILP-1 in the synovial tissue

Synovial samples were embedded in paraffin after fixation in formalin. The 5 µm sections were cut, deparaffinized and treated with 3% H₂O₂ to inactivate endogenous peroxidase. The sections were then treated with Dako REAL Antibody Diluent (S2022; Dako Denmark A/S, Glostrup, Denmark) to block non-specific binding. After blocking, the sections were incubated with the rabbit polyclonal antibody to CILP-1 (courtesy of Prof. Dick Heinegård's group, Lund University, Sweden) overnight at 4°C. Primary antibody concentration was 500 ng/ml. Visualization of the primary antibody was performed using the commercial kit "Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse" (K5007; Dako Denmark A/S, Glostrup, Denmark). Washing steps in-between were done in phosphate buffered saline (PBS) which contained 0.07% of Tween 20 as the detergent. Toluidine blue (Applichem, Darmstadt, Germany) was used for background staining. No immunohistochemical staining was noted in negative controls where the primary antibody was incubated with the corresponding CILP-1 peptide.

Semi-quantitative assessment of immunohistochemical staining of CILP-1 in the synovial tissue

Samples were evaluated semi-quantitatively under a microscope by two examiners. The following grading was used: no staining = 0, mild staining = 1 (less than five blood vessels with stained cells in the slide), moderate staining = 2 (more than five blood vessels with stained cells in the slide).

Evaluation of histological synovitis

Formalin-fixed paraffin-embedded sections stained with haematoxylin-eosin and by Van

Gieson method were used by a clinical pathologist to evaluate histological synovitis. Histological synovitis was evaluated according to the system suggested by Loeuille et al. [10]. Six parameters were studied: 1) number of synovial lining cells, 2) subsynovial infiltration by lymphocytes and plasma cells, 3) surface fibrin deposition, 4) congestion related to blood vessel vasodilatation and/or proliferation, 5) fibrosis, and 6) perivascular edema. Each parameter was scored from 0 (normal synovial tissue) to 3 (severe synovitis). Average grading of six parameters were used to give the final synovitis score as follows: 0 = none; 1 = mild; 2 = moderate; 3 = severe.

Statistics

Nonparametric correlation (Spearman r) test (GraphPadInStat software) was used to assess the correlation between semi-quantitative assessment of immunohistochemical staining for CILP-1 and histological synovitis score.

Immunofluorescent double staining of CILP-1 and CD68 in synovial macrophages

Synovial samples were embedded in paraffin after fixation in formalin. The 5 µm sections were cut, deparaffinized and treated with 3% H₂O₂ to inactivate endogenous peroxidase. The sections were then treated with Dako REAL Antibody Diluent (S2022; Dako Denmark A/S, Glostrup, Denmark) to block non-specific binding. After blocking, the sections were incubated with a mixture of the rabbit polyclonal antibody to CILP-1 (courtesy of Prof. Dick Heinegård's group, Lund University, Sweden) and mouse monoclonal antibody to CD68 (marker for synovial macrophage) (ab955, Abcam) overnight at 4°C. Visualization of the primary antibodies was performed using a mixture of two fluorescent secondary antibodies: the goat polyclonal secondary antibody to mouse IgG-H&L (DyLight® 488) and the donkey polyclonal secondary antibody to rabbit IgG H&L (DyLight® 594) (ab96879 and ab96921 respectively, Abcam). To reduce the autofluorescence of the tissue the sections were treated with 0.1% Sudan Black B (199664; Sigma-Aldrich) in 70% ethanol for 20 minutes at room temperature after secondary antibody incubation. Washing steps in-between were done in PBS which contained 0.07% of Tween 20. The sections were mounted in a glycerolphosphate buffer mixture (1:1) and sealed with a coverslip. No immuno-

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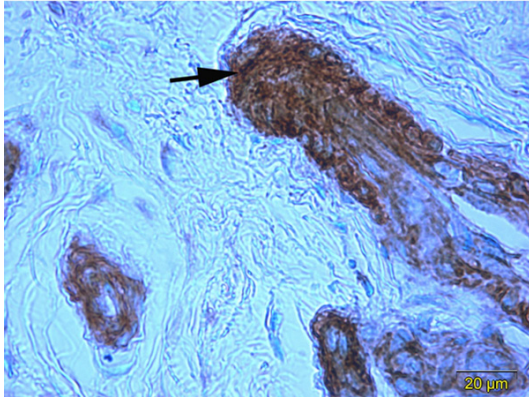


Figure 1. Staining for CILP-1 (brown staining) shown in blood vessels. Staining is the most intense in the media of the blood vessel which consists mostly of smooth muscle cells (shown with the black arrow).

