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# SMALL FRAGMENTS OF CARTILAGE OLIGOMERIC MATRIX PROTEIN IN SYNOVIAL FLUID AND SERUM AS MARKERS FOR CARTILAGE DEGRADATION

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SUMMARY

We determined the tissue distribution of cartilage oligomeric matrix protein (COMP) in man and evaluated COMP in synovial fluid (SF) and serum. COMP was purified from human articular cartilage. Polyclonal antibodies were used to detect COMP in tissue cryosections and protein extracts. COMP was determined quantitatively and qualitatively in SF and serum by competitive enzyme-linked immunosorbent assay and immunoblotting. Knee joint SF was taken from nine cadaveric and six living controls, 52 patients with osteoarthritis (OA), 85 patients with rheumatoid arthritis (RA) and 60 patients with other forms of inflammatory arthritis. The degradative potential of SF on native COMP was tested *in vitro*. The highest concentrations of COMP were measured in articular cartilage and meniscus, the lowest in rib and trachea. Compared with controls, the concentrations of COMP in SF and serum were elevated in 36 and 50% of the patients. A total of 84% of patients with RA and 60% of patients with other forms of inflammatory arthritis showed significant amounts of low-molecular-weight COMP fragments (50–70 kDa) in SF. In contrast, SF fragments were present in only 21% of the OA patients. Furthermore, 13% of SF taken from patients with RA or other forms of inflammatory arthritis were able to degrade COMP *in vitro*. Using inhibitors, the involvement of serine proteinases could be demonstrated in only 8% of the cases. Based on these results, the absolute levels of COMP in SF and serum, and its fragmentation pattern in SF, seem to be promising as markers of joint tissue metabolism.

KEY WORDS: Cartilage oligomeric matrix protein (COMP), Osteoarthritis, Rheumatoid arthritis, Proteinases.

THE various components of articular cartilage extracellular matrix interact intensively with each other and with the chondrocytes. One of these macromolecules is the non-collagenous glycoprotein cartilage oligomeric matrix protein (COMP) [1–3].

COMP, as revealed by electron microscopy of rotary shadowed and negatively stained particles, consists of five arms containing a peripheral globular domain, a flexible strand and a central assembly domain where the five arms are connected in a cylindrical structure [1, 4]. COMP belongs to the thrombospondin family [4, 5] and has been isolated from cartilage [1, 2, 6], but is also present in much lower amounts in other tissues (e.g. bovine tendon [7]). Immunohistochemistry of sections of adult human articular cartilage showed the distribution of COMP to be localized to the interterritorial matrix, with increased staining in the deeper zones [8]. The physiological role of COMP is still unclear; however, evidence exists indicating that COMP interacts with chondrocytes [4, 8]. During limb development in mice, COMP is detected at a specific stage of endochondral ossification, i.e. after the appearance of type II collagen [3].

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In human joint fluid, increased amounts of COMP have been reported after knee injury, in early-stage osteoarthritis (OA) [9] and in active reactive arthritis [10]. In contrast, in patients with advanced destruction of the joints due to rheumatoid arthritis (RA) or reactive arthritis, COMP is decreased [11]. Similarly, in serum, increased levels of COMP occur in early stages of OA [12], RA [13] and in reactive arthritis [10]. RA patients with rapidly progressive joint destruction show initially elevated levels of COMP that subsequently decrease along with less active disease [13]. RA patients with benign courses of their disease have normal serum levels of COMP [11, 13].

It is uncertain whether the distribution of COMP in different cartilages is similar in the human and bovine systems. We report here that COMP has a broad distribution in human connective tissues. In previous studies [9–13], COMP was evaluated as a marker for cartilage degradation in human joint diseases. Purified COMP from bovine articular cartilage was employed in the assays and/or used in order to obtain polyclonal antibodies. In the present paper, we report results using isolated COMP from human articular cartilage and polyclonal antibodies to human COMP. Using these reagents, we quantified COMP in human tissues and evaluated COMP as a marker in OA, RA and other forms of arthritis. In addition, we report novel findings of different COMP fragment patterns in synovial fluids of these patients.

## PATIENTS AND METHODS

*Patients and controls*

Knee joint synovial fluid was taken from nine deceased individuals at the department of forensic medicine, with institutional approval, within 20 h after death; they had no history or macroscopic signs of joint disease (three women/six men, $33 \pm 11$ yr old). In addition, synovial fluids were obtained from six living healthy controls, 52 patients with OA, 85 with RA and 60 with other forms of inflammatory arthritis [29 unclassified mono- or polyarthritis, 17 reactive arthritis, seven arthritis with ankylosing spondylitis, four psoriatic arthritis, two arthritis with Reiter's syndrome and one juvenile chronic arthritis], 37% of them being HLA-B27 positive [determined by sHLA STAT®B27 enzyme-linked immunosorbent assay (ELISA), SangStat Med. Corp., see ref. 14 for details]. All OA and RA patients fulfilled the respective ACR criteria [15, 16]. Mean age was $39 \pm 9$ yr in living controls (one woman/five men), $63 \pm 9$ yr in OA (27 women/25 men), $46 \pm 13$ yr in RA (49 women/36 men) and $39 \pm 11$ yr in other forms of arthritis (19 women/41 men).

Three types of synovial fluids were analysed: (1) whole samples; (2) cell-free samples (centrifuged at 400 *g* for 30 min); (3) cell-free samples with additional ultracentrifugation (29 000 *g*, 5 min, 22–24°C). These fluids were kept at –80°C until pre-treatment with hyaluronidase. At room temperature, 10 $\mu$l of hyaluronidase (100 U in 0.05 M sodium acetate buffer, pH 5.8) from bovine testes (Sigma) were added to 100 $\mu$l of synovial fluid. After incubation at 37°C for 2 h, the synovial fluids were stored at –20°C until examination. In order to exclude the possibility that this pre-treatment affects the COMP pattern obtained by SDS–PAGE and immunoblotting, samples were treated in the following manner and compared with each other: using glass or plastic tubes, in the presence or absence of heparin, with or without centrifugation, and finally digested with or without hyaluronidase. The fresh (cell-free) samples were used for the *in vitro* tests mentioned below.

