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ANALYSIS OF CARTILAGE OLIGOMERIC MATRIX PROTEIN (COMP) IN SYNOVIAL FIBROBLASTS AND SYNOVIAL FLUIDS

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SUMMARY

We investigated the expression of cartilage oligomeric matrix protein (COMP) in normal and rheumatoid arthritis (RA) synovial fibroblasts. *In situ* hybridization (ISH) was conducted on synovial specimens from five RA patients applying specific probes for COMP or fibroblast collagen type I. ISH was combined with immunohistochemistry, applying antibodies to the macrophage marker CD68. Ribonuclease protection assay (RPA) and rapid amplification of 3′-cDNA ends (3′-RACE) were performed on total RNA from normal and RA synovial fibroblast cultures. Protein extracts from fibroblasts and culture supernatants were compared with synovial fluids and protein extracts from isolated chondrocytes by Western blot utilizing polyclonal and monoclonal antibodies (18-G3 mAb) to COMP. COMP mRNA was detected in fibroblasts of RA synovium by ISH, and in normal and RA synovial fibroblast cultures by RPA. 3′-RACE demonstrated sequence homology of chondrocyte and synovial fibroblast COMP along the coding sequence. COMP protein was detected in synovial fibroblasts and culture supernatants by immunoblot. Using polyclonal antibodies, the major portion of COMP from fibroblasts and culture supernatants was present as low-molecular-weight (LMW) bands, corresponding to those found in synovial fluids. These LMW COMP bands, however, were not detected in any of the cells or tissues tested using 18-G3 mAb. In protein extracts from chondrocytes and in COMP purified from cartilage, these LMW bands could not be detected. In conclusion, the data suggest that certain forms of COMP detected in synovial fluid are secreted from synovial fibroblasts and could be distinguished by specific mAbs from COMP secreted by chondrocytes.

KEY WORDS: Cartilage oligomeric matrix protein (COMP), Synovial fibroblasts, Synovial fluid, Rheumatoid arthritis.

CARTILAGE oligomeric matrix protein (COMP) is a pentameric non-collagenous glycoprotein belonging to the heterogeneous family of thrombospondins [1]. COMP is found abundantly in synovial fluid, reaching concentrations of up to 250 μg/ml. Several investigators suggested that COMP is mainly produced by articular chondrocytes, and concluded that COMP levels in synovial fluid and serum might be related to cartilage damage [2–4]. Polyclonal antisera against bovine or human COMP have been produced to establish a test system to monitor cartilage metabolism in joint diseases, including osteoarthritis (OA) and rheumatoid arthritis (RA) [5–7]. In OA, an early increase of COMP in serum was reported to be associated with aggressive disease progression [7]. Interestingly, most recent data, using chondroscopy as a parameter of cartilage destruction, revealed that serum levels of COMP were not significantly associated with the progression of cartilage destruction in OA [8]. Moreover, in RA and other inflammatory arthritides, it has been suggested that, in addition to the absolute levels of COMP in synovial fluid and serum, the fragmentation pattern of COMP in synovial fluid is of diagnostic importance [9]. This particular pattern was thought to be due to enzymatic degradation of COMP within the cartilaginous matrix [10] and/or the synovial fluid [9] of inflamed joints. However, other studies have also localized COMP in both bovine and equine tendons [11, 12], as well as in human meniscus and cruciate ligament [9]. Consequently, COMP may not be regarded as a cartilage-specific protein. Recently, preliminary studies reported an expression of COMP protein and mRNA in synovium or synovial fibroblasts [13–15].

The results of the present study confirm and complete these reports. In addition, immunoblot characteristics of COMP from cultured synovial fibroblasts and culture supernatants were compared with those of COMP in synovial fluids, articular cartilage and isolated chondrocytes.

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METHODS

Synovial tissue samples

Synovial tissue samples were collected from five RA patients undergoing wrist and metacarpophalangeal joint arthroplasty. All patients fulfilled the revised criteria of the American College of Rheumatology for the diagnosis of RA [16]. Three normal synovial samples were collected: one from the knee of a patient undergoing diabetic-leg amputation and two from the knee of a fresh cadaver from the Department of Forensic Medicine. Samples were immediately embedded in OCT TissueTek medium (Miles Inc.), snap frozen, and stored at -80°C, or digested enzymatically to obtain fibroblast cultures.

