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Novel COMP neoepitopes identified in synovial fluids from patients with joint diseases using affinity chromatography and mass spectrometry*

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**Running title: Identification of COMP neoepitopes.*

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Keywords: affinity purification, biomarker, COMP, ELISA development, joint disease, mass spectrometry, neoepitope.

CAPSULE

Background: To understand molecular processes underlying cartilage destruction we analyzed COMP fragments released into synovial fluid in joint diseases.

Results: Twelve novel COMP neoepitopes have been identified.

Conclusion: The release of COMP neoepitopes provides means for monitoring disease progression.

Significance: Based on the specificity, selectivity and sensitivity of each neoepitope a

new generation biomarkers for cartilage destruction can be developed.

ABSTRACT

To identify patients at risk for progressive joint damage there is a need for early diagnostic tools to detect molecular events leading to cartilage destruction. Isolation and characterization of distinct cartilage oligomeric matrix protein (COMP) fragments derived from cartilage and released into synovial fluid will allow discrimination between different pathological conditions and monitoring of disease progression. Early detection of disease and processes in the tissue as well as an

understanding of the pathologic mechanisms will also open for novel treatment strategies. Disease specific COMP fragments were isolated by affinity chromatography of synovial fluids from patients with rheumatoid arthritis (RA), osteoarthritis (OA) or acute trauma (AT). Enriched COMP fragments were separated by SDS-PAGE followed by in-gel digestion and mass spectrometric identification and characterization. Using the enzymes trypsin, chymotrypsin and Asp-N for the digestions an extensive analysis of the enriched fragments could be accomplished.

Twelve different neopeptides were identified and characterized within the enriched COMP fragments. For one of the neopeptides, S⁷⁷, an inhibition ELISA was developed. This ELISA quantifies COMP fragments clearly distinguishable from total COMP. Furthermore, fragments containing the neopeptide S⁷⁷ were released into the culture medium of cytokine (TNF- α and IL-6/sIL-6R) stimulated human cartilage explants.

The identified neopeptides provide a complement to the currently available commercial assays for cartilage markers. Through neopeptide assays, tools to pin-point disease progression, evaluation methods for therapy and means to elucidate disease mechanisms will be provided.

INTRODUCTION

Destruction of articular cartilage and changes of the underlying bone are key characteristics of joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). These pathological conditions resulting in tissue degradation constitute a major medical, social and economic problem (1). In order to minimize permanent tissue damage caused by pathological cartilage degeneration, it is important to diagnose such conditions at an early stage (2).

In progressive joint diseases, the degradation of extracellular matrix (ECM) proteins and proteoglycans leads to irreversible alterations in the properties of the collagen network. In

addition, the imbalance in the turnover of matrix proteins often results in increased proteolysis of molecules bound to and exposed at the surface of collagen fibers such as fibromodulin, decorin and cartilage oligomeric matrix protein (COMP) (3-5). During the last decade efforts have been made to find suitable biological markers that enable early detection of pathological cartilage degeneration (6,7).

One such biological marker is COMP. Elevated serum levels of COMP are associated with ongoing joint destruction in rheumatoid arthritis (8-10). COMP is cleaved and released from the cartilage tissue into synovial fluid in both OA, RA and other forms of inflammatory arthritis (6,10-13) and it is well established that COMP can be used as a marker of cartilage turnover (14).

COMP was primarily found in cartilage (15) but it has also been found in other tissues such as synovium and tendon (16,17). It is a pentameric protein of 524 kDa (15) where the monomers are joined together by a coiled-coil domain in the N-terminal. Each N-terminal domain is followed by four EGF repeat domains, eight thrombospondin type-III-domains and a C-terminal globular domain (18). The C-terminal domain is involved in interactions with other proteins in the extracellular matrix such as collagen I, II and IX (19-21). Each of the five globular C-terminal domains binds collagen with $K_D \approx 10^{-9}$ and thereby catalyzes collagen fibril assembly (19). Many proteases have been shown to degrade COMP but the specific cleavage sites within COMP as well as the newly formed N- and C-terminals have so far not been described.

In this work we have identified 12 novel COMP neopeptides and can hereby describe the newly formed N- and C-terminal ends. These neopeptides were identified through affinity enrichments of knee joint synovial fluids from patients with acute trauma, OA and RA followed by mass spectrometric identification and characterization of the enriched COMP fragments.

By using an *in vitro* model of joint disease we have successfully demonstrated the presence of the COMP neopeptide S⁷⁷ as a released fragment from cartilage explants. We have subsequently verified that the same cleavage occurs *in vivo* by showing the presence of neopeptide S⁷⁷ in the synovial fluid from 16 different patients with acute knee pain. Furthermore, an inhibition ELISA was developed for the neopeptide S⁷⁷ that specifically distinguished and quantified this neopeptide from total COMP.

EXPERIMENTAL PROCEDURES

Materials

Ammonium bicarbonate (NH₄HCO₃), dithiothreitol (DTT), formic acid, iodoacetamide (IAM), N-ethylmaleimide (NEM), Trifluoroacetic acid (TFA) and Triton-X100 were purchased from Sigma-Aldrich (St. Louis, MO USA). Anhydrous sodium acetate (NaAc) was purchased from Merck (Darmstadt, Germany). Sodium chloride (NaCl) from Scharlab S.L (Barcelona, Spain). Albumin bovine Fraction V, pH 7.0 (BSA) was purchased from SERVA Electrophoresis GmbH (Heidelberg Germany). The HPLC grade acetonitrile was from Rathburn (Walkerburn, Scotland). Trypsin was purchased from Promega (Madison, WI USA), Chymotrypsin and Asp-N from Roche (Mannheim, Germany). Stagetips were homemade according to (22) from 47-mm Empore C18 extraction discs (3M, Minneapolis, MN USA).