Serum was obtained from 35 healthy subjects (18 women/17 men, $42 \pm 11$ yr old), 16 of the 52 OA, 41 of the 85 RA and 20 of the 60 other arthritic patients (nine reactive arthritis, eight unclassified mono- or polyarthritis and three psoriatic arthritis).

*Purification of matrix proteins*

Native human COMP was purified from articular cartilage according to DiCesare *et al.* [6]. In summary, COMP was purified from knee joint articular cartilage of three individuals (21- to 52-yr-old men) by extraction with ethylenediaminetetraacetic acid disodium salt (EDTA, Fluka)-containing buffer, affinity chromatography on wheat germ agglutinin (Sigma) coupled to CNBr-activated Sepharose (Pharmacia), gel filtration on Sepharose CL4B (Pharmacia) and removal of thrombospondin-1 by affinity chromatography on heparin coupled to Sepharose CL6B (Pharmacia).

*Antibodies to human COMP*

Polyclonal antibodies against human COMP were raised in rabbits. Antibody specificity was tested by immunoblot. They were used to detect proteins in tissue sections by immunohistochemistry, as well as in hyaluronidase-treated synovial fluid and in serum by competitive ELISA and by qualitative immunoblotting.

*Immunohistochemistry*

Tissues were obtained within 20 h after death from a 45-yr-old male individual: normal articular cartilage was harvested from the femoral and tibial plateau, cartilage from the trachea and rib, annulus fibrosus, nucleus pulposus, meniscus and cruciate ligament. Samples were frozen instantaneously on dry ice in Tissue-Tek® (Miles Inc.). Sections (5 $\mu$m) were cut on a cryostat and mounted on gelatin-coated slides and air dried. To increase antibody penetration, sections were digested for 1 h with 40 mU/ml chondroitinase ABC (ICN Immunobiological) in TBS [50 mM Tris–HCl, 150 mM NaCl (pH 7.4), Fluka] containing 0.01% (w/v) bovine serum albumin (BSA, Sigma). Endogenous peroxidase was blocked by incubation in methanol containing 1% (v/v) $H_2O_2$ and non-specific antibody binding was reduced by treatment with 1% (w/v) BSA in TBS. Sections were treated with the anti-COMP antiserum or non-immune rabbit serum (as control) for 1 h, followed by horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (Dakopatts) for 45 min. All antibodies were diluted in TBS containing 1% BSA. Slides were developed with 0.025% (w/v) 3-amino-9-ethylcarbazole (Sigma) and 0.02% (v/v) $H_2O_2$.

*Analytical extraction of human tissues*

Tissues were extracted with 4 M guanidine–HCl (Sigma) in 50 mM sodium phosphate buffer (pH 7.4) (10 ml/g wet tissue) containing proteinase inhibitors [10 mM EDTA, 2 mM phenylmethylsulphonyl fluoride (PMSF, Sigma) and 2 mM *N*-ethylmaleimide (NEM, Fluka)], by a sequence of homogenization (Polytron homogenizer), extraction (16 h at 4°C) and centrifugation (17 000 *g*, 30 min at 4°C).

*Competitive ELISA*

Determination of COMP was carried out on polystyrene microtitre plates (Nunc). The plates were coated with 1 $\mu$g/ml human COMP diluted in TBS, kept overnight at 4°C and blocked with 1% (w/v) BSA in TBS for 2 h at 22–24°C. On a separate plate, the tissue extracts or biological fluids were diluted in TBS (1:50 to 1:400 for extracts; 1:10 to 1:40 for serum; 1:80 to 1:320 for synovial fluid). The samples and standards (purified human COMP) were preincubated with an equal volume of specific antiserum (diluted 1:300 in TBS with 1% BSA) overnight at 4°C. The solutions were transferred to the coated plates and incubated for 1 h at 22–24°C. The plates were washed with TBS containing 0.05% (v/v) Tween-20 (Merck) to remove the unbound antibody/antigen complexes. The bound anti-COMP antibodies

were incubated with HRP-conjugated anti-rabbit IgG, diluted 1:200 in TBS with 1% BSA, for 1 h at 22–24°C. The wells were washed and bound antibodies visualized by using 0.25 mg/ml 5-amino-2-hydroxybenzoic acid (Sigma) (pH 6.0) as substrate, in the presence of 0.00024% (v/v) $H_2O_2$. The reaction was stopped with 2 N NaOH and the absorbance was read at 492 nm. For each of the three dilutions, the COMP concentration was derived from the linear range of a standard curve [i.e. $\mu$g/ml COMP on a log scale (*x*-axis) *vs* absorbance (*y*-axis)], using the following equation:

$$\text{concentration} = 10^{(\text{absorbance}-b)/a}$$

where $a$ is the slope of the curve and $b$ is the intersection with the *y*-axis. The concentration (in $\mu$g/ml) was multiplied by the dilution factor. The average of the values obtained from three dilutions was only considered if the coefficient of variation was $< 20\%$.

*Immunoblotting*

Fifty microlitres of tissue extracts (after precipitation of protein with ethanol), 50 $\mu$l of serum or 10 $\mu$l of synovial fluid (whole or cell free), as well as molar mass standards, were diluted in sample buffer (including 10 mM EDTA, 2 mM PMSF and 2 mM NEM) and applied to 4–15% gradient polyacrylamide gels (Protogel, National Diagnostics), under unreduced or reduced conditions (2.5% v/v 2-mercaptoethanol, Sigma), and run according to Laemmli [17]. The samples were electrophoretically transferred to nitrocellulose (Schleicher & Schuell) according to Towbin *et al*. [18], and antigens revealed using the antiserum of interest and by enhanced chemiluminescence (ECL, Amersham). The relative intensity of bands was determined (ONE-Dscan, Scanalytics) from scans using video images of the immunoblots [Fuji HC1000 video camera ($5 \times 10^6$ d.p.i.) and Access Image]. The synovial fluids of six healthy and nine cadaveric individuals, 46 of the 50 OA, 77 of the 87 RA and 40 of the 51 other arthritic patients were analysed.

