Identification of a complex between fibronectin and aggrecan G3 domain in synovial fluid of patients with painful meniscal pathology

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Abstract

Objectives: We previously described a panel of four cytokines biomarkers in knee synovial fluid for acute knee pain associated with meniscal pathology. The cytokine biomarkers included interferon gamma (IFN-γ), interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein-1 beta (MIP-1β). Validation studies using other immunologic techniques confirmed the presence of IL-6, MCP-1 and MIP-1β, but not IFN-γ. Therefore we sought the identity of the IFN-γ signal in synovial fluid.

Methods: Knee synovial fluid was collected from patients with an acute, painful meniscal injury, as well as asymptomatic volunteers. A combination of high-pressure chromatography, mass spectrometry and immunological techniques were used to enrich and identify the protein components representing the IFN-γ signal.

Results: A protein complex of fibronectin and the aggrecan G3 domain was identified in the synovial fluid of patients with a meniscal tear and pain that was absent in asymptomatic controls. This protein complex correlated to the IFN-γ signal. A novel enzyme-linked immunosorbent assay (ELISA) was developed to specifically identify the complex in synovial fluid.

Conclusions: We have identified a protein complex of fibronectin and aggrecan G3 domain that is a candidate biomarker for pain associated with meniscal injury.

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Introduction

Degenerative joint disease and joint injury are associated with increased turnover of articular cartilage proteins, inflammation and alterations to other joint tissue proteins [1,2]. Degenerative joint disease in the knee is often idiopathic, however it has also been strongly associated with prior injury such as meniscal damage. The inflammatory milieu induced by such injury may therefore lay the groundwork for future degeneration and osteoarthritis. The profile of inflammatory proteins within synovial fluid after acute knee injury may represent diagnostic or prognostic biomarkers for the degenerative joint disease or osteoarthritis that may ensue.

Expression and fragmentation of the extracellular matrix protein fibronectin has been shown to occur in the synovial fluid of arthritic patients and joint injury [3,4]. Fibronectin fragment induced knee injury in an animal model results in further cartilage damage and loss of proteoglycans [5]. Fibronectin also induces microglial activation and stimulation of cytokine production and activation of matrix metalloproteases [6]. It is well known that inflammatory cytokines are associated with fibronectin and its fragments in the pathophysiology of degenerative joint disease [7].

Aggrecan, a high molecular weight proteoglycan present in articular cartilage, undergoes extensive degradation and turnover during normal cartilage metabolism, aging and joint diseases [8]. Aggrecanases are activated during cartilage degradation and diseases [9]. Recently, it was demonstrated that patterns of aggrecan fragments differ between acute injury and chronic degeneration relative to healthy controls [10]. Therefore both fibronectin and aggrecan exhibit increased fragmentation in degenerative joint conditions and after articular cartilage damage. It is possible that fibronectin, aggrecan and their fragments interact in the synovial fluid to facilitate signaling cascades that augment joint and cartilage degeneration.
We recently described a panel of four cytokines biomarkers for acute knee pain associated with meniscal pathology [11], and one cytokine in the epidural space of spinal disc disease [12]. The cytokine biomarker panel was identified from synovial fluid using multiplex inflammatory cytokine profiling and included interferon gamma (IFN-γ), interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein-1 beta (MIP-1β). Validation studies using other immunologic techniques confirmed the presence of IL-6, MCP-1 and MIP-1β, but not IFN-γ.

To further determine the identity of the IFN-γ signal in the multiplex inflammatory cytokine panel, we used a combination of column chromatography and mass spectrometry to enrich and determine the amino acid sequence identity of the IFN-γ signal. Additional immunoassays were used to confirm the sequence identity. Our study identified a protein complex from knee synovial fluid containing fibronectin and aggrecan. In addition, this protein complex was present in a painful knee with meniscal pathology but absent from asymptomatic healthy volunteers.

Materials and methods

Subjects and synovial fluid collection

Our prospective study included 15 adult patients without rheumatoid arthritis who were diagnosed with painful intra-articular derangement of the knee by history, physical examination and magnetic resonance imaging (MRI) who elected for arthroscopic debridement following a failure of non-operative pain management. Inclusion criteria were an age of 18 years or older, knee pain of recent onset (less than 6 months), and physical examination findings and MRI results consistent with intra-articular pathology. Exclusion criteria were an age of less than 18 years, a history (within 3 months) of an intra-articular injection of a corticosteroid, and a past or current history of autoimmune disease (such as rheumatoid arthritis). The mean±standard deviation age was 77.2±5.2 years, and there were 7 males and 8 females in the study group.