Human clinical samples

Synovial fluid was aspirated from knees of patients with acute knee pain with or without acute trauma (seeking care at the emergency room within the first week), established OA and established RA, Table 1. The use of patient samples was approved by The Ethical Committee in Lund (411/2005). The samples were stored at -80°C prior analysis.

Human donor knee cartilage from the tibial plateau (19-year-old-male, modified Collins (23) grade-0 (normal)) was obtained from the Gift of

Hope Organ and Tissue Donor Network (Elmhurst, IL, USA), approved by the Office of Research Affairs at Rush-Presbyterian-St. Luke's Medical Center and the Committee on Use of Humans as Experimental Subjects at MIT.

Antibodies

The mouse monoclonal antibody (2D3) towards the N-terminal domain of COMP was previously described in (24,25). The mouse monoclonal antibody towards the thrombospondin type-III-domain (TSP-III) was developed in-house (26). The C-terminal antibody used in western blots was a peptide antibody, towards the last 15 amino acids in human COMP, raised in goat (27).

Neopeptide antibodies were raised using immunogenic peptides containing the 5-mer neopeptide cleavage site followed by three glycine residues and one cysteine coupled to KLH (GenScript (Piscataway, NJ USA)). For antibody affinity purification the peptide CDACGMQQS⁷⁷ (internal cysteine alkylated) was immobilized to SulfoLink coupling resin according to manufacturer's protocol (Thermo Scientific, Rockford, IL USA). The column was equilibrated with HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) prior to incubation for 1h at room temperature with crude neopeptide sera. The column was washed with 15 ml HBS and 2 ml HBS with 1 M NaCl before elution of antibodies using 3 M potassium thiocyanate (KSCN). The eluate was immediately desalted on a PD10 column (GE healthcare, Buckinghamshire, UK) equilibrated with HBS.

Affinity enrichment of COMP from synovial fluid

For affinity enrichments mouse monoclonal antibodies towards the N-terminal domain (2D3) or towards the TSP-III domain were coupled to MiniLeak gel according to manufacturer's protocol (KemEnTec, Denmark). Synovial fluid from patient with acute trauma was diluted with two volumes of PBS (20 mM phosphate, 150 mM NaCl, pH 7.4) containing 0.8 % (w/v) SDS

and incubated for 2 h at room temperature. Excess of SDS was neutralized by addition of one volume 4 % Triton-X100 in PBS and incubated overnight at room temperature. In small scale enrichment of synovial fluid without SDS the same fragments were enriched (data not shown) and thus SDS was omitted. Synovial fluid from patients with OA and RA were incubated with 5 mM NEM prior to centrifugation for 20 min 1000 x g at room temperature. To diminish unspecific binding to the MiniLeak gel the synovial fluid samples were first passed through a column containing MiniLeak gel without any bound antibody. The flow through was then applied to the affinity column with the N-terminal antibody and subsequently the flow through from the N-terminal affinity column was applied to the affinity column with the TSP-III domain antibody. The columns were washed with HBS (10 mM HEPES, 150 mM NaCl, pH 7.4), HBS with 0.5 M NaCl and finally with HBS. Bound proteins were eluted using 0.1 M citrate pH 3 and immediately neutralized with 1.5 M Tris pH 8.8. Eluted fractions were precipitated with ethanol overnight at 4°C and collected by centrifugation (13200 x g, 4°C, 30 min). The pellets were re-precipitated in ethanol with 50 mM NaAc for 4 h at -20°C and collected by centrifugation (13200 x g, 4°C, 30 min) prior to SDS-PAGE.

SDS-PAGE and Western Blot

The precipitate pellets were dissolved in SDS-PAGE sample buffer (28) without reducing agent and separated on 4-16 % gradient SDS-polyacrylamide gels. Triplicate bands from each eluate were digested with trypsin, chymotrypsin and Asp-N as described below.

In screening of synovial fluids, samples were diluted ten times with non-reducing sample buffer and run on 4-16 % SDS-PAGE. Following electrophoresis, proteins were transferred to PVDF membranes (Thermo Scientific, Rockford, IL USA) at 100 V for 1 h in 25 mM Tris, 192 mM Glycine, 10 % Methanol. Membranes were blocked for 1 h at room temperature in 3 % w/v

BSA (SERVA Electrophoresis GmbH) in T-TBS (10 mM Tris, 150 mM NaCl, 0.05 % Tween, pH 7.4). Primary antibodies were diluted in T-TBS with 3 % (w/v) BSA (monoclonal antibodies 10 000 times dilution and polyclonal antibodies 1 000 times dilution) and incubated either for 1 h at room temperature or overnight at 4°C. As secondary antibody either goat-anti-mouse or donkey-anti-rabbit were used (Jackson ImmunoResearch, West Grove, PA USA), both were diluted 10 000 times in T-TBS with 3% BSA. Immunoblots were visualized using SuperSignal®WestDura Extended Duration Substrate (Thermo Scientific, Rockford, IL USA) on a luminescence image analyzer (Fuji Film LAS-1000).