*Incubation of purified human COMP with synovial fluids*

After ethanol precipitation, purified COMP was resuspended in 25 mM Tris–HCl (pH 7.5) (Fluka) (100 $\mu$g/ml). Ten microlitres of cell-free synovial fluid taken from 37 patients with RA or other inflammatory arthritides were diluted 1:10 in Tris–HCl. Five microlitres of each sample were incubated with either 5 $\mu$l of 25 mM Tris–HCl or 5 $\mu$l of 100 $\mu$g/ml purified COMP, and 10 $\mu$l of 25 mM Tris–HCl (or 10 $\mu$l of protease inhibitors, see below) for 4 h at 37°C, in the presence of 2 mM each of $CaCl_2$ and $MgCl_2$ (Fluka) (except when EDTA was used as inhibitor). Twenty microlitres of sample buffer were added and 10 $\mu$l of the mixture were applied to a 4–15% gradient polyacrylamide gel. Fresh synovial fluids were compared with those stored at –80°C. We tested the effects of the following fresh prepared proteinase inhibitors: 10 mM EDTA, 2 mM NEM (Fluka), 10 mM PMSF, 1 mM iodoacetamide, 2 mM 3,4-dichloroisocoumarin and 10 mM 1,10-phenanthroline (Sigma). The immunoblotting was performed and analysed as described above.

*Statistics*

All available subjects were studied and thus many important covariates (e.g. age and sex) may not be balanced between the groups of individuals. Means $\pm$ S.D. and medians are shown. The Kruskal–Wallis test, Mann–Whitney *U*-test (using Bonferroni corrections), $\chi^2$ test or paired Student's *t*-test were employed as appropriate. Pearson's linear correlation was used. The level of significance is indicated in the text.

## RESULTS

*Distribution of COMP*

The immunohistochemical staining revealed a broad connective tissue distribution of COMP (representative example shown in Table I). High amounts of COMP were detected in meniscus and in articular cartilage. A homogeneous staining was obtained throughout these tissues. The weakest stainings were obtained in rib and trachea.

TABLE I
Distribution of COMP in various tissues taken from a 45-yr-old man*

| Tissues | Immunohistochemistry COMP | Immunoblots COMP | Quantification of COMP by ELISA |
|---|---|---|---|
| Meniscus | + + + | + + + | 3.47 mg/g (wet weight) |
| Tibial cartilage | + + + | + + + | 3.40 |
| Femoral cartilage | + + + | + + + | 2.17 |
| Annulus fibrosus | + + | + + | 1.62 |
| Nucleus pulposus | + + | + + | 1.61 |
| Cruciate ligament | + + | + | 0.93 |
| Rib | + | ± | 0.26 |
| Trachea | + | – | 0.04 |

Staining: + + +, strong; + +, intermediate; +, low; ±, trace; –, negative.
*The distribution of COMP in the examined human tissues was studied qualitatively by immunohistochemistry of tissue sections as well as by immunoblots of crude extracts. Human tissues were extracted with 4 M guanidine–HCl and the concentration of COMP determined by competitive ELISA.

The immunoblots of the extracts showed the specific COMP bands with a gradient in intensity corresponding to the staining obtained by immunohistochemistry: large amounts could be detected in meniscus and articular cartilage, intermediary levels in annulus fibrosus, nucleus pulposus and knee joint ligament, but undetectable amounts in rib and trachea (Table I).

Analysis of the tissue extracts by ELISA confirmed the results quantitatively. The maximal amounts of COMP in the extracts were 3.5 and 3.4 mg/g tissue (wet weight) found in meniscus and articular cartilage of the tibial plateau (Table I). The lowest tissue concentrations of COMP were found in rib and tracheal extracts with 0.26 and 0.04 mg/g, respectively, showing a 100-fold difference in amounts of COMP between the different cartilages.

*COMP in human biological fluids*

The ELISA methods employing either bovine or human COMP showed different results (the Pearson's correlation coefficient reached 0.64 only). In our hands, the use of bovine material (i.e. bovine COMP for immunization, coating and standard curves) leads to an overestimation (of ~30%) of the amount of COMP in human biological fluids. In the present study, we used purified human COMP and raised polyclonal antibodies against this antigen in rabbits. Figure 1a shows the inhibition of the antibody/antigen interaction after pre-incubation of the antiserum with purified human COMP, synovial fluid and serum. The logarithmic transformation of the *x*-axis allows a linear relationship to be obtained with the absorbance between 0.05 and 2.5 $\mu$g/ml COMP ($r = 0.98$). The logarithmic transformation of both COMP concentration and absorbance allows a linear relationship to be obtained between 0.05 and 20 $\mu$g/ml ($r = 0.97$). The slopes obtained for synovial fluid and serum were similar to those shown by the standard curve (this was not the case when bovine COMP was employed). This indicates that the intact COMP standard and the samples (containing COMP fragments) react similarly with the antibodies. Control experiments showed that neither cross-reactivity of the polyclonal antibodies with recombinant human thrombospondin-1 (Fig. 1b) nor interference of rheumatoid factors (data not shown) constitute major problems.

*Synovial fluid levels of COMP*

Pre-treatment of synovial fluids with hyaluronidase, which increased homogeneity, was essential in order to prevent an overestimation of >100% of the level measured by ELISA. However, once the synovial fluid had been hyaluronidase treated, no significant difference was found between the whole, cell-free and ultracentrifuged samples.

