Novel Cartilage Oligomeric Matrix Protein (COMP) Neoepitopes Identified in Synovial Fluids from Patients with Joint Diseases Using Affinity Chromatography and Mass Spectrometry*

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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 of biomarkers for cartilage destruction can be developed.

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 the way for novel treatment strategies. Disease-specific COMP fragments were isolated by affinity chromatography of synovial fluids from patients with rheumatoid arthritis, osteoarthritis, or acute trauma. 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 neoepitopes were identified and characterized within the enriched COMP fragments. For one of the neoepitopes, Ser77, an inhibition ELISA was developed. This ELISA quantifies COMP fragments clearly distinguishable from total COMP. Furthermore, fragments containing the neoepitope Ser77 were released into the culture medium of cytokine (TNF-α and IL-6/soluble IL-6 receptor)-stimulated human cartilage explants. The identified neoepitopes provide a complement to the currently available commercial assays for cartilage markers. Through neoepitope assays, tools to pinpoint disease progression, evaluation methods for therapy, and means to elucidate disease mechanisms will be provided.

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). 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 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 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).

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The abbreviations used are: OA, osteoarthritis; AT, acute trauma; RA, rheumatoid arthritis; COMP, cartilage oligomeric matrix protein; sIL-6R, soluble IL-6 receptor; TSP-III, thrombospondin type III domain; HBS, HEPES-buffered saline; MMP, matrix metalloproteinase.
COMP is 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) in which where the monomers are joined together by a coiled coil domain in the N terminus. 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 collagens I, II, and IX (19–21). Each of the five globular C-terminal domains binds collagen with a $K_D$ $=$ $10^{-9}$ 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 termini have so far not been described.

In this work, we identified 12 novel COMP neoepitopes and hereby describe the newly formed N- and C-terminal ends. These neoepitopes 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 neoepitope$^3$ Ser$^{77}$ as a released fragment from cartilage explants. We have subsequently verified that the same cleavage occurs in vivo by showing the presence of neoepitope Ser$^{77}$ in the synovial fluid from 16 different patients with acute knee pain. Furthermore, an inhibition ELISA was developed for the neoepitope Ser$^{77}$ that specifically distinguished and quantified this neoepitope from total COMP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ammonium bicarbonate (NH$_4$HCO$_3$), dithiothreitol (DTT), formic acid, iodoacetamide, N-ethylmaleimide, trifluoroacetic acid (TFA), and Triton X-100 were purchased from Sigma-Aldrich. Anhydrous sodium acetate was purchased from Merck. Sodium chloride (NaCl) was 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, UK). Trypsin was purchased from Promega (Madison, WI). Chymotrypsin and Asp-N were from Roche Applied Science. Stage tips were homemade according to Ref. 22 from 47-mm Empore C$_18$ extraction discs (3M, Minneapalis, MN).

**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 1st week), established OA, and established RA (Table 1). The use of patient samples was approved by The Ethical Committee in Lund, Sweden (411/2005). The samples were stored at $-80$ °C prior to analysis.

Human donor knee cartilage from the tibial plateau (19-year-old male, modified Collins scale (23) grade 0 (normal)) was obtained from the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL). The procedure was 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 the Massachusetts Institute of Technology.

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

Neoepitope antibodies were raised using immunogenic peptides containing the 5-mer neoepitope cleavage site followed by three glycine residues and one cysteine coupled to keyhole limpet hemocyanin (GenScript, Piscataway, NJ). For antibody affinity purification, the peptide CDACGMQQS$^{77}$ (internal cysteine was alkylated) was immobilized to SulfoLink coupling resin according to the manufacturer’s protocol (Thermo Scientific, Rockford, IL). The column was equilibrated with HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) prior to incubation for 1 h at room temperature with crude neoepitope sera. The column was washed with 15 ml of HBS and 2 ml of HBS with 1 M NaCl before elution of antibodies using 3 M potassium thiocyanate. The eluate was immediately desalted on a PD10 column (GE Healthcare) equilibrated with HBS.

**Affinity Enrichment of COMP from Synovial Fluid**—For affinity enrichments, mouse monoclonal antibodies toward the N-terminal domain (2D3) or toward the TSP-III domain were coupled to MiniLeak gel according to the manufacturer’s protocol (KemEnTec, Denmark). Synovial fluid from a patient with acute trauma was diluted with 2 volumes of PBS I (20 mM phosphate, 150 mM NaCl, pH 7.4) containing 0.8% (w/v) SDS and incubated for 2 h at room temperature. Excess SDS was neutralized by addition of 1 volume of 4% Triton X-100 in PBS I 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 fluids from patients with OA and RA were incubated with 5 mM N-ethylmaleimide prior to centrifugation for 20 min at 1000 × 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, HBS with 0.5 M NaCl, and finally 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 (13,200 × g, 4 °C, 30 min). The pellets were reprecipitated in ethanol with 50 mM