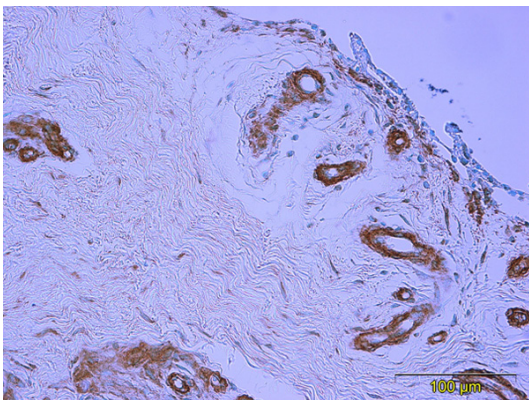


Figure 2. Staining for CILP-1 (brown staining) shown in blood vessels and cells. The staining can be noted in the round cells located in the intima of synovial membrane (possibly synovial macrophages or type A cells).

histochemical staining was noted in negative controls where the primary antibody was omitted.

Results

Detection and immunohistochemical description of CILP-1 in synovial membrane

The immunohistochemical study demonstrated moderate staining of CILP-1 in 10 synovial membranes of 30 and mild staining in 8 samples. 12 samples had no staining for CILP-1. In the synovial membranes staining was noted in several but not all blood vessels. Staining in blood vessels occupied the entire vessel wall from the endothelium to the media and adven-

titia. Staining tended to be weaker in the endothelium; however, if the vessel wall was stained, an intense reaction was always seen in the medial layer, as seen in **Figure 1**.

Besides the blood vessels of synovial membrane, immunohistochemical staining of CILP-1 was noted in cells. CILP-1 positive cells were noted in both synovial intima and subintima as seen in **Figure 2**.

Significant staining could be noted in round cells in synovial intima which could be described as type A synoviocytes or A-cells (synovial macrophages in intima).

In order to specify if the CILP-1 staining was indeed located in macrophages, immunofluorescent double staining was used, where the tissue sections were incubated with the mixture of CILP-1 antibody and the antibody to CD68 (marker for synovial macrophage chosen in accordance with a paper by Bartok and Firestein [11]).

Immunofluorescent staining for CD68 and CILP-1 was noted in round cells which could be considered synovial macrophages (**Figure 3**). To our knowledge, nobody has described the presence of CILP-1 in synovial macrophages before.

Staining for CILP-1 was also noted in blood vessels and cells of subsynovial connective tissue (**Figure 2**).

Histological degree of synovitis

Moderate synovitis was detected in 2 samples, 24 samples had mild synovitis and 4 samples had no synovitis. No cases of severe synovitis were noted.

Correlation between the semi-quantitative score of CILP-1 immunohistochemical staining and the score of synovitis

There was no significant correlation between the semi-quantitative score of CILP-1 staining and score of synovitis (Spearman $r = -0.1$; 95% CI -0.45 to 0.28 ; Two tailed p -value = 0.60).

Discussion

The cartilage intermediate layer protein 1 (CILP-1) has been described, as the name implies, in the articular cartilage. However, we found CILP-1 to be localized also in blood vessels of synovial membrane and also in synovial