Institutional Review Board (IRB) approval was obtained for the study, and knee synovial fluid was collected upon informed patient consent by needle aspiration. Synovial fluid was placed in polystyrene tubes containing a protease inhibitor (10 mM AEBSF, Sigma Aldrich, St. Louis, MO) and stored at −80 °C. Prior to use, the synovial fluid was treated with 5 mg/ml Hyaluronidase and clarified by centrifugation at 5000 g.

Multiplex cytokine assay

Multiplex immunoassay was performed using Bio-Rad’s Bio-Plex 200 with Bio-Plex human cytokine 4, 17, and 27 multiplex panels. The assay was performed as recommended by the manufacturer.

Antibodies and chemicals

Horseradish peroxidase (HRP) labeled anti-fibronectin antibody was obtained from US Biological, Swampscott, MA; anti-aggrecan G3 domain antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA; human fibronectin was obtained from BD Biosciences, San Jose, CA; all other chemicals were obtained from Sigma Aldrich.

HPLC and protein purification

High performance liquid chromatography (HPLC) assays and purification were performed on a Bio-Rad BioLogic DuoFlow HPLC system. Size exclusion chromatography (SEC) was performed using two Bio-Rad SEC400-5 columns in series. SEC separation was performed in isocratic mode using 50 mM Tris/HCl, 100 mM NaCl pH 7.0 buffer.

Anion exchange chromatography (AEC) was performed using a Bio-Rad UNO Q1 column. Buffer A: 50 mM Tris/HCl pH 7.0; Buffer B: 50 mM Tris/HCl, 1.0 M NaCl pH 7.0. The protein was loaded in 90% buffer A/10% buffer B and eluted with a linear gradient from 90% buffer A/10% buffer B to 70% buffer A/30% buffer B in 20 min.

Mass spectrometry

Ammonium bicarbonate (1 M) was added to solution sample to buffer pH to 8. Cysteine residues were reduced and alkylated with 2.5 mM TCEP and 20 mM iodoacetamide. Sample was digested using 2 ng of trypsin at 37 °C for 3 h. Digestion mixtures were loaded onto a precolumn (360 mm od×100 mm id fused silica, Polymicro Technologies, Phoenix, AZ) packed with 3 cm irregular C18 (5–15 mm non-spherical, YMC, Inc., Wilmington, NC) and washed with 0.1 M HOAc for 5 min before switching in-line with the resolving column (7 cm spherical C18, 360 mm×100 mm). Once the columns are in-line, the peptides are gradient eluted with a gradient of 0–100%A in 30 min where A is 0.1 M HOAc in nanopure H2O and B is 0.1M HOAc in 80% MeCN. All samples were analyzed using a Thermo Electron LTQ or LTQ-Orbitrap (San Jose, CA). Electrospray was accomplished using an Advion Triversa Nanomate (Advion Biosystems, Ithica, NY) with a voltage of 1.7 kV and a flow rate of 300 nL/min. The mass spectrometer was operated using data dependent scanning with the top 5 most abundant ions in each spectrum being selected for sequential MS/MS experiments. All MS/MS spectra were searched with Sequest using appropriate human database (ipi database v3.32). Database search results are tabulated and visually inspected using Scaffold (Proteome Software, Portland, OR).

Affinity chromatography

Anti-aggrecan G3 domain antibody was immobilized on Affi-Gel according to the manufacturer’s (Bio-Rad) recommended procedure to obtain an affinity chromatography for the purification of aggrecan G3 related molecules. The eluted peak from the AEC column containing the Bio-plex IFN-γ signal was further enriched on the aggrecan G3 affinity column. The column was equilibrated with 10 mM phosphate, 150 mM NaCl buffer pH 7.8. The AEC fractions were pooled and concentrated using spin filter with 30 kDa molecular weight cutoff. The column was then washed with the same buffer and eluted with 15 mM phosphate buffer pH 3.0. The eluted fraction was assayed for aggrecan and fibronectin proteins by slot blot analysis.

Gel electrophoresis and Western blotting

Gel electrophoresis was performed using Tris/HCl 18% or 4–20% polyacrylamide gradient gels (Bio-Rad). The samples were treated with SDS sample buffer at room temperature and loaded on the gel immediately. The gel was run at constant voltage of 200 V for 1 h using 20 mM Tris base, 192 mM Glycine, 0.1% SDS, pH 8.3 as running buffer. The gel was stained with silver stain (Bio-Rad Silver Stain Kit) according to the manufacturer’s recommended procedure.