Mass spectrometry sample preparation

After staining overnight with Blue silver stain (29) bands of interest were excised and reduced with 10 mM DTT at 56°C for 30 min and alkylated with 50 mM iodoacetamide for 30 min at room temperature (dark). Bands were digested overnight at 37°C with either 20 ng/µl of Trypsin in 25 mM NH₄HCO₃, 50 ng/µl of Chymotrypsin in 25 mM NH₄HCO₃, or 40 ng/µl of Asp-N in 50 mM phosphate buffer pH 8. Peptides were extracted consecutively with 1 % TFA, twice with 50 % acetonitrile in 0.1 % TFA and with 100 % acetonitrile. After drying the extracted peptides were dissolved in 10 µl 0.1 % TFA and purified on C18 Stage Tips (22).

Mass spectrometry

Purified peptide samples were re-dissolved in 0.2 % formic acid and analyzed on an Esquire HCT IonTrap (Bruker Daltonik, Bremen, Germany) as described in (30). Database searches were performed using MASCOT (version 2.1) MS/MS Ions Search in the UniProtKB (2010_09) database. MASCOT search parameters included: carbamidomethylation of cysteine as fixed modification, deamidation (Asn and Gln) and oxidation (Met) were considered as variable modifications. Other MASCOT search parameters were: monoisotopic masses, ±0.4 Da peptide mass tolerance, ±0.4 Da fragment mass

tolerance, max miss cleavage of 3, ion score minimum 20 and taxonomy *Homo sapiens*. Database searches using semi-style cleavages were used to identify peptides containing a neoepitope end. The peptide ends not representing a tryptic, chymotryptic or Asp-N cleavage are referred to as the neoepitopes. To remove false positive neoepitopes caused by unspecificity of the used proteases recombinant COMP was digested with trypsin, chymotrypsin and Asp-N. Only for chymotrypsin were three neoepitopes found in the control samples (data not shown) and these were removed from the resulting summary of neoepitopes.

Neoepitope S⁷⁷ inhibition ELISA and total COMP ELISA

An inhibition ELISA was developed for the neoepitope S⁷⁷ and was used to quantify synovial fluid samples from patients with RA, OA and acute trauma. The peptide CDACGMQQS⁷⁷ (internal cysteine alkylated) was crosslinked to BSA using MBS (Thermo Scientific, Rockford, IL USA) according to manufacturer's protocol. All incubations were performed at room temperature. Coating concentrations and buffers were optimized (data not shown) and binding plates were prepared by coating 96-well microtiter plates (NUNC-ImmunoPlates, Maxisorp, Nunc Intermed Ltd, Copenhagen, Denmark) with 50 µl of BSA-crosslinked peptide (12,5 ng/ml in PBS (150 mM NaCl, 10 mM potassium phosphate, pH 7.4) overnight.

Coated wells were washed with 0.15 M sodium chloride and 0.05 % (w/v) Tween 20 and blocked with 120 µl of 2 mg/ml BSA in PBS pH 7.4 for 1h. As a generous gift from Dr. Tobias Gustavsson (The Novo Nordisk foundation, Center for Protein Research, University of Copenhagen, Denmark) a protein fragment containing the N-terminal part of COMP (amino acid 20-77) and ending at neoepitope S⁷⁷ was provided. The protein fragment, labelled NT-S⁷⁷, was reduced and alkylated as described above prior to use. Standard NT-S⁷⁷ (2 µg/ml to 15.6 ng/ml) and synovial fluids (diluted 40 times) were diluted in a solution of 0.5 % BSA, 0.8 %

(w/v) SDS, 10 mM EDTA, in incubation buffer (0.14 M NaCl, 8 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM KCl, pH 7.4) and incubated overnight in a sterilin plate (Bibby Sterilin Ltd., U.K.). One volume of affinity purified neoepitope S⁷⁷ antibody (0.1 ng/ml), in 4% Triton X-100 in 10 mM sodium phosphate pH 7.4, was added to the sterilin plates. After 1h pre-incubation 50 µl of the mixture was added to the coated wells of the NUNC plate. The plate was incubated for 1h prior to washing and bound antibodies were detected by the addition of 50 µl of swine-anti-rabbit IgG-alkaline phosphatase (DAKO A/S, Denmark) diluted 1:1000 in 2 mg/ml BSA, incubation buffer with 0.05 % Tween-20. After 1h incubation the plates were washed and 100 µl of substrate was added (1mg/ml p-nitrophenyl phosphate in 1M diethanolamine pH 9.8 containing 0.5 M MgCl₂). The absorbance of each sample and standard was measured at 405 nm in duplicate by a microplate reader (Expert96, AsysHitech, Austria). The Mikrowin 200 software program (AsysHitech, Austria) was used to plot the calibration curve and to calculate the amount of COMP neoepitope in the samples.

Total COMP was measured in the same synovial fluid samples (diluted 40 or 80 times) according to manufacturer's protocol (AnaMar AB, Lund, Sweden).

Cartilage explants

Full thickness human knee cartilage explants (3 mm diameter, ~1.5 mm thick) were obtained using a dermal punch. Disks were maintained in high-glucose DMEM supplemented with 10 mM HEPES buffer, 1% insulin-transferrin-selenium (10 µg/ml, 5.5 µg/ml and 5 ng/ml, respectively), 0.1 mM nonessential amino acids, 0.4 mM proline, 20 µg/mL ascorbic acid, 100 units/mL penicillin G, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B in a 37°C, 5% CO₂ incubator prior to treatment. After two days of equilibration, groups of cartilage explants were cultured with an added cytokine cocktail consisting of 100 ng/mL TNFα, 50 ng/mL IL-6, and 250 ng/mL sIL-6R, as described previously

(31). Control explants were maintained in culture medium as above with no cytokines added. Medium changes were carried out every three days, and used medium was collected for analysis at each time point between day 3 and day 21 during the 21-day culture.