Compared with living healthy controls, significantly elevated synovial fluid levels of COMP were found in OA (Table II, Fig. 2). Cadaveric individuals also showed higher levels compared with living

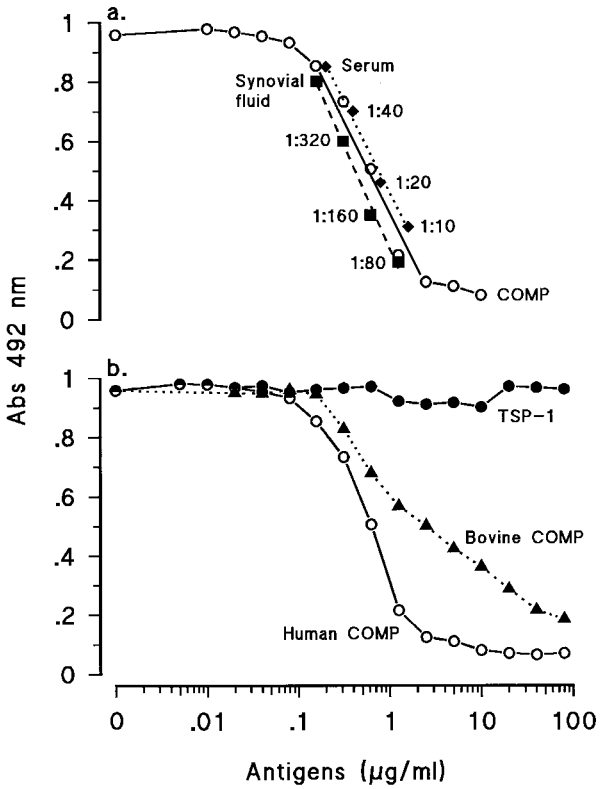


FIG. 1.—Competitive COMP-ELISA using purified human articular COMP and antiserum against this antigen (a). Representative examples of inhibition with purified human COMP (○), as well as with hyaluronidase-treated synovial fluid (■, 108 $\mu$g/ml COMP) and serum (◆, 8.5 $\mu$g/ml COMP) taken from a patient with rheumatoid arthritis. The results obtained with the usual dilutions of synovial fluid (1:80, 1:160, 1:320) or serum (1:10, 1:20, 1:40) are indicated. Cross-reactivity is observed with bovine COMP (▲), but not with human thrombospondin-1 (●, TSP-1) (b).

controls. A great inter-individual variability was observed (coefficients of variation: 30% in controls and 56–66% in patients). High amounts of COMP (i.e. > 50 $\mu$g/ml, the maximum value in living controls) were measured in 36% of the patients. In the group with other forms of inflammatory arthritides, high levels of COMP were mainly found in the synovial fluids of HLA-B27 negative patients [HLA-B27 negative ($n = 38$), $68 \pm 45$ $\mu$g/ml; HLA-B27 positive ($n = 22$), $42 \pm 20$ $\mu$g/ml ($P < 0.05$)].

*Serum levels of COMP*

In patients, the COMP levels in serum were only $14 \pm 6\%$ of the amount determined in synovial fluid (Table II). Nevertheless, the serum and synovial fluid levels of COMP correlate with each other: $r = 0.52$ for all patients ($P < 0.01$), between 0.84 in OA ($P < 0.01$) and 0.50 in RA ($P < 0.05$).

Compared with healthy controls, the serum levels of COMP are increased in all three patient groups (Table II). High levels of COMP (i.e. >5 $\mu$g/ml, the maximum level in controls) were found in 47% of OA, 57% of RA and 47% of patients with other inflammatory arthritis.

TABLE II
Levels of COMP in synovial fluid and serum (means ± S.D. [median]) in controls and patients with osteoarthritis, rheumatoid arthritis or other forms of inflammatory arthritides*

| | (*n*) | Synovial fluid COMP (μg/ml) | (*n*) | Serum COMP (μg/ml) | Ratio: serum/ synovial fluid |
|---|---|---|---|---|---|
| Living controls | (6) | 33 ± 10 [24] | (35) | 1.7 ± 1.4 [0.9] | ≅0.07 |
| Cadaveric controls | (9) | 53 ± 17 [47]† | | – | – |
| Osteoarthritis | (52) | 52 ± 29 [45]† | (16) | 5.7 ± 3.2 [4.6]† | 0.12 ± 0.04 [0.12] |
| Rheumatoid arthritis | (85) | 48 ± 32 [35] | (41) | 7.2 ± 3.7 [4.2]‡ | 0.16 ± 0.06 [0.16] |
| Other arthritides | (60) | 59 ± 39 [42] | (20) | 4.3 ± 2.0 [4.2]† | 0.14 ± 0.07 [0.15] |

*No difference found between men and women. However, significant differences were found between HLA-B27 positive and HLA-B27 negative (see Results).
Significant differences compared with living controls (Mann–Whitney *U*-test): †$P < 0.05$; ‡$P < 0.01$.

*Immunoreactive COMP fragments in synovial fluids*

The electrophoretic pattern of COMP fragments was compared for the different patient groups using the Kruskal–Wallis test with a significant level of $\alpha = 0.05$, followed by pair comparisons employing the Mann–Whitney *U*-test which, according to the Bonferroni correction, was considered at a significant level of $\alpha < 0.0083$ (Table III). Figure 3 shows the heterogeneity in molecular mass of immunoreactive COMP molecules: under non-reducing conditions, 1–4% of intact pentamers (450–500 kDa), 33–40% of oligomeric reduction-sensitive fragments (150–450 kDa), 12–24% of monomer-like molecules (90–150 kDa, including the α band) and 32–44% of monomeric non-reduction-sensitive fragments (50–90 kDa, including the β band and smaller fragments) were detected, on average, in controls and patients. Between the groups of subjects, these proportions were not significantly different.

Under non-reducing conditions, the major immunoreactive COMP molecules among the 50–150 kDa monomers and fragments migrated between 70 and 100 kDa (Table IIIa, Fig. 3a). On average, they constitute 89 and 81% of the molecules in controls and OA. The shape of the band suggests at least two groups of molecules: the 90–100 kDa proteins were designated α bands and the 70–90 kDa proteins were called β fragments. In general, the β fragments represented the major group (60 and 50% in controls and OA, respectively). In addition, one or two bands of low-molecular-weight fragments (50–70 kDa) can be detected.