For Western analysis, proteins were transferred to nitrocellulose membrane. The transfer was performed at constant voltage of 100 V for 40 min using Towbin buffer. The membranes from Western or slot blot analysis was blocked with 20 mM Tris, 0.5 mM NaCl, 1% casein pH 7.4 overnight and developed using anti-aggrecan G3, anti-fibronectin or anti-interferon-γ antibodies.

ELISA assay

Enzyme-linked immunosorbent assay (ELISA) plates were coated with anti-aggrecan G3 domain antibody in PBS/tween 20/thimerosal and blocked with 1% BSA overnight at 4 °C. Samples were incubated at room temperature for 1 h followed by 6 washes (using Bio-Rad’s Bio-
Plex Pro II wash station). HRP-labeled anti-fibronectin antibody was then added and the plate was incubated at room temperature for 1 h followed by 6 washes. Tetramethylbenzidine (TMB) substrate was added and incubated at room temperature in the dark for 5 min. The reaction was stopped with sulfuric acid and the plate was read using Bio-Rad’s Benchmark Plus microplate spectrophotometer at 450 nm. All assays were performed in triplicate. Human fibronectin at 1 µg/mL is used as negative control.

Results

By cytokine profiling of synovial fluid, we previously identified significant increase in the concentrations of IL-6, MCP-1, MIP-1β and IFN-γ in patients with cartilage degeneration pathology [11]. In the current study, we measured levels of these same cytokines in the knee undergoing arthroscopic debridement as described in Material and methods.

The knee synovial fluid from 5 of these patients in pain with meniscal pathology that exhibited the highest levels of all 4 cytokine biomarkers (IFN-γ, IL-6, MCP-1, and MIP1-β) was used for the subsequent analysis. While immunoblot confirmed the presence of IL-6, MCP-1 and MIP1-β, we could not confirm the presence of IFN-γ by immunoblot or ELISA using multiple IFN-γ antibodies and commercially available ELISA kits (data not shown). We therefore sought other methodologies to enrich for the IFN-γ signal detected by the Bio-Plex assay and determine the protein identity corresponding to this cytokine signal.

Size exclusion chromatography performed via high performance liquid chromatography (SEC-HPLC) analysis of the synovial fluid displayed a complex protein elution profile (Fig. 1). Direct comparisons between a symptomatic and non-symptomatic subject demonstrate the difference in the presence or absence of high molecular weight proteins (Fig. 1). As expected, silver stained polyacrylamide gels for SEC fractions 9–18 showed high molecular species in the early fractions and lower molecular weight species in the later fractions (Fig. 2). We assayed column fractions for the presence of the 4-cytokine biomarkers using the Bio-plex assay. IFN-γ signal resided in fractions 13, 14 and 15, elution time 32–38 min (Table 1). This elution time is characteristic of proteins 400–700 kDa molecular mass. The other three cytokines were present in fractions 17–19, a mass range more typical of the respective cytokine proteins. The molecular mass of the IFN-γ signal was confirmed by fractionating the clarified synovial fluid using spin filters. The IFN-γ signal was retained on a spin filter with a 300 kDa cutoff membrane and flowed through a spin filter with a 1000 kDa cutoff membrane (data not shown).

Identification of fibronectin–aggrecan complex

To determine the protein identity corresponding to the IFN-γ signal, we pooled SEC fractions 14 and 15 and digested the proteins with trypsin for subsequent amino acid sequence analysis by liquid chromatography based mass spectrometry (LC-MS/MS). A predominant protein identified from these fractions corresponded to fibronectin. We identified 15 unique peptides representing 320 of 2386 amino acids (13.4% coverage) of human fibronectin (Swiss-Prot identification number P02751) sequence (Fig. 3). While other proteins were identified in these SEC fractions, fibronectin is an extracellular matrix protein previously implicated in cartilage joint disease [7]. The molecular mass of fibronectin in its dimeric form is approximately 524 kDa in-line with the SEC fractions containing the IFN-γ signal have approximately 400–700 kDa molecular mass. Since human fibronectin did not cross react with INF-γ antibody labeled beads in the Bio-Plex assay (data not shown), additional proteins may interact with fibronectin within these SEC fractions that give rise to the antibody cross reactivity.