Cell cultures

After enzymatic digestion of the synovial samples, cells were grown in Dulbecco's modified Eagle medium (DMEM) and 10% fetal calf serum (FCS) [17]. Cells were harvested after 3–8 passages. Flow cytometric analysis using fluorescein-conjugated anti-fibroblast monoclonal antibody AS02 (Dianova, Hamburg, Germany) confirmed that >95% of the cells were fibroblasts. Myeloid leukaemia U-937 cells were grown in RPMI-1640 supplemented with 10% FCS.

Articular cartilage, synovial fluid and antibodies

Normal femoral cartilage was obtained from knees shortly after death and extracted with guanidine–HCl as described previously [9]. Native human COMP was purified from femoral cartilage according to DiCesare *et al.* [4]. Both were used as standards in the immunoblotting procedure. Polyclonal antibodies to purified COMP were raised in rabbits and shown not to cross-react with thrombospondin-1 [9]. The murine monoclonal antibody to native COMP (18-G3) has been developed and characterized earlier [18]. Human chondrocytes were isolated as described by Häuselmann *et al.* [19]. Chondrocytes were kept at −80°C until RNA and protein isolation. Synovial fluids were obtained from a healthy individual and two patients with RA, and immediately centrifuged at 450 *g* for 30 min. Synovial fluid cells and cell-free samples were kept at −80°C.

RNA and protein isolations

Total RNA was isolated from cells according to the method developed by Chomczynski and Sacchi [20]. DNA in the organic phase was removed by precipitation with ethanol. Protein was recovered by precipitation with isopropyl alcohol, washed in 0.3 M guanidine–HCl and dissolved in 1% sodium dodecyl sulphate (SDS). Supernatants of synovial fibroblast cultures were lyophilized and the pellet dissolved in 1% SDS. Proteinase inhibitors [10 mM EDTA, 2 mM phenylmethylsulphonyl fluoride (Sigma) and 2 mM *N*-ethylmaleinimide (Fluka)] were added during the preparation of cell protein and culture supernatants.

Cloning of COMP cDNA fragment from chondrocytes

A COMP cDNA fragment (498 bp) was obtained by reverse transcription-polymerase chain reaction (RT-PCR) of chondrocyte RNA and subsequently cloned into a plasmid vector. First-strand cDNA synthesis was performed with oligo$(dT)_{12-18}$ primer (Gibco BRL) and M-MuLV reverse transcriptase (Boehringer Mannheim), the other reagents being from Stratagene (Basel, Switzerland), according to the manufacturer's protocol. The following primers for human COMP were used: forward 3′-CCC CGA GTC CGC TGT ATC AA-5′; reverse 3′-CCA CAT CCT CCT GCC CTG AG-5′. cDNA was amplified by PCR under standard conditions using *Pfu* DNA polymerase (Stratagene). For PCR, we used a three-step protocol with 35 cycles and an annealing temperature of 58°C. Denaturation was performed at 94°C for 1 min and extension at 72°C for 2 min. The final extension step was 10 min. The PCR product was ligated into the PCR-Script Amp SK+ vector (Stratagene) according to the manufacturer's protocol. The vector was transfected into Epicurian *Escherichia coli* XL1-Blue MRF′ Kan competent cells. The identity of the cloned cDNA fragment was confirmed by commercial dideoxy sequencing.

Rapid amplification of 3′-cDNA ends (3′-RACE)

3′-RACE was performed on total RNA from isolated chondrocytes and RA synovial fibroblasts. First-strand cDNA was synthesized using an oligo dT anchor primer and AMV reverse transcriptase according to the manufacturer's recommendations (Boehringer Mannheim). cDNA was amplified by *Pfu* DNA polymerase applying the anchor primer and the upper primers f588 or f30. The primer sequences were f588 3′CCT CAA CCC CTG CGT CAA TAC AA-5′ and f30 3′-CTT CTT GCG TCC GCC ACA GCC CCT-5′. The amplification was conducted with 35 cycles and an annealing temperature of 60°C. Denaturation was performed at 94°C for 1 min and extension at 72°C for 2 min. The reaction products were run on a 1% agarose gel and visualized by ethidium bromide staining. DNA molecular weight marker IV (Boehringer Mannheim) was used as standard. Reaction products were analysed by commercial dideoxy sequencing.