| TABLE 1  
Cohort demographics | AT | Knee pain | OA | RA |
|-----------------|---|---------|---|---|
| $n$             | 19| 16      | 20| 20|
| Mean age (years)| 31.2| 43.9   | 67.7| 64.3|
| Age range (years)| 13–65| 16–65 | 55–85| 41–83|
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sodium acetate for 4 h at −20 °C and collected by centrifugation (13,200 × 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 synovial fluids, samples were diluted 10× with non-reducing sample buffer and separated by 4–16% SDS-PAGE. Following electrophoresis, proteins were transferred to non-reducing sample buffer and separated by 4–16% SDS-PAGE. Following electrophoresis, proteins were transferred to non-reducing sample buffer and separated by 4–16% SDS-PAGE. Following electrophoresis, proteins were transferred to non-reducing sample buffer and separated by 4–16% SDS-PAGE.

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Neoepitope Ser77 Inhibition ELISA and Total COMP ELISA—An inhibition ELISA was developed for the neoepitope Ser77 and was used to quantify synovial fluid samples from patients with RA, OA, and acute trauma. The peptide CDACGMQQS77 (internal cysteine was alkylated) was cross-linked to BSA using m-maleimidobenzoyl-N-hydroxysuccinimide ester (Thermo Scientific) according to the 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-Immu-no plates, Maxisorp, Nunc Intermed Ltd., Copenhagen, Denmark) with 50 μl of BSA-cross-linked peptide (12.5 ng/ml in PBS II (150 mM NaCl, 10 mM potassium phosphate, pH 7.4) overnight.

Coated wells were washed with 0.15 M sodium chloride, 0.05% (w/v) Tween 20 and blocked with 120 μl of 2 mg/ml BSA in PBS II for 1 h. A protein fragment containing the N-terminal part of COMP (amino acids 20–77) and ending at neoepitope Ser77 was a generous gift provided by Dr. Tobias Gustavsson (The Novo Nordisk Foundation, Center for Protein Research, University of Copenhagen, Copenhagen, Denmark). The protein fragment, labeled NT-Ser77, was reduced and alkylated as described above prior to use. Standard NT-Ser77 (2 μg/ml to 15.6 ng/ml) and synovial fluids (diluted 40×) 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., UK). One volume of affinity-purified neoepitope Ser77 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 1-h preincubation, 50 μl of the mixture was added to the coated wells of the Nunc plate. The plate was incubated for 1 h 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 1-h incubation, the plates were washed, and 100 μl of substrate was added (1 mg/ml p-nitrophenyl phosphate in 1 M 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) 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×) according to the 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-transfer- sulin-selenium (10 μg/ml, 5.5 μg/ml, and 5 μg/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, 0.25 μg/ml amphotericin B in a 37 °C, 5% CO2 incubator prior to treatment. After 2 days of equilibration, groups of cartilage explants were cultured with an added cytokine mixture consisting of 100 ng/ml TNF-α, 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 3 days, and used...
RESULTS

Identification and Characterization of COMP Neoepitopes in Synovial Fluids—Using mouse monoclonal antibodies toward 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 12 novel neoepitopes (Table 2). Peptides ending with an amino acid other than those formed by the proteases used (trypsin, chymotrypsin, and Asp-N) are referred to as neoepitopes. The ending amino acids of the neoepitopes are superscripted. The highest MS/MS ion score is presented, and the number of bands in which the neoepitope was identified is shown in parentheses. CT, chymotrypsin, UV, trypsin, and Asp-N are superscripted.

| Peptide identified by MS | Synovial fluid | Digested enzyme | Neoepitope amino acid | Ion score (n) |
|-------------------------|----------------|-----------------|-----------------------|---------------|
| NTVMECDACGMQSS | VR | AT | Ser77 | 64 (1) |
| LH | 91CA | CFPCGVAVC | Cys91 | 33 (1) |
| IQ | 105TSGG | | Cys105 | 41 (2) |
| PN | 195SCTG | | Ser195 | 32 (4) |
| QCVPQGFGVVDQASG | QT | | Gly202 | 22 (1) |
| PE | 252SVEVLTDFR | | Asn252 | 57 (1) |
| IDVPENAE | VTLTDFR | RA | Asp-N | 52 (1) |
| VLT | 252SVEVLTDFR | | Asn252 | 43 (3) |
| EIVQTMNSDPGLAVG | YTA | OA | Ser77 | 64 (1) |
| EIVQTMNSDPGLAVGYAT | 577 | NG | Asp-N | 38 (2) |
| HT | 658GDTESQVRLWK | RA | Gly658 | 26 (1) |