To further characterize proteins within these SEC fractions, we performed Western blot analysis with antibodies to fibronectin and other potential binding partners representing various cartilage and...
synovial fluid proteins. A representative silver stain SDS-polyacrylamide gel containing SEC fractions 14 and 15 is shown in Fig. 4A. We determined that protein bands immunoreactive for fibronectin and aggrecan G3 co-migrated in the gel (Figs. 4B and C). A polyclonal antibody to IFN-γ also weakly immunolabeled the same protein band (Fig. 4D).

**Enrichment of the fibronectin/aggrecan complex by anion exchange chromatography and anti-aggrecan G3 affinity column chromatography**

To further validate our SEC results and obtain amino acid sequence information for fibronectin interacting proteins, we enriched for the Bio-plex IFN-γ signal by anion exchange chromatography (AEC) of synovial fluid from a patient suffering from a painful meniscus tear. The AEC elution profile is shown in Fig. 5A and all fractions were analyzed by Bio-plex cytokine assay to identify fractions containing the IFN-γ signal. Slot blot analysis demonstrated the presence of both fibronectin and aggrecan G3 in these same fractions (data not shown).

An ELISA was developed to identify the presence of a fibronectin/aggrecan G3 protein complex in synovial fluid. The ELISA format was a modification of the classical sandwich ELISA, where the capture antibody recognized aggrecan G3 domain and the detection antibody was HRP-labeled anti-fibronectin antibody. We used this ELISA and determined the presence of a fibronectin–aggrecan protein complex in the AEC fractions (Table 2). The fibronectin–aggrecan G3 complex was present in AEC fractions 19–20 (Fig. 5A), corresponding to the Bio-plex IFN-γ signal.

AEC fractions 19 and 20, containing the fibronectin–aggrecan protein complex, were pooled and concentrated using a 30 kDa molecular weight cutoff spin filter. This sample was further purified using the aggrecan G3 affinity column as described in Materials and methods, the elution profile is shown in Fig. 5B. We noted a large peak of protein that failed to bind to the affinity column (fractions 2–5), a peak of proteins that eluted off the column at pH 3.0 (fractions 6–19). The eluted proteins were analyzed by silver stain (Fig. 5B). A representative silver stain SDS-polyacrylamide gel containing SEC fractions 14 and 15 is shown in Fig. 4A. We determined that protein bands immunoreactive for fibronectin and aggrecan G3 co-migrated in the gel (Figs. 4B and C). A polyclonal antibody to IFN-γ also weakly immunolabeled the same protein band (Fig. 4D).

**Table 1**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IFN-γ</th>
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<tr>
<td>8</td>
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<tr>
<td>9</td>
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<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
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</tr>
<tr>
<td>14</td>
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</tr>
<tr>
<td>15</td>
<td>8.4</td>
</tr>
<tr>
<td>16</td>
<td>1.7</td>
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</tr>
<tr>
<td>19</td>
<td>0</td>
</tr>
</tbody>
</table>

SEC fractions obtained from symptomatic subject shown in Fig. 1 were assayed for IFN-γ cytokine levels by the multiplex Bio-plex assay (Bio-rad). IFN-γ levels are shown for fractions containing the peak IFN-γ signal.
A final peak of proteins that eluted during the re-equilibration of the column (fractions 35–38). Due to protein aggregation and loss of protein–protein interaction that typically occurs at low pH, we used a slot blot analysis to explore the presence or absence of fibronectin and aggrecan G3 in these affinity column fractions (Fig. 6). We detected both fibronectin and aggrecan in flow-through fractions 2–5 suggesting saturation of the column, no aggrecan or fibronectin in the pH 3.0 elution fractions 18–24, but the presence of both aggrecan and fibronectin in fractions 35–38. Fibronectin tends to aggregate at pH 3.0 and we believe this explains why the eluted peak containing fibronectin and aggrecan appears at the buffer front during the re-equilibration of the column.

**Table 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Absorption units (450 nm)</th>
<th>Average absorption</th>
<th>CV%</th>
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<td>1 µg fibronectin, negative control</td>
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<td>0.03 0.04 0.04 0.04</td>
<td>0.04 0.04 0.04 0.04</td>
<td>17.7</td>
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<tr>
<td>Positive control</td>
<td>10</td>
<td>2.69 2.45 2.70 2.61</td>
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<td>5.4</td>
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<tr>
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<td>1.51 1.53 1.53 1.51</td>
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</tr>
<tr>
<td>fx. 12</td>
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<td>0.04 0.04 0.05 0.04</td>
<td>0.04 0.04 0.05 0.04</td>
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</tr>
<tr>
<td>fx. 13</td>
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<td>0.04 0.04 0.04 0.04</td>
<td>2.4</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>13.8</td>
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<tr>
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<td>0.05 0.04 0.05 0.05</td>
<td>13.5</td>
</tr>
<tr>
<td>fx. 18</td>
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<td>0.12 0.12 0.10 0.12</td>
<td>14.4</td>
</tr>
<tr>
<td>fx. 19</td>
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<td>26.7</td>
</tr>
<tr>
<td>fx. 20</td>
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<td>0.31 0.41 0.31 0.31</td>
<td>30.3</td>
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<tr>
<td>fx. 21</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>0.04 0.04 0.05 0.04</td>
<td>7.3</td>
</tr>
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</table>