Statistical methods

Data from neopeptide S⁷⁷ ELISA did not show a Gaussian distribution so all statistical tests used were non-parametric. Data is presented as median values with interquartile range. P value < 0.05 was considered to be statistically significant. Analyses were performed using unpaired Mann-Whitney U test in GraphPad Prism Version 6 (GraphPad Software Inc, La Jolla, CA USA).

RESULTS

Identification and characterization of COMP neopeptides in synovial fluids.

Using mouse monoclonal antibodies towards the N-terminal coiled-coil domain and the thrombospondin type-III domain affinity enrichments of synovial fluids from patients with joint disease were performed. Mass spectrometric characterization of the enriched COMP fragments resulted in the identification of twelve novel neopeptides, Table 2. Peptides ending with an amino acid other than those formed by the used proteases (trypsin, chymotrypsin and Asp-N) is referred to as neopeptides. The ending amino acids of the neopeptides are superscripted.

In summary two neopeptides were identified in synovial fluid from patient with acute trauma; S⁷⁷ and F⁵³¹, six in synovial fluid from patient with OA; ⁹¹C, G²⁰², ⁵²³N, ⁵⁵⁵N, Y⁵⁷⁴ and F⁵⁷⁷, and five in synovial fluid from patient with RA; T¹⁰⁵, ¹⁹⁵S, Q²²⁶, ⁵⁵⁵N, ⁶⁵⁸G. Six of the neopeptides contains a new C-terminal end (S⁷⁷, G²⁰², Q²²⁶, F⁵³¹, Y⁵⁷⁴ and F⁵⁷⁷) and six contains a new N-terminal end (⁹¹C, ¹⁰⁵T, ¹⁹⁵S, ⁵²³N, ⁵⁵⁵N and ⁶⁵⁸G). Furthermore six of the neopeptides (S⁷⁷, ⁹¹C, ¹⁰⁵T, ¹⁹⁵S, G²⁰² and Q²²⁶) are located between the N-terminal and the thrombospondin-type-III-

domains whereas the other six neopeptides (⁵²³N, F⁵³¹, ⁵⁵⁵N, Y⁵⁷⁴, F⁵⁷⁷, ⁶⁵⁸G) are located within or in close proximity with the C-terminal globular domain.

A representative SDS-PAGE and western blot of enriched COMP fragments from OA synovial fluid with the excised bands used for identification of the neopeptides is shown in Figure 1. COMP was detected with high sequence coverage (18-54%) in all bands except the very weak band G which only contained keratin. In the 300 kDa band (labelled C) the neopeptides G²⁰², Y⁵⁷⁴ and F⁵⁷⁷ were identified. The 70 kDa band (labelled L) resulted in identification of neopeptides ⁹¹C, ⁵²³N, Y⁵⁷⁴ and F⁵⁷⁷. As can be seen, some of the neopeptides (Y⁵⁷⁴ and F⁵⁷⁷) were identified in both bands C and L. In band L it was clearly shown that this fragment does not contain the N-terminal domain, as seen by the enrichment of this fragment on the TSP-III column but not the N-terminal domain column and by the western blot of the eluted fractions, Figure 1. The most N-terminally detected peptide in this band contained the neopeptide ⁹¹C, indicating that the N-terminal has been cleaved off at or before this site.

Only one neopeptide, ⁵⁵⁵N, was identified in both OA and RA. However it cannot be ruled out that all neopeptides could occur in all diseases but at different levels depending on the disease state.

Synovial fluids from patients with acute knee pain contain the neopeptide S⁷⁷.

Synovial fluids from 16 patients were analyzed by western blots using neopeptide antibody S⁷⁷. No difference was seen in terms of the volume of aspirated synovial fluid (ranging from 20-135 ml). A band of approximately 40 kDa is detectable in all samples, Figure 2. This band corresponds to cleavage at S⁷⁷ within all five monomers of the COMP pentamer. Since the gels and blots were analyzed at non-reduced conditions the N-terminal coiled-coil domain remains intact and migrates as a cleaved pentamer of 40 kDa. For several of the samples a

band at 100 kDa is also visible, corresponding to fragmentations at S⁷⁷ at four out of five monomers within the COMP pentamer. A band at 15 kDa is also detected for some of the samples; these bands probably reflect further fragmentations within the coiled-coil domain resulting in reduced molecular weight of the migrating molecule.

Quantification of neopeptide S⁷⁷ and total COMP in synovial fluid from patients with different joint diseases.

An inhibition ELISA was developed to measure the amounts of neopeptide S⁷⁷ in synovial fluid of patients with acute trauma (n = 19), OA (n = 20) and RA (n = 20), Figure 3. A. The median values of neopeptide S⁷⁷ for the respective patient group were; 8.41 µg/ml (interquartile range 5.76-18.12) in acute trauma, 4.52 µg/ml (3.42-9.92) for OA and 1.23 µg/ml (0.70-1.62) for RA.

Also total COMP, as measured using the AnaMar assay, was determined, Figure 3. B. The median values of total COMP for the respective patient group were; 170.5 U/l (128.0-200.7) in acute trauma, 110.3 U/l (95.12-125.4) for OA and 51.15 U/l (37.23-74.16) for RA.