Preparation of cDNA templates and riboprobes

Templates were prepared by linearization of purified plasmids. Antisense and sense RNA probes for COMP, collagen type I and collagen type II [21], and GAPDH were obtained by *in vitro* transcription. For *in situ* hybridization, probes were labelled with digoxigenin (DIG)-11-UTP, the ratio of labelled:unlabelled UTP being 1:2. For ribonuclease protection assay, probes were labelled with [α-^{32}P]UTP. The efficacy of the transcription and the integrity of the probe were verified on a 4% polyacrylamide gel under denaturating conditions.

In situ hybridization

In situ hybridization of synovial tissue was conducted as described by Kriegsmann *et al.* [22], with minor modifications. Frozen sections (4–6 μm) were fixed in 3% buffered paraformaldehyde and acetylated in 0.25% acetic anhydride/0.1 M triethanolamine–HCl. After pre-hybridization, the sections were incubated with the DIG-labelled RNA antisense probe overnight at 52°C. Unbound probe was digested for 45 min at 37°C with 10 μg/ml RNase A (Boehringer Mannheim). Sections were incubated in 2 × SSC, 50% formamide for 15 min at 50°C, and washed in 1 × SSC/0.1% SDS, 0.25 × SSC/0.1% SDS and 0.1 × SSC/0.1% SDS each for 15 min at 50°C.

Detection of hybridized probes was performed immunologically by using anti-DIG antibody Fab fragments linked to alkaline phosphatase and NBT/BCIP substrate (Boehringer Mannheim, Rotkreuz, Switzerland). Non-specific binding was blocked with

2% normal horse serum. Endogenous alkaline phosphatase was blocked by levamisole added to the substrate.

As negative controls, sections were hybridized with the sense probes or collagen type II probe.

Immunohistochemical double labelling

For double labelling of synovial specimens, after *in situ* hybridization, immunohistochemistry was performed applying the anti-monocyte/macrophage antibodies (anti-CD68, Dako) in an APAAP technique [22]. Non-specific binding sites were blocked with 4% milk/2% normal goat serum. As negative controls, the primary antibody was replaced by a mouse antibody of the same isotype and concentration (Dako).

Ribonuclease protection assay (RPA)

RPA was performed using reagents from Ambion (AMS Biotechnology, Lugano, Switzerland) according to the manufacturer's protocol. α-^{32}P-labelled probes were separated on a denaturing urea/4% polyacrylamide gel and eluted. Appropriate amounts of the GAPDH and COMP probes were hybridized to 8–15 μg of total RNA overnight at 42°C. Unprotected RNA was digested with RNase A/T1 and the samples run on a denaturing urea/4% polyacrylamide gel.

Western blot

SDS–PAGE was performed according to the protocol of Laemmli [23]. Four to 15% gradient polyacrylamide gels were run under non-reduced conditions or in the presence of 2.5% (v/v) 2-mercaptoethanol. For the range 14–94 kDa, low-molecular-weight (LMW) standards (Pharmacia) were used (i.e. 94 kDa, phosphorylase b; 67 kDa, albumin; 43 kDa, ovalbumin). In addition, in the non-reduced gel, purified human COMP was used for identification of the 500 kDa band. The samples and standards were transferred to nitrocellulose by electrophoresis according to Towbin *et al.* [24], the standard stained with Ponceau S solution (0.02% in 0.3% trichloroacetic acid; Serva) and antigens detected using polyclonal or monoclonal antibodies to COMP, peroxidase-conjugated swine anti-rabbit IgG (Dako) or goat anti-mouse IgG (Jackson Immunoresearch) and by enhanced chemiluminescence (ECL, Amersham).

RESULTS

RPA

A cDNA fragment of COMP was cloned from human chondrocytes by RT-PCR. Using a riboprobe derived from this fragment, we performed an RPA on total RNA prepared from fibroblast cultures of one normal and three RA synovial samples. We also included total RNA from the myelomonocytic cell line U937.

The RPA on Fig. 1 revealed distinct signals in two of the synovial cells and faint signals in the other two. Applying at least the same amount of total RNA from U937 cells, as determined by co-hybridization with a GAPDH probe, no signal for COMP transcripts could be detected.