Under reducing conditions, two bands between 70 and 100 kDa constitute 91 and 75% of the immunoreactive COMP molecules in controls and OA (Table IIIb, Fig. 3b). These two major bands (also designated α and β) resemble those obtained with purified human COMP and probably constitute intact and/or partially degraded monomers. In addition, three groups of well-defined small fragments (γ, δ and ε, 50–70 kDa) can be detected. The cadaveric individuals showed more small fragments than the living controls [e.g. under reducing conditions: cadavers ($n = 9$), total 11 ± 4%; living

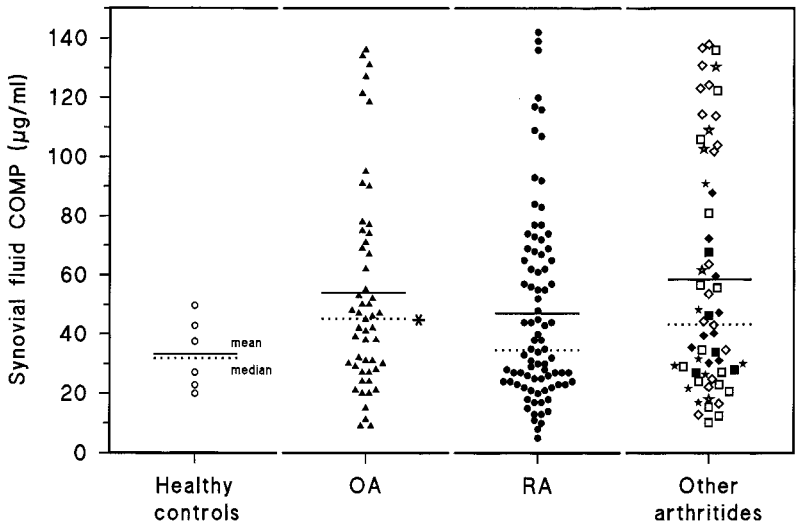


FIG. 2.—Synovial fluid levels (means and medians) of COMP in healthy controls, osteoarthritis (OA), rheumatoid arthritis (RA) and other forms of inflammatory arthritis (◆, unclassified arthritis; ■, reactive arthritis; ★, others; closed and open symbols: HLA-B27 positive and negative, respectively). The levels are significantly elevated in OA (*$P < 0.05$ compared with controls); 39% of the patients have synovial fluid levels of COMP > 50 μg/ml.

TABLE III

Relative proportions among 50–150 kDa COMP monomer and fragments in synovial fluids (mean ± S.D. [median]) taken from controls, patients with osteoarthritis (OA), rheumatoid arthritis (RA) and other forms of inflammatory arthritis*

| Molecular weight (kDa) | Living controls ($n$ = 6) | Cadaveric controls** (9) | OA (52) | RA (85) | Other arthritides (60) |
|---|---|---|---|---|---|
| (a) Non-reducing conditions | | | | | |
| 100–150 (smear) | 5 ± 2% [5] | 8 ± 3 [8]‡ | 13 ± 8% [9] | 13 ± 6% [9]† | 13 ± 8% [9]† |
| 90–100 (usual α band) | 20 ± 5 [20] | 35 ± 3 [37]‡ | 31 ± 6 [32]‡ | 27 ± 7 [27]† | 28 ± 6 [28] |
| 80–90 (usual fragments β) | 73 ± 4 [74] | 52 ± 7 [55]‡ | 50 ± 11 [51]†‡ | 38 ± 13 [35]† | 45 ± 13 [43]† |
| 50–70 (low MW fragments) | 1 ± 1 [1] | 4 ± 2 [4]‡ | 6 ± 6 [4]‡ | 22 ± 14 [20]† | 14 ± 10 [12]†‡ |
| (b) Reducing conditions | | | | | |
| 100–150 (smear) | 0 ± 0 [0] | 1 ± 1 [1]‡ | 3 ± 2 [3] | 3 ± 3 [2]† | 4 ± 4 [1] |
| 90–100 (usual α band) | 53 ± 3 [53] | 46 ± 5 [48]‡ | 28 ± 13 [34]‡ | 21 ± 12 [24]† | 24 ± 12 [26]† |
| 70–90 (usual fragments β) | 46 ± 2 [46] | 41 ± 2 [40] | 47 ± 13 [46] | 39 ± 13 [40] | 36 ± 13 [36] |
| 60–70 (small fragments γ) | 1 ± 1 [1] | 7 ± 3 [8]‡ | 12 ± 10 [10] | 17 ± 12 [17]† | 18 ± 14 [16]† |
| 50–60 (small fragments δ) | 0 ± 0 [0] | 2 ± 1 [2]‡ | 5 ± 3 [5]‡ | 10 ± 6 [9]† | 10 ± 8 [8]† |
| < 50 (small fragments ε) | 1 ± 1 [1] | 2 ± 2 [2]‡ | 4 ± 3 [3]‡ | 9 ± 13 [5]† | 8 ± 9 [5]† |

MW, molecular weight.

*No difference found between men and women. However, significant differences were found between HLA-B27 positive and HLA-B27 negative (see Results).

**For statistical comparison, the living and cadaveric controls are combined ($n$ = 15).

Significant differences (Kruskal–Wallis test, followed by Mann–Whitney $U$-test with Bonferroni correction): †$P < 0.0083$ compared with controls; ‡$P < 0.0083$ compared with RA.