By comparing the median values of neopeptide versus total COMP between the three patients groups we could see that the neopeptide assay was distinguishable from the total COMP assay. Using the total COMP assay the highest levels were present in the AT group, approximately 3.3 and 1.5 fold higher than the RA and OA group respectively. There was also 2.2 fold higher levels in the OA versus the RA group.

With the neopeptide S⁷⁷ assay the AT group also had the highest levels 6.9 and 1.9 fold higher than the RA and OA group respectively. There was also 4.5 fold higher levels in the OA versus the RA group.

The coefficient of variation for the total COMP levels were 28% (AT), 20% (OA) and 36% (RA) while for the neopeptide assay they were 98% (AT), 85% (OA) and 53% (RA).

COMP neopeptide S⁷⁷ fragments are released from cytokine stimulated human cartilage explants.

Human cartilage explant plugs were incubated with cytokines (TNF-α and IL-6/sIL-6R) and the COMP fragments released into the incubation media were analysed by western blots using antibodies towards the N-terminal domain, the C-terminal domain or the neopeptide S⁷⁷, Figure 4. The use of antibodies towards both the N-terminal and the C-terminal shows how intact the released molecules are, whereas the neopeptide S⁷⁷ antibody shows the presence of this fragment. This experiment was repeated on 3 additional plugs taken from different locations within the same normal tibial plateau and all showed similar results.

The control sample contains a pool of media without added cytokines collected from all timepoints during the 21 day culture (Figure 4, labelled C). The release of COMP from cartilage explants into the surrounding medium is very limited during normal conditions. However two weak high molecular weight (HMW) bands above 250 kDa are detected with both the N-terminal and the C-terminal antibody.

For cytokine stimulated cartilage the release of intact COMP is detected at day 3 and 6 with both N- and C-terminal antibodies as double bands above 250 kDa, Figure 4. A and B. At day 9 N-terminal fragments are seen at approximately 300 kDa, 200 kDa and 100 kDa. These fragments start to appear at day 9 and persist until day 21 but show strongest staining, indicating highest amount released, at day 12. It is also clearly visible that the intact HMW COMP double bands show the strongest staining at day 12 and then decrease. This is also seen with the C-terminal antibody where intact double HMW bands are seen at day 3 to 9, with highest abundance at day 9 and then decrease. At day 9 a C-terminally containing fragment is detected at 70 kDa which increases until day 15 and then starts to decrease. At day 12 two C-terminal containing bands appear at 300 kDa and 200 kDa that remain until day 21.

Using the neoepitope antibody S⁷⁷ two fragments, at 200 and 100 kDa, are clearly visible from day 12 to day 21, Figure 4. C. The size of the fragments indicate that the COMP pentamer has been cleaved at position S⁷⁷ in three out of five monomers for the 200 kDa band and in four of the five monomers for the 100 kDa band. A weak band at 300 kDa that could represent cleavage in two of the five monomers is indicated at day 15. The results from day 12 and 15 are depicted in Figure 4D with a cartoon image representing the cleavages within the COMP pentamer. The glycosaminoglycan release was also measured, using the dimethyl methylene blue assay, showing that approximately 30-50% was released before the demonstrated release of the S⁷⁷ epitope.

Structural effects on the COMP pentamer caused by cleavage at the neoepitope sites.

Six of the identified neoepitopes (S⁷⁷, ⁹¹C, ¹⁰⁵T, ¹⁹⁵S, G²⁰² and Q²²⁶) are located between the N-terminal and the thrombospondin- type-III-domains, these neoepitopes could result in fragmentations of COMP disrupting its pentamer organization. The other six identified neoepitopes (⁵²³N, F⁵³¹, ⁵⁵⁵N, Y⁵⁷⁴, F⁵⁷⁷, ⁶⁵⁸G) are located within or in close proximity with the C-terminal globular domain of COMP. Cleavages at the neoepitope sites could interfere with several of the interactions between COMP and other proteins, since these interactions mainly occur with the C-terminal globular domain (19), Figure 5. The disulphide bridge between C⁵²⁰ and C⁷⁴¹, in the C-terminal domain of COMP, complicates the involvement of cleavages within the C-terminal domain since cleavages can occur between these two amino acids without affecting the migration properties of the COMP molecule as analyzed under non-reduced SDS-PAGE. However, the advantage of analyzing released COMP fragments under non-reducing conditions is to give an indication of how intact the released COMP molecules are.

DISCUSSION

Using affinity enrichments we have identified and characterized COMP fragments present in synovial fluids of patients with joint disease. Within these COMP fragments we describe the presence of twelve novel neoepitopes.

It is a well-established fact that COMP fragments are present in synovial fluids in a variety of joint disease conditions (6,10,12) Characteristics of these COMP fragments have previously been described using mouse monoclonal epitope mapping (32,33). However the present report for the first time defines ending amino acids, neoepitopes, within these COMP fragments.

By identifying the COMP neoepitopes we could raise peptide antibodies specific for these neoepitopes. This gives us the unique possibility to quantify these neoepitopes in synovial fluids using inhibition ELISA. By removing the requirement of a capture antibody, novel fragmentations present in synovial fluids can be determined. Furthermore the characterization of these neoepitopes provides important information regarding the specificity of the degrading enzymes causing these cleavages.

We have developed an inhibition ELISA towards one of these neoepitopes, S⁷⁷. This neoepitope is unique, both as compared to other human thrombospondins as well as to COMP from different species, which makes it suitable as a biomarker specific to human cartilage degradation.

The highest amounts of both total COMP and neoepitope S⁷⁷ were found in acute trauma synovial fluids, followed by OA and finally RA. The increased variability (CV) found with the neoepitope assay could reflect that this assay is more sensitive for various stages of disease making it a strong candidate for further biomarker evaluation.