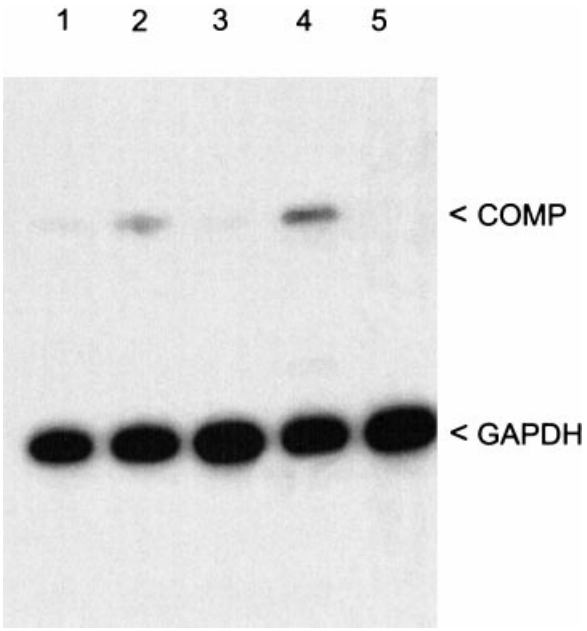

FIG. 1.—Ribonuclease protection assay: 8–12 μg of total RNA from one normal (lane 1), three RA synovial fibroblast cultures (lanes 2–4) and U937 cell line (lane 5) were co-hybridized with α-^{32}P-labelled COMP and GAPDH riboprobes. Unprotected RNA was digested with RNase. Hybridization products, which were not digested, were run on a denaturing polyacrylamide gel. Signals for COMP were detected in all four fibroblast samples (strong signal for two RA samples, weak signal for one RA and the normal fibroblast sample). No signal was detected in the U937 cells. The signals for the GAPDH standard are similar for all samples.

3′-RACE

Amplification of the 3′-cDNA ends applying specific primers reaching up to the 5′-cDNA end (f30 position bp 30, f588 position bp 588) and subsequent sequencing revealed identical amplification products for either isolated chondrocytes or RA synovial fibroblasts. Applying the primer f30, the amplification product covers almost the total coding sequence (bp 30–2299, GenBank No. G602449).

In situ hybridization and double labelling

To localize and determine cell types other than fibroblasts within the synovium expressing COMP mRNA, *in situ* hybridization was performed on synovial specimens obtained from five RA patients using riboprobes derived from the same templates as for the RPA. Serial sections were processed with antisense probes for COMP collagen type I (a fibroblast marker) and collagen type II (a chondrocyte marker).

As presented in Fig. 2a–d, we were able to co-localize COMP mRNA-expressing cells with collagen I mRNA-expressing fibroblasts in the synovial sublining. In the lining areas of RA synovium, little or no expression of either could be detected. Cells within lymphocytic infiltrates or capillary endothelium were also negative for both probes. Double labelling, as shown in Fig. 3, revealed that expression of COMP mRNA and the macrophage marker CD68 were mutually exclusive.