In summary, two neoepitopes were identified in synovial fluid from a patient with acute trauma (Ser77 and Phe531), six were identified in synovial fluid from a patient with OA (91Cys, G202, 523Asn, 555Asn, Tyr574, and Phe577), and five were identified in synovial fluid from a patient with RA (Thr105, 195Ser, Gly202, and Gln226). Six of the neoepitopes contain a new C-terminal end (Ser77, Gly202, Gln226, Phe531, Tyr574, and Phe577), and six contain a new N-terminal end (91Cys, 105Thr, 195Ser, 523Asn, 555Asn, and 658Gly). Furthermore, six of the neoepitopes (Ser77, 91Cys, 105Thr, 195Ser, Gly202, and Gln226) are located between the N-terminal and the thrombospondin type III domains, whereas the other six neoepitopes (523Asn, Phe531, 555Asn, Tyr574, Phe577, and 658Gly) are located within or in close proximity to the C-terminal globular domain.

A representative SDS-polyacrylamide gel and Western blot of enriched COMP fragments from OA synovial fluid with the excised bands used for identification of the neoepitopes are shown in Fig. 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 (labeled C), the neoepitopes Gly202, Tyr574, and Phe577 were identified. The 70-kDa band (labeled L) resulted in identification of neoepitopes 91Cys, 523Asn, Tyr574, and Phe577. As can be seen, some of the neoepitopes (Tyr574 and Phe577) 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 (Fig. 1). The most N-terminally detected peptide in this band contained the neoepitope 91Cys, indicating that the N terminus has been cleaved off at or before this site.

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

FIGURE 1. Representative image of COMP fragments in OA synovial fluid. COMP fragments from OA synovial fluid were affinity-enriched using mouse monoclonal antibodies toward the N-terminal domain (A) and toward the TSP-III of COMP (B). Eluates were separated by non-reducing 4–16% SDS-PAGE and either stained with blue silver (29) or blotted to PVDF membranes (marked WB) followed by visualization using the N-terminal antibody for the N terminus-enriched eluate or using the TSP-III antibody for the TSP-III-enriched eluate. In-gel digests of the enriched fragments were analyzed by mass spectrometry as described under “Experimental Procedures.” Bands are labeled A–L, and neoepitopes were identified in bands labeled C and L.
Identification of COMP Neoepitopes

Synovial Fluids from Patients with Acute Knee Pain Contain the Neoepitope Ser77—Synovial fluids from 16 patients were analyzed by Western blots using neoepitope antibody Ser77. No difference was seen in terms of the volume of aspirated synovial fluid (ranging from 20 to 135 ml). A band of ~40 kDa is detectable in all samples (Fig. 2). This band corresponds to cleavage at Ser77 within all five monomers of the COMP pentamer. Because the gels and blots were analyzed under non-reducing 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, corresponding to fragmentations at Ser77 at four of five monomers within the COMP pentamer, is also visible. 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 Neoepitope Ser77 and Total COMP in Synovial Fluid from Patients with Different Joint Diseases—An inhibition ELISA was developed to measure the amounts of neoepitope Ser77 in synovial fluid of patients with acute trauma (n = 19), OA (n = 20), and RA (n = 20) (Fig. 3A). The median values of neoepitope Ser77 for the patient groups were 8.41 µg/ml (interquartile range, 5.76–18.12) for acute trauma, 4.52 µg/ml (interquartile range, 3.42–9.92) for OA, and 1.23 µg/ml (interquartile range, 0.70–1.62) for RA.

Total COMP as measured using the AnaMar assay also was determined (Fig. 3B). The median values of total COMP for the patient groups were 170.5 units/liter (interquartile range, 128.0–200.7) in acute trauma, 110.3 units/liter (interquartile range, 95.12–125.4) for OA, and 51.15 units/liter (interquartile range, 37.23–74.16) for RA.

By comparing the median values of neoepitope versus total COMP among the three patient groups, we could see that the neoepitope assay was distinguishable from the total COMP assay. Using the total COMP assay, the highest levels were present in the acute trauma (AT) group (~3.3- and 1.5-fold higher than the RA and OA groups, respectively). There were also 2.2-fold higher levels in the OA versus the RA group.

With the neoepitope Ser77 assay, the AT group also had the highest levels (6.9- and 1.9-fold higher than the RA and OA groups respectively). There were also 4.5-fold higher levels in the OA versus the RA group. The coefficients of variation for the total COMP levels were 28 (AT), 20 (OA), and 36% (RA), and for the neoepitope assay, they were 98 (AT), 85 (OA), and 53% (RA).