The release of COMP fragments containing neoepitope S⁷⁷ was furthermore determined in human knee cartilage explants. Within this

experiment this neoepitope is clearly prominent in the cytokine stimulated medium. This was also verified through an extensive mass spectrometric analysis of the cartilage explant medium comparing untreated controls to trauma-stimulated cartilage explants¹. These results indicate that the protease causing the neoepitope S⁷⁷ cleavage is activated or upregulated by the presence of the cytokines TNF- α and IL-6/sIL-6R. This suggests the possibility of selective inhibition trials to prevent the cleaving at this neoepitope site.

From the present data on synovial fluids we cannot yet confirm whether the proteolytic processing of COMP occurred within the cartilage or after release of total COMP into the synovial fluid, where accumulated proteases could then act. However, based on the kinetics of the release of neoepitope S⁷⁷ demonstrated in the cartilage explant studies (Figure 4), it is most likely that degradation of COMP into fragments is initiated within the cartilage tissue and then released into the medium.

Our data clearly shows that COMP is released, both as intact pentamer and fragmented, from cytokine-stimulated human cartilage explants. In the search for biomarkers of joint disease involving the detection of released fragments from cartilage, the presence of neoepitope S⁷⁷ represents a strong candidate since it clearly is distinguishable from intact COMP.

An eukaryotic expression system used for purification of pentameric recombinant COMP has previously shown the presence of a 100 kDa fragment starting at amino acid ⁷⁸V. These authors suggest that a protease present either within the cells or in the culture medium is able to cleave COMP at this amino acid (21). This is consistent with the cleavage at amino acid S⁷⁷ observed in the present study.

Purified COMP has been shown to be a substrate for several MMPs such as MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1), MMP-9 (gelatinase B) and MMP-13 (collagenase 3) (34). COMP fragments containing the N-terminal domain or the EGF-domain have previously been shown in synovial fluids from patients with OA, RA and ACL injury, as bands of 80 and 100 kDa on western blots (35). Also ADAMTS-7 (36) and ADAMTS-12 (37) have been shown to cleave COMP by binding to the EGF domains, forming fragments of 100 kDa. Furthermore IL-1 α stimulation of bovine nasal cartilage shows release of COMP fragments of 110 kDa (38). Even though many proteases have been shown to degrade COMP, the specific cleavage sites within COMP have not been identified. Our identified COMP neoepitopes can be of use for determination of the proteases involved in cartilage degradation and their involvement in the disease progression. The involvement of the released COMP neoepitopes and their role in the disease propagation is also of great importance (1).

The main focus of analyzing COMP fragments in joint disease was to identify and characterize protein neoepitopes created by the pathological process that can be separated from normal tissue turnover. Assays for quantifying these neoepitopes can be used in diagnostic approaches, disease monitoring and evaluation of treatment and therapy.

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FOOTNOTES

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³The abbreviations used are: AT, acute trauma; OA, osteoarthritis; RA, rheumatoid arthritis; COMP, cartilage oligomeric matrix protein; SF, synovial fluid.

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TABLES

Table 1. Cohort demographics.

	AT	knee pain	OA	RA
n	19	16	20	20
Age mean (years)	31.2	43.9	67.7	64.3
Age range (years)	13-65	16-65	55-85	41-83

Table 2. COMP neopeptides identified in synovial fluid from patients with AT, OA and RA. The end of the identified peptide which corresponds to a neopeptide is superscripted. The highest MS/MS ion score is presented and the number of bands the neopeptide was identified in is shown in brackets.

Peptide identified by MS	Synovial fluid	Digesting enzyme	Neopeptide amino acid	Ion score (n=)
NTVMECDACGMQQS ⁷⁷ ↓VR	AT	Trypsin	S ⁷⁷	64 (1)
LH↓ ⁹¹ CAPGFCFPGVACIQTESGAR	OA	Trypsin	⁹¹ C	33 (1)
IQ↓ ¹⁰⁵ TESGARCPCPAGF	RA	CT	¹⁰⁵ T	41 (2)
PN↓ ¹⁹⁵ SVCINTRGSF	RA	CT	¹⁹⁵ S	32 (4)
QVCTDINECETGQHNCVPNSVCINTRG ²⁰² ↓SF	OA	Trypsin	G ²⁰²	22 (1)
QCGPCQPGFVGDQASGCQRRQA ²²⁶ ↓RF	RA	CT	Q ²²⁶	48 (3)
PE↓ ⁵²³ NAEVTLTDFR	OA	Trypsin	⁵²³ N	57 (1)
IDVCPENAEVTLTDF ⁵³¹ ↓RA	AT	Trypsin	F ⁵³¹	52 (1)
VL↓ ⁵⁵⁵ NQGREIVQTMNS	RA, OA	Asp-N	⁵⁵⁵ N	43 (3)
EIVQTMNSDPGLAVGY ⁵⁷⁴ ↓TA	OA	Trypsin	Y ⁵⁷⁴	38 (2)
EIVQTMNSDPGLAVGYTAF ⁵⁷⁷ ↓NG	OA	Trypsin	F ⁵⁷⁷	38 (2)
HT↓ ⁶⁵⁸ GDTESQVRLW	RA	Asp-N	⁶⁵⁸ G	26 (1)

FIGURE LEGENDS

FIGURE 1. Representative image of COMP fragments in OA synovial fluid. COMP fragments from OA synovial fluid were affinity enriched using mouse monoclonal antibodies towards: **A.** the N-terminal domain and towards: **B.** the thrombospondin type-III-domain (TSP-III) of COMP. Eluates were run on a non-reduced 4-16 % SDS-PAGE either stained with Blue Silver (29) or blotted to PVDF membranes (marked WB), followed by visualization using the N-terminal antibody for the N-terminally enriched eluate or using the TSP-III antibody for the TSP-III enriched eluate. In-gel digests of the enriched fragments were analyzed with mass spectrometry as described in materials and methods and neoepitopes were identified in bands labelled C and L.