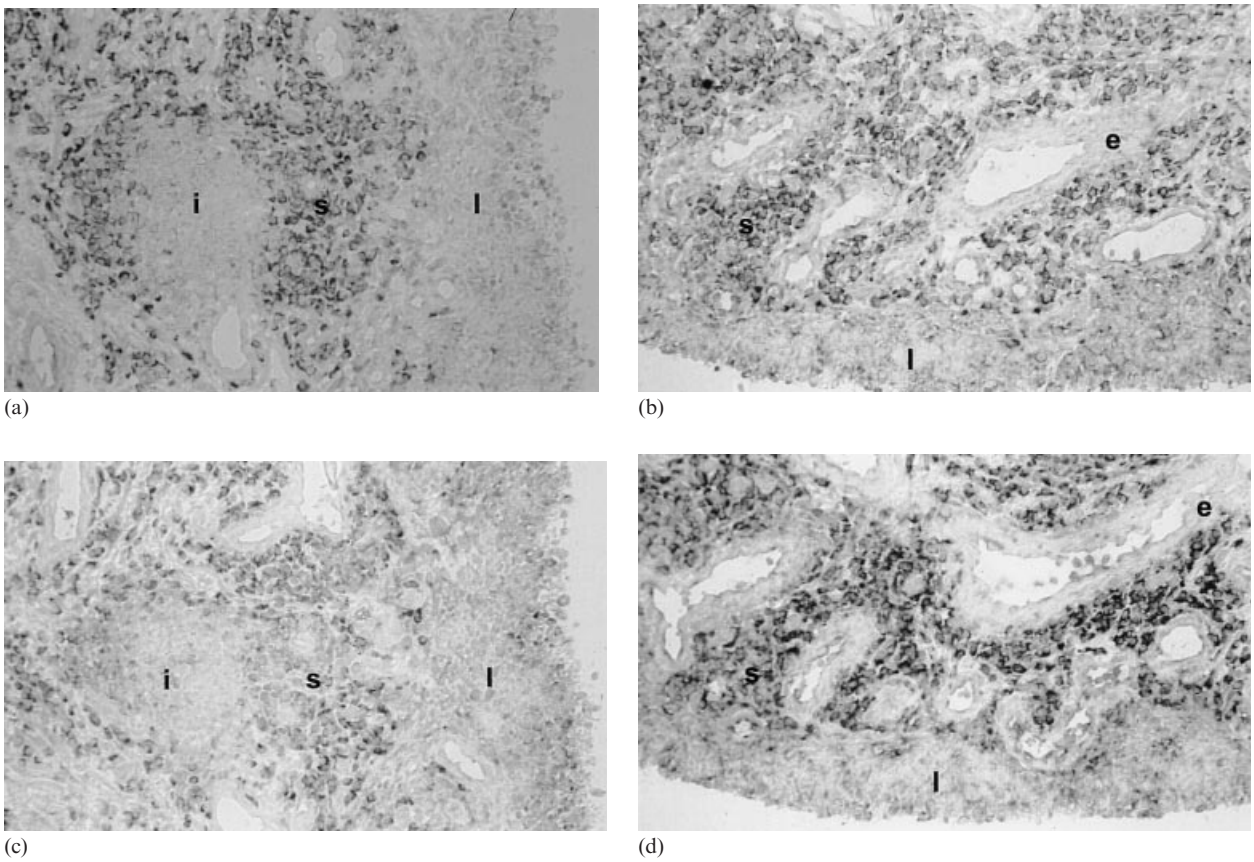

FIG. 2.—*In situ* hybridization: serial sections of RA synovial samples examined using riboprobes for COMP, collagen type I as fibroblast marker and cartilage collagen type II as negative control. COMP mRNA was detected in numerous cells in the synovial sublining (s), but only in a few cells of the lining cell layer (l) (a and b). In contrast, no cells within the lymphocytic infiltrates (i) and endothelium (e) stained positive. Expression of COMP mRNA was co-localized with the expression of collagen type I (c and d).

No signal was detected in synovium with either sense probes for COMP and collagen I or antisense probe for collagen type II.

Western blot

Immunoanalysis was performed of protein extracts from human fibroblasts cultured from normal ($n = 3$) and RA synovium ($n = 3$), and of culture supernatants ($n = 4$), as well as of protein extracted from isolated human chondrocytes and COMP purified from normal human femoral cartilage. We applied polyclonal antibodies known to bind human COMP specifically.

In immunoblots under non-reducing conditions, COMP in cartilage extracts or in protein preparations of human chondrocytes was detected as the expected band at 450–500 kDa representing the pentameric protein. Protein extracts from human synovial fibroblasts, as shown in Fig. 4a, presented this band, too. However, the major portion consisted of LMW bands at 80 and 90 kDa (α1 and β1, respectively). Using reducing conditions, as presented in Fig. 4b, immunoreactive COMP can be seen as the two characteristic 90 and 80 kDa bands (α2 and β2) in all preparations (cartilage, chondrocytes, fibroblasts and synovial fluid). In contrast to cartilage and chondrocyte preparations, synovial fibroblasts expressed more 90 kDa (α2) than 80 kDa (β2) fragments. No qualitative difference was found in COMP of fibroblasts from 'healthy' individuals or patients with RA. Neither in myelomonocytic cells U937 nor in cells obtained from synovial fluid could COMP be detected.

The murine monoclonal antibody (18-G3), as shown in Fig. 5a, recognized almost exclusively native COMP from cartilage and isolated chondrocytes in its pentameric form. Regarding fibroblast COMP, this antibody bound small amounts of the pentameric form, as did the polyclonal antiserum. The LMW bands (α1 and β1), which represent the major portion of COMP detected with the polyclonal antiserum, could only be detected in small amounts with the monoclonal antibody in the non-reduced gel. In the synovial fluid from a 'healthy' individual, the monoclonal antibody recognized exclusively some oligomeric forms, but not the smaller bands, which could be detected with the polyclonal antiserum. In RA ($n = 3$, data not shown), the monoclonal antibody detected less oligomeric forms. However, as shown in Fig. 5b, the monoclonal antibody, under reducing conditions, did not detect any 90 kDa (α2) or 80 kDa (β2) bands either in synovial fibroblasts, which were clearly recognized by this antibody in cartilage and chondrocytes, and to a lesser extent in synovial fluid.