Knee synovial fluid from a patient in pain with a meniscal injury was applied to an anion exchange column (AEC) and eluted with a linear salt gradient as described in Materials and methods. The fibronectin/aggrecan G-3 ELISA was used to analyze the AEC fractions for the presence of the protein complex. Fibronectin at 1 µL/mL was used as negative control. A pool of positive patient samples was used as positive control. Starting sample is the patient sample prior to AEC fractionation.

Finally, we used the novel ELISA to the fibronectin–aggrecan G3 complex to measure its level in the synovial fluid of 15 symptomatic subjects undergoing arthroscopic debridement of the knee. The optical density of the ELISA immunoassay for the symptomatic group ranged from 0.87 to 15.43 (mean 7.6; std.dev. 4.2).

**Discussion**

It is well accepted that disc and joint cartilage degeneration is associated with aging. However, it is not known why the degeneration is painful in some individuals and not in others. Aggrecan, the major
component of cartilage, and fibronec tin degradation were observed in both groups and considered part of the cartilage generation/degeneration cycle. We recently described a panel of four cytokines (IFN-γ, MCP-1, IL-6 and MIP-1β) that correlated to pain in patients with meniscal injury of the knee [11]. In this study, we have identified a complex of fibronec tin with an aggrecan fragment that is associated with painful cartilage degeneration or damage. The presence of this protein complex correlated to the IFN-γ signal in synovial fluid detected by multiplex analysis.

Since we could not confirm the presence of IFN-γ in synovial fluid by immunoblot or ELISA specific to IFN-γ, we enriched for the Bio-plex IFN-γ signal by column chromatography. It was evident from size exclusion chromatography and spin filters with nominal molecular weight cutoff that the protein(s) responsible for the IFN-γ signal had an apparent molecular mass of 400–600 kDa, much larger than the molecular mass of 25 kDa. LC-MS/MS of the partially purified IFN-γ signal demonstrated the presence of fibronec tin that was confirmed by western and ELISA assays. Fibronec tin alone did not show a positive signal in the Bio-Plex IFN-γ assay. Aggrecan is a multidomain protein where the C-terminal domain (G3) consists of an EGF, lectin and CRP sequences [13]. Age-related increases in aggrecan G3 have been shown to occur in articular cartilage [14], and increased aggrecan fragments have been observed after knee injury and in osteoarthriti s [15]. The known interaction of fibronec tin with lectins suggests that a complex could be formed between fibronec tin and aggrecan G3 domain. Western blot analysis of the partially purified IFN-γ signal by SEC-HPLC showed one band immunopositive for fibronec tin and fibronec tin in the synovial fluid of healthy volunteers is a prelude to cartilage degeneration. One potential mechanism involves the early generation of aggrecan fragments that may associate with full-length fibronec tin to contribute to cartridge degradation [19,20]. Full-length fibronec tin at levels of 15–80 ng/mL was detected in 60% of the synovial fluid samples obtained from our healthy control subjects. Fibronec tin fragments were not detected in the synovial fluid of any healthy control subject by Western blot or column chromatography. At this time, we do not know if the presence of fibronec tin in the synovial fluid of healthy volunteers is a prelude to cartilage degeneration. Further studies are necessary to explore the relationship between the fibronec tin–aggrecan G3 complex identified in this study and the continued fragmentation of fibronec tin, aggrecan and other structural proteins during cartilage degenerative disorders.

In conclusion, we have identified a protein complex between fibronec tin and aggrecan G3 domain present in the synovial fluid of patients with meniscal degeneration/injury and pain, and this complex may play a role in the development of future degenerative joint disease.

Acknowledgment

We would like to thank Dr. Jennifer Busby, Kristie Rose and Valerie Cavett at Scripps Florida, Jupiter FL, for performing the LC/MS/MS experiments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2010.04.069.

References