FIGURE 2. The neoepitope S⁷⁷ is present in synovial fluid from patients with acute knee pain. Western blots of synovial fluid from knee pain patients using COMP neoepitope antibody S⁷⁷. The presence of neoepitope S⁷⁷ is clearly visible in all analyzed samples as a band migrating at 40 kDa. For several of the samples bands of 100 kDa and 15 kDa are also visible. Samples are distributed based on aspirated synovial fluid volume, ranging from 20 ml (sample 1) to 135 ml (sample 16). Sample 3, 7 and 11 had an acute trauma.

FIGURE 3. ELISA quantification of neoepitope S⁷⁷ and total COMP in synovial fluid from patients with joint disease. **A.** An inhibition ELISA developed for neoepitope S⁷⁷ was used to quantify the amounts of neoepitope present in synovial fluids from knee patients with acute trauma (AT), OA and RA. Logarithmic median values with whiskers at the interquartile range are presented. Mann-Whitney P values between the groups were; AT vs OA, p=0.0377; OA vs RA, p= < 0.0001, AT vs RA, p= < 0.0001. **B.** Total amount of COMP was measured using the AnaMar COMP assay. Mann-Whitney P values between the groups were; AT vs OA, p=0.0005; OA vs RA, p= < 0.0001, AT vs RA, p= 0.0001.

FIGURE 4. Identification of COMP fragmentations in cytokine stimulated human cartilage explants. Explants were incubated with cytokines (TNF- α and IL-6/sIL-6R) and the COMP fragments released to the incubation media were analysed with western blots using antibodies towards; **A.** The N-terminal domain, **B.** The C-terminal domain, **C.** The neoepitope S⁷⁷ antibody. Sample labelled C represent pooled control media collected at all-time points. Molecular weight markers (kDa) are indicated. **D.** Enlargement of the results from day 12 and 15 with a cartoon image representing the cleavages within the COMP pentamer.

FIGURE 5. Schematic presentation of the identified neoepitopes in COMP. Monomeric view of the domains in COMP; the N-terminal coiled-coil domain, the four EGF-domains; the eight thrombospondin type-III-domains and the C-terminal globular domain. Epitopes for the antibodies used for affinity enrichments (α -N-terminal and α -TSP-III domain) are illustrated on top of the monomer. The cysteine bridge between Cys520 and Cys740 within the C-terminal domain is marked. Boxes showing the neoepitope amino acid and arrows indicating locations are marked. Neoepitopes identified in acute trauma (AT) are boxed and marked with dotted lines, OA with broken lines, RA with lines and identified in both OA and RA is marked with thick box. The ending amino acids of the neoepitopes are superscripted; S⁷⁷ represent the newly formed C-terminal whereas ⁹¹C represent a newly formed N-terminal.

FIGURE 1.

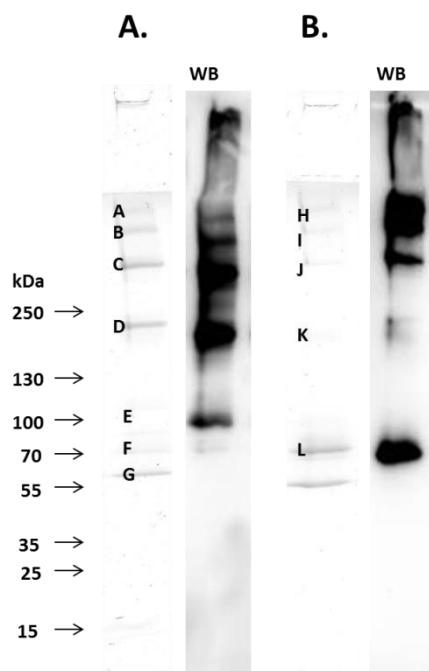


FIGURE 2.

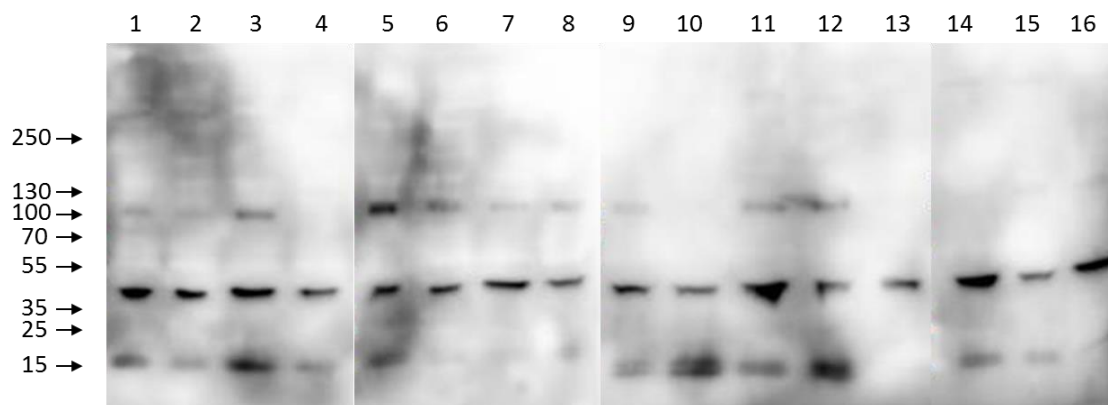


FIGURE 3.