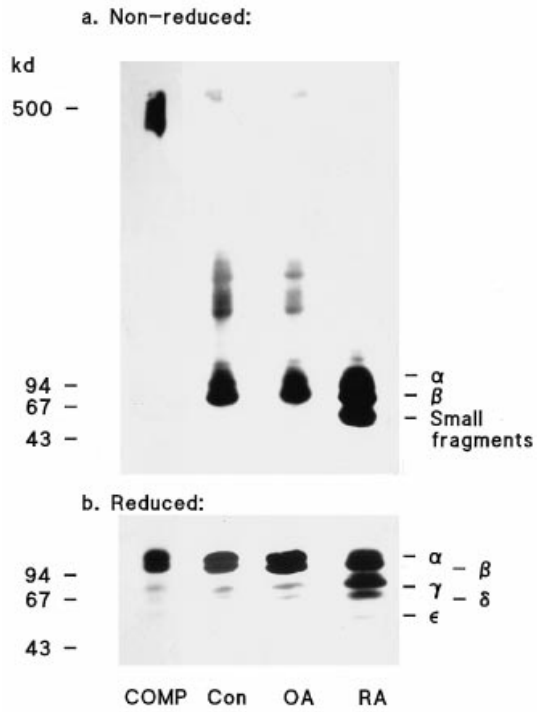


FIG. 3.—Representative immunoblots of hyaluronidase-treated synovial fluid COMP in a healthy control, osteoarthritis (OA) and rheumatoid arthritis (RA). The migration positions of purified human COMP are indicated on the left. Under non-reducing conditions (a), pentamer, oligomers, as well as α and β bands are usually obtained. In 82% of patients with RA, additional low-molecular-weight fragments are detected. In 4% of patients with RA, an extensive degradation occurs (shown on the right). Under reducing conditions (b), α and β bands are usually found. In RA, the disappearance of the α band is associated with the generation of small γ, δ, ε COMP fragments.

controls ($n$ = 6), 1.1 ± 1.0% ($P < 0.05$)] (Table III). Nevertheless, combination of the two groups showed significant differences to the patient groups.

*Immunoreactive COMP fragments in serum*

Figure 4 shows that small fragments (50–90 kDa), but no α band, can be found in serum.

*Disappearance of the large α bands (90–100 kDa) in synovial fluid from patients*

Under non-reducing conditions, in RA synovial fluid (but not in OA), the proportion of the large α band is significantly decreased (Table IIIa). Under reducing conditions, all groups of patients show a significantly reduced relative amount of the α band (Table IIIb). Table IV shows the decreased proportions of the α bands in all groups of patients, if compared with controls (under reducing conditions only).

Under reducing conditions, elevated amounts of the β fragments were detected in 39% of OA patients

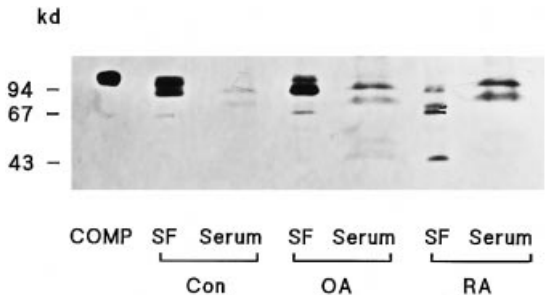


FIG. 4.—Representative immunoblots of synovial fluid (SF) and serum COMP in healthy controls, patients with osteoarthritis (OA) and rheumatoid arthritis (RA), under reducing conditions. Lane 1: purified human COMP (5 μl of 200 μg/ml); 2: normal human SF (Con) (5 μl); 3: control serum (25 μl); 4: OA SF; 5: OA serum; 6: RA SF; 7: RA serum. In serum, mostly small fragments (50–90 kDa), but no α band, can be detected.

TABLE IV
Occurrence (%) of altered COMP patterns in osteoarthritis (OA), rheumatoid arthritis (RA) and other inflammatory arthritides

| | | Incidence (%) | | | | |
|---|---|---|---|---|---|---|
| | Criterion | Living controls ($n=6$) | Cadaveric controls (9)* | OA (52) | RA (85) | Other arthritides (60) |
| (a) Non-reducing conditions | | | | | | |
| Decreased $\alpha$ band | $<11$** | 17 | 0 | 0 | 3 | 0 |
| Increased $\beta$ fragment | $>78$*** | 0 | 0 | 0 | 0 | 0 |
| Low MW fragments | $>9$** | 0 | 11‡ | 21‡ | 84† | 60† |
| (b) Reducing conditions | | | | | | |
| Decreased $\alpha$ band | $<36$** | 0 | 11‡ | 67†‡ | 88† | 85† |
| Increased $\beta$ fragment | $>50$*** | 0 | 0 | 38†‡ | 18 | 12 |
| Increased $\gamma$ fragment | $>10$** | 0 | 0‡ | 42†‡ | 65† | 67† |
| Increased $\delta$ fragment | $>4$** | 0 | 11‡ | 48†‡ | 78† | 72† |
| Increased $\varepsilon$ fragment | $>5$** | 0 | 11‡ | 17‡ | 35† | 47† |

*For statistical comparison, the living and cadaveric controls are combined ($n = 15$).
**Among the 50–150 kDa COMP molecules: mean + 2 S.D. of controls.
***Among the 50–150 kDa COMP molecules: maximum value in controls (if lower than the mean plus 2 S.D.).
Significant differences ($\chi^2$ test, with Bonferroni correction): †$P < 0.0083$ with controls; ‡$P < 0.0083$ with RA.

(Table IVb). Only in OA did the increase in the $\beta$ fragments accompany the disappearance of the large $\alpha$ bands ($r = -0.50$ under non-reducing conditions, $r = -0.61$ under reducing conditions, $P < 0.001$ for both).

*Low-molecular-weight COMP fragments in synovial fluid of inflammatory arthritides*

In contrast to OA, under non-reducing conditions, the relative proportions of low-molecular-weight fragments (among the 50–150 kDa COMP molecules) were significantly increased in RA and other inflammatory arthritides (Table IIIa, Figs 3a and 5). Accordingly, a high incidence of patients with large amounts of low-molecular-weight COMP fragments was detected in RA and other forms of inflammatory arthritis (Table IVa).