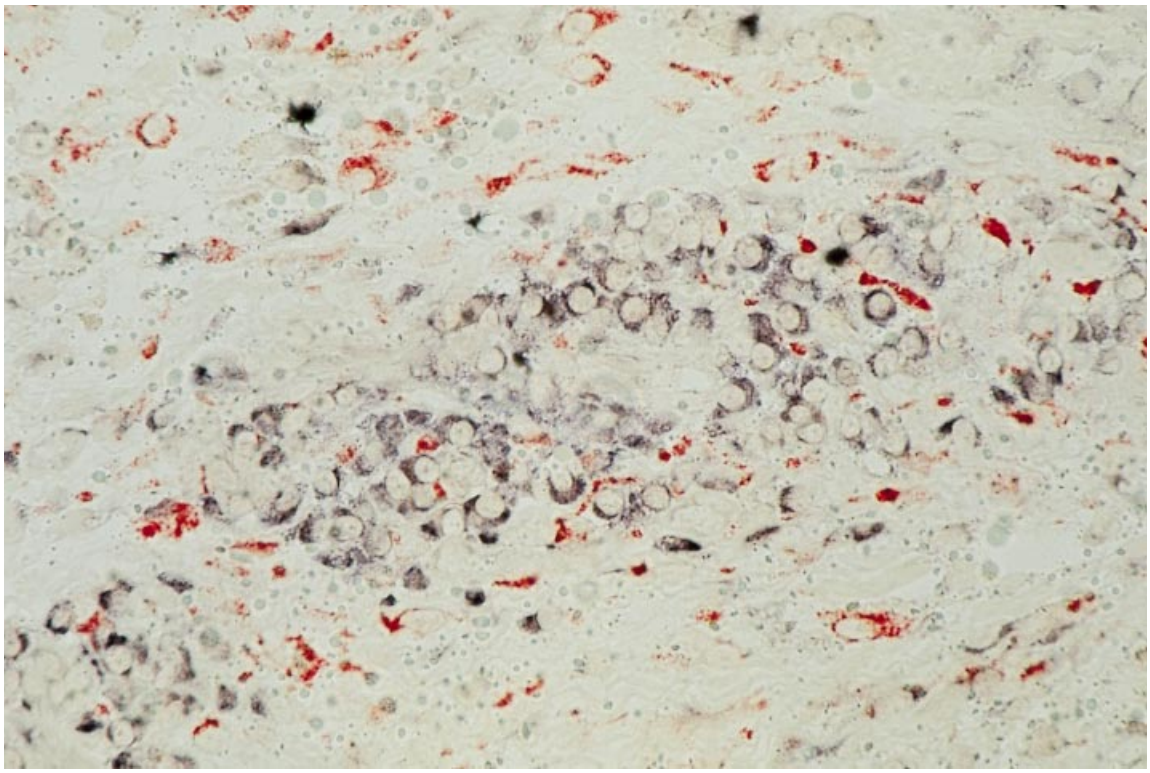

FIG. 3.—*In situ* hybridization and double labelling: RA synovial sample examined using riboprobes for COMP mRNA and anti-CD68 monoclonal antibody. Expression of COMP mRNA and the macrophage marker CD68 were mutually exclusive.

As illustrated by Fig. 6, supernatants from synovial fibroblast cultures presented the same immunoblot characteristics as the fibroblast preparations. Thus, pentameric forms of COMP, as well as LMW α1 and β1 bands, were detected in both synovial fibroblasts and culture supernatants. The α1 and β1 bands were similar to those found in synovial fluids.

DISCUSSION

This study supports previous reports showing the production of COMP protein and/or mRNA in synovium *in situ* and synovial fibroblasts *in vitro* [13–15]. These observations are completed and summarized as follows. Distinct COMP mRNA expression was detected in the synovial sublining. Lymphocytic infiltrates and capillary endothelium showed no or only little COMP mRNA expression. As shown by double labelling, expression of COMP mRNA and the macrophage marker CD68 were mutually exclusive. On the other hand, COMP mRNA-expressing cells were strictly co-localized to cells expressing collagen type I mRNA—in synovium, a marker