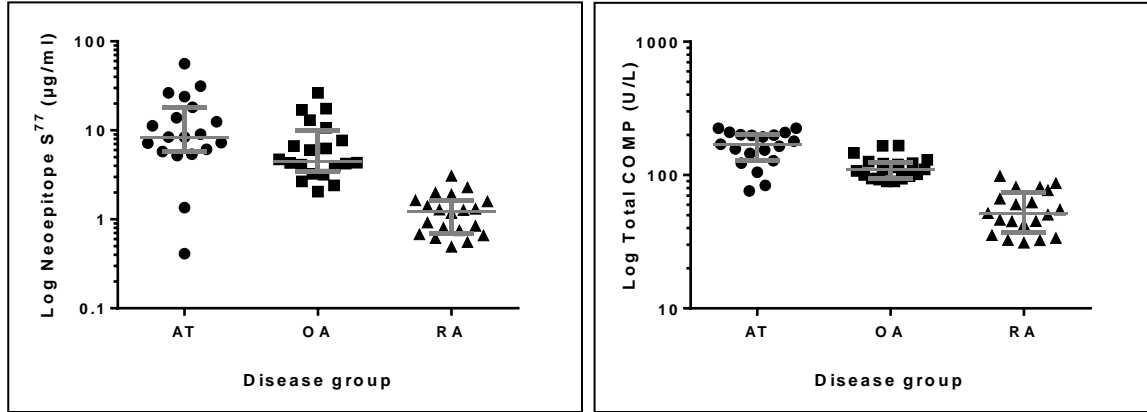


FIGURE 4.

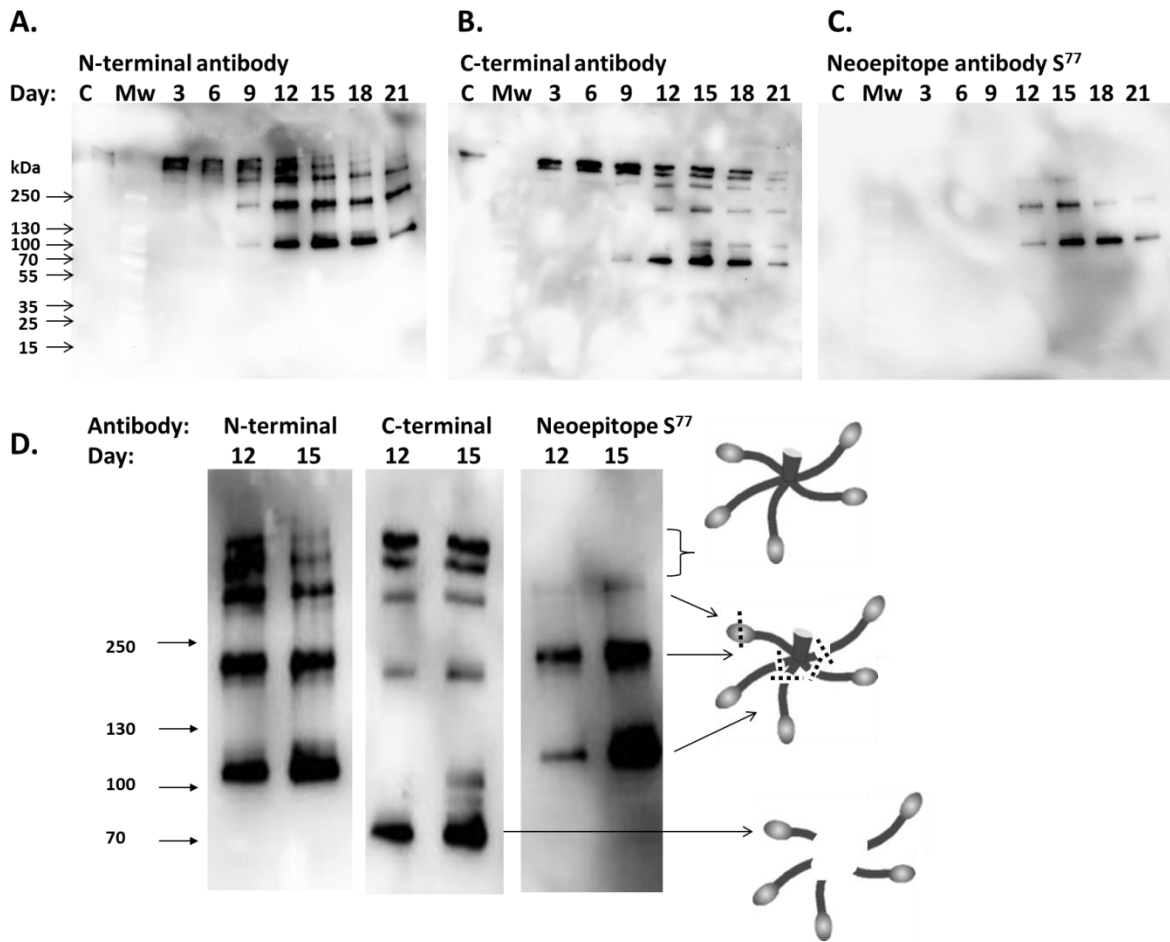


FIGURE 5.