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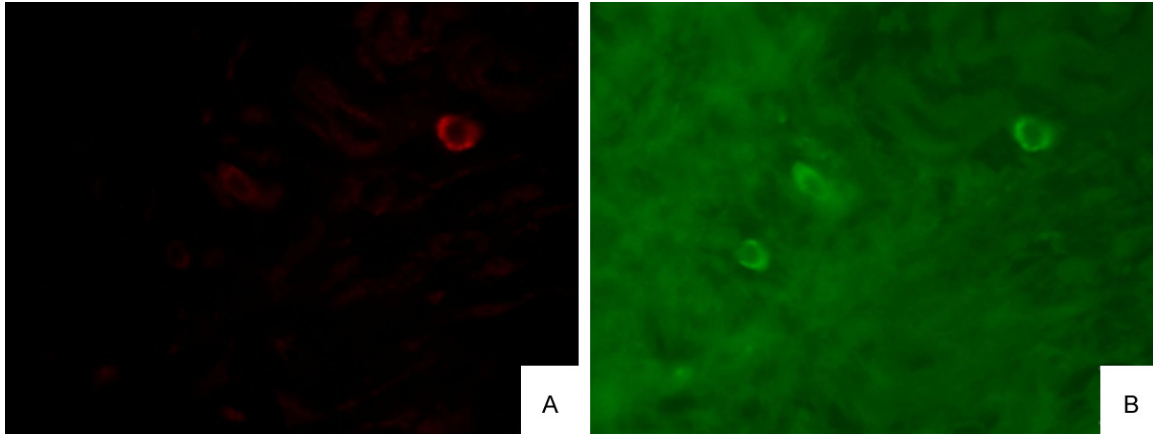


Figure 3. Staining for CILP-1 (red staining, panel A) was shown to be co-localized with staining for CD68 (green staining, panel B), which is a marker for synovial macrophages. Both pictures on **Figure 3** were taken using 100 × objective.

macrophages. There have been reports, where CILP has been detected outside the cartilage, e.g. muscle, heart, adipose tissue, synovial fluid and synovial lesions [4, 6-9]. Yet to the best of our knowledge, localization of CILP-1 in synovial macrophages has not been previously demonstrated.

The synovial membrane is a vascularized loose connective tissue that lines the joint capsule and consists of specific synovial cells -the macrophages (A-cells) and the fibroblast-like B-cells [12]. According to our histological evaluation, positive staining for CILP-1 was seen in all layers of blood vessel walls. However, the most intense staining was seen in the medial layer, thus demonstrating strong staining of smooth muscle cells, which are the dominant cells of the medial layer. As not all blood vessels stain positively for CILP-1, it can be speculated that the pattern of vessels may regionally differ, i.e. newly formed vessels may have different staining characteristics; however, this speculation needs additional investigations.

Positive staining was also noted in the cells histologically corresponding to the description of macrophages. Co-localization of staining for CILP-1 and CD68 was clearly evident, indicating that CILP-1 is expressed in synovial macrophages, which can be synoviocytes A, or the macrophages that may have migrated into synovial membrane during synovitis [13].

By showing CILP-1 expression in the synovial membrane of OA patients, this report suggests

that CILP-1 may have roles or functions other than a structural extracellular matrix molecule in cartilage and that CILP-1 could be involved in pathogenetic processes of OA and synovitis. For example, it has also been shown that CILP-1 has an inhibitory effect on insulin-like growth factor 1 [14], which suggest that CILP-1 is involved in metabolic activity. Since staining for it was observed in macrophages, it may be that CILP-1 could play a role in the immunological component of synovitis or OA [15]. However, since we were unable to demonstrate correlation between semi-quantitative score of CILP-1 immunohistochemical staining and the score of synovitis, this study does not offer any evidence for this. Although the semi-quantitative evaluation of immunohistochemical staining may have its limitations in estimating the protein content of the tissue, the authors believe that it would be sufficient method if robust and significant changes are to be shown.

In summary, we detected CILP-1 in the synovial membranes of patients with knee complaints and demonstrated that CILP-1 is localized in the synovial blood vessels and macrophages. Further research should also aim for co-localization of immunohistochemical staining for CILP-1 and a proper neovascularization marker to see if CILP-1 staining would be different in newly formed blood vessels in synovial membrane.

Acknowledgements

This study was supported by target financed project No. SF0180012s11 from the Estonian

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Ministry of Education and Research and by the NanoDiaRA project, grant No. 228929, funded by the EC 7th Framework Programme FP7-NMP-2008-L. The authors would like to thank Prof. Dick Heinegård's group, Lund University, for providing the antibody for CILP-1.

Disclosure of conflict of interest

None.

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