COMP Neoepitope Ser77 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 analyzed by Western blots using antibodies toward the N-terminal domain, the C-terminal domain, or the neoepitope Ser77 (Fig. 4). The use of antibodies toward both the N-terminal and the C-terminal domains shows how intact the released molecules are, whereas the neoepitope Ser77 antibody shows the presence of this fragment. This experiment was repeated on three 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 time points during the 21-day culture (Fig. 4, labeled C). The release of COMP from cartilage explants into the surrounding medium is very limited during normal conditions. However, two weak high molecular mass bands above 250 kDa are detected with both the N-terminal and the C-terminal antibodies.

For cytokine-stimulated cartilage, the release of intact COMP is detected at days 3 and 6 with both N- and C-terminal antibodies as double bands above 250 kDa (Fig. 4, A and B). At day 9, N-terminal fragments are seen at ~300, 200, 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 high molecular weight 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 high molecular weight bands are seen at days 3–9 with highest abundance at day 9 and then decrease. At day 9, a C terminus-containing fragment is detected at 70 kDa that increases until day 15 and then starts to decrease. At day 12, two C terminus-containing bands appear at 300 and 200 kDa that remain until day 21.

Using the neoepitope antibody Ser77, two fragments at 200 and 100 kDa are clearly visible from day 12 to day 21 (Fig. 4C). The size of the fragments indicates that the COMP pentamer has been cleaved at position Ser77 in three of five monomers for the 200-kDa band and in four of 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 days 12 and 15 are depicted in Fig. 4D with a schematic image representing the cleavages within the COMP pentamer. The glycosaminoglycan release was also measured using the dimethyl methylene blue assay, which showed that ~30–50% was released before the demonstrated release of the Ser77 epitope.

Structural Effects on the COMP Pentamer Caused by Cleavage at the Neoepitope Sites—Six of the identified neoepitopes (Ser77, 91Cys, 105Thr, 199Ser, Glh202, and Glh226) are located between the N-terminal and the thrombospondin type III domains. These neoepitopes could result in fragmentations of COMP that disrupt its pentamer organization. The other six identified neoepitopes (523Asn, Phe531, 553Asn, Tyr574, Phe577, and 638Gly) are located within or in close proximity...
to the C-terminal globular domain of COMP. Cleavages at the neoepitope sites could interfere with several of the interactions between COMP and other proteins because these interactions mainly occur with the C-terminal globular domain (19) (Fig. 5). The disulfide bridge between Cys520 and Cys741 in the C-terminal domain of COMP complicates the involvement of cleavages within the C-terminal domain because cleavages can occur between these two amino acids without affecting the migration properties of the COMP molecule as analyzed by non-reducing SDS-PAGE. However, the advantage of analyzing released COMP fragments under non-reducing conditions is that it gives 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. We describe the presence of 12 novel neoepitopes within these COMP fragments.

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...
Identification of COMP Neoepitopes

FIGURE 5. Schematic presentation of the identified neoepitopes in COMP. Shown is a 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 Cys542 and Cys593 within the C-terminal domain is marked. Boxes showing the neoepitope amino acid and arrows indicating locations are marked. Neoepitopes identified in AT are boxed and marked with dotted lines, those in OA are marked with broken lines, those in RA are marked with lines, and that identified in both OA and RA is marked with a thick box. The ending amino acids of the neoepitopes are superscripted; Ser77 represents the newly formed C terminus, whereas Cys91 represents a newly formed N terminus.

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 toward one of these neoepitopes, Ser77. This neoepitope is unique as compared with both other human thrombospondins and COMP from different species, making it suitable as a biomarker specific to human cartilage degradation.

The highest amounts of both total COMP and neoepitope Ser77 were found in acute trauma synovial fluids followed by OA and finally RA. The increased variability (coefficient of variation) 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 Ser77 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 with trauma-stimulated cartilage explants.4 These results indicate that the protease causing the neoepitope Ser77 cleavage is activated or up-regulated by the presence of the cytokines TNF-α and IL-6/sIL-6R. This suggests the possibility of selective inhibition trials to prevent the cleavage 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 Ser77 demonstrated in the cartilage explant studies (Fig. 4), it is most likely that degradation of COMP into fragments is initiated within the cartilage tissue, and then release into the medium occurs.

Our data clearly show that COMP is released both as intact pentamer and fragments 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 Ser77 represents a strong candidate because it clearly is distinguishable from intact COMP.

A eukaryotic expression system used for purification of pentameric recombinant COMP has previously shown the presence of a 100-kDa fragment starting at amino acid Val79Val. 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 Ser77 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 anterior cruciate ligament 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-1α stimulation of bovine nasal cartilage shows release of COMP fragments of 110 kDa (38). Although 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 disease propagation are 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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