*Small $\gamma$, $\delta$ and $\varepsilon$ COMP fragments in synovial fluid of inflammatory arthritides*

Under reducing conditions, the proportions of $\gamma$, $\delta$ and $\varepsilon$ fragments were increased in patients with RA and other forms of inflammatory arthritis, but much less in OA (Table IIIb, Fig. 3b). The occurrence of elevated levels of the 60–70 kDa $\gamma$ fragments was 65% in RA and 67% in patients with other forms of inflammatory arthritides, but only 43% in OA (Table IVb). Furthermore, increased proportions of the 55–60 kDa $\delta$ fragments were found in the great majority (72–78%) of patients with RA and other

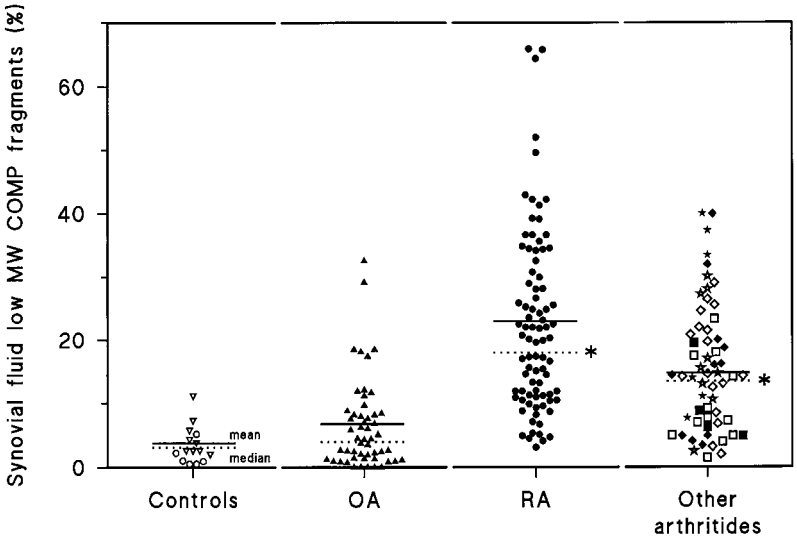


FIG. 5.—Synovial fluid levels of low-molecular-weight (MW) COMP fragments (% of 50–70 kDa among 50–150 kDa COMP molecules) under non-reducing conditions as determined by densitometry, in living (○) and cadaveric (▽) controls, osteoarthritis (OA), rheumatoid arthritis (RA) and other forms of inflammatory arthritis (◆, unclassified arthritis; ■, reactive arthritis; ★, others; closed and open symbols: HLA-B27 positive and negative, respectively). The levels of low-molecular-weight fragments are elevated in RA and other inflammatory arthritides, but not in OA (*$P < 0.01$ compared with controls or OA).

inflammatory arthritides, but only in 48% of patients with OA. Similarly, elevated levels of the smallest $\varepsilon$ fragments were detected in patients with RA and other inflammatory arthritides (35–47%), but much less in OA patients (17%).

Subgroups of patients could be identified on the basis of genetic markers. For example, among the cases with various inflammatory arthritides, the degradation process was more pronounced in HLA-B27-positive than in HLA-B27-negative patients [e.g. HLA-B27 positive ($n = 22$): $\gamma$ $20 \pm 10\%$, $\delta$ $12 \pm 7\%$, $\varepsilon$ $10 \pm 7\%$, total $42 \pm 18\%$; HLA-B27 negative ($n = 38$): $\gamma$ $15 \pm 9\%$, $\delta$ $8 \pm 4\%$, $\varepsilon$ $5 \pm 2\%$, total $28 \pm 12\%$ ($P < 0.05$)].

Under non-reducing conditions, in RA (but not in OA and in other forms of inflammatory arthritides), the decrease of $\alpha$ bands was associated with the appearance of low-molecular-weight fragments ($r = -0.57$, $P < 0.001$).

Under reducing conditions, in RA and other inflammatory arthritides (but not in OA), highly significant correlations were obtained between the decreased proportion of $\alpha$ bands and the increased total level of $\gamma$, $\delta$ and $\gamma$ fragments ($r = -0.84$ and $-0.78$, respectively, $P < 0.001$ for both).

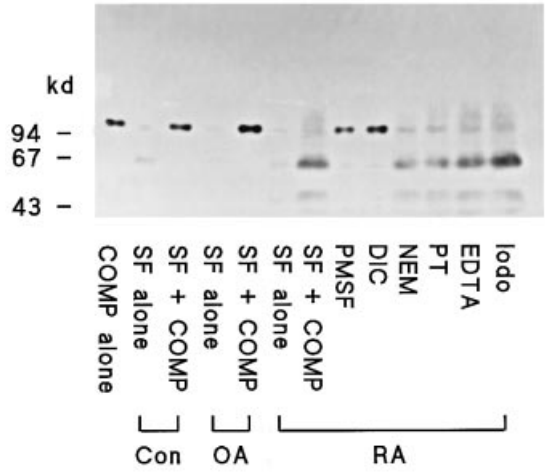


FIG. 6.—Immunoblots showing the degradative potential of cell-free synovial fluids (SF) on native COMP *in vitro*. Lane 1: purified human COMP (5 $\mu$l of 50 $\mu$g/ml); 2: normal human SF (Con) (5 $\mu$l of a 1:10 dilution); 3: purified COMP plus normal SF (5 $\mu$l of 50 $\mu$g/ml COMP and 5 $\mu$l of diluted sample); 4: osteoarthritis (OA) SF; 5: purified COMP plus OA SF; 6: rheumatoid arthritis (RA) SF; 7: purified COMP plus RA SF; 8–13: the latter mix in the presence of 10 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM 3,4-dichloroisocoumarin (DIC), 2 mM *N*-ethylmaleimide (NEM), 10 mM 1,10-phenanthroline (PT), 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA) or 1 mM iodoacetamide (Iodo). The solutions were incubated for 4 h at 37°C. The immunoblot was run under reducing conditions. The diluted SF showed only a weak background of endogenous COMP. Co-incubation of exogenous COMP with SF taken from patients with RA or other inflammatory arthritides produces a detectable degradation in 13% of the cases only. For the present RA SF, the degradation process is blocked by PMSF or DIC (lanes 8 and 9), suggesting an action of serine proteinases.

A complex relationship exists between the values obtained under non-reducing and reducing conditions. For example, it may be expected that the values for the $\gamma$ and $\beta$ components would be the same under the two conditions, and that the low-molecular-weight fragments can be separated into at least three groups of small COMP fragments ($\gamma$ 60–70 kDa, $\delta$ 55–60 kDa and $\varepsilon < 55$ kDa). The statistical analysis shows that the relationship is more complex. Under reducing conditions, the bands also include the peptides originating from oligomers and oligomeric fragments which are not considered in the analysis of the 50–150 kDa molecules obtained under non-reducing conditions.

*The COMP fragments are not produced ex vivo*

Control experiments in OA, RA and reactive arthritis show that the small COMP fragments did not appear as a consequence of the pre-treatment of synovial fluids with hyaluronidase. The presence or absence of heparin, as well as the type of tubes (plastic or glass), had no influence on the COMP patterns. However, the removal of cells and large debris by centrifugation was essential; without centrifugation, ~25% of the synovial fluid specimens from patients with RA and other inflammatory arthritides had COMP patterns that were more degraded in appearance. In such cases, the degradation could be at least in part inhibited by 10 mM PMSF, 2 mM 3,4-dichloroisocoumarin, 2 mM NEM and/or 1 mM iodomethacine (i.e. inhibitors of serine and cysteine proteinases).

*Production of COMP fragments in vitro*

In 13% (5/37) of patients with RA or other inflammatory arthritides, co-incubation of purified human COMP with the corresponding cell-free synovial fluids produced degradation patterns similar to those found *in vivo*. Within 4 h, small fragments were generated. Storage of the samples at –20 or –80°C before the co-incubation did not alter their degradation potential. In the example shown in Fig. 6, as well as in two other patients, the degradation process could be blocked with 10 mM PMSF and 2 mM 3,4-dichloroisocoumarin (i.e. inhibitors of serine proteinases), but not with NEM, iodoacetamide, EDTA or 1,10-phenanthroline. In two other cases, the inhibitors tested were less effective.

## DISCUSSION

The tissue distribution and concentration of COMP in man have not been previously examined, largely due to the lack of appropriate antigen and/or antiserum. Assays have been performed in biological fluids to monitor joint diseases [9–13]. An ELISA has been reported with polyclonal antibodies to bovine COMP and human COMP for coating and standardization [11, 12]. Here, we show by immunohistochemistry, immunoblots and ELISA that high amounts of COMP are found in articular cartilage and meniscus. This observation supports the conten-

tion that COMP can be used as a metabolic marker of joint tissues in human diseases.

In the present study, determination of COMP in tissue extracts and biological fluids was carried out using native COMP purified from human articular cartilage and polyclonal antibodies raised against human COMP. There was no cross-reactivity of the antiserum with human thrombospondin-1, which shares important amino acid sequence homologies with COMP [4, 5]. The absolute levels we measured are slightly lower than those reported earlier [9, 12], which could result as a consequence of the different methodologies employed, i.e. the use of the human antigen for coating and/or antibody production.

The levels of COMP in synovial fluid are not significantly different between the three groups of patients. However, a high inter-individual variation was observed, with a few low levels and many elevated concentrations (13 and 35% of the patients, respectively). No difference was found between men and women.

In synovial fluid, published reports have indicated increased amounts of COMP in early-stage OA [13] and in reactive arthritis [10]. Thus, the high COMP levels measured in the present study probably result mainly from the degradation of the articular cartilage. The occurrence of low levels of COMP in all of our patient groups resembles the published findings in advanced stages of RA [11]. Provided that COMP is liberated at a constant rate, its concentration will depend on the volume of the synovial fluid. Low COMP levels could appear because of either an elevated clearance from the joint space, previous degradation and loss of cartilage or a dilution of the protein. Thus, the assessment of synovial fluid constituents as concentrations is an inaccurate measurement [19]; the ratio between different markers (e.g. proteoglycan [11]) is a useful way of overcoming this criticism.

As previously reported by others [10, 12, 13], the sera of patients with OA, RA or other forms of inflammatory arthritis show more COMP immunoreactivity when compared with controls. The level of COMP in serum is constantly lower than that in synovial fluid. As reported for patients with early-phase reactive arthritis [10], a positive correlation between serum and synovial fluid also exists in our patient groups.

We observed qualitative differences in synovial fluids of a few patients with OA and most cases with RA (or other forms of inflammatory arthritis). Under non-reducing conditions, 84% of RA patients and 60% of patients with other inflammatory arthritides show large amounts of low-molecular-weight COMP fragments (50–70 kDa), whereas their occurrence is much lower in controls (7%) and OA (21%). This can be explained by the degree of the degradation process and the extent of synovitis, which are different in OA and RA (and other inflammatory arthritides).

It is postulated that certain enzymes are responsible for the degradation of cartilage, particularly serine proteinases [20], cysteine proteinases [21, 22] and metalloproteinases [23, 24]. In most situations, an excess of enzyme inhibitors is present in human synovial fluid [19, 25, 26]. If the imbalance between active proteinases and their inhibitors is of importance in the degradation of cartilage matrix *in vivo*, this should also be observed by the use of purified components *in vitro*. However, our data suggest that synovial fluid enzymes are not responsible for the degradation patterns obtained. Whether the degradation already occurs within the cartilage matrix by proteinases synthesized by inflammatory cells of the synovial tissue (and penetrating into the cartilage) or by chondrocytes is under investigation [27].

In serum, mostly fragments (50–90 kDa) are detected. As a consequence, patients with high levels of COMP in serum might represent individuals with a very active degradation of their articular cartilage. Since early OA is often unnoticed by the patient and the physician, the serum level of COMP might be of great value in the early diagnosis of this joint disease. Nevertheless, the possible role of COMP as a marker of joint tissue metabolism in early aggressive RA has to be elucidated further. In addition to its relevance in the early diagnosis of joint diseases, future examinations have to validate the concept of whether both quantitative and qualitative evaluations of COMP could be used as a marker of cartilage degradation and for monitoring disease activity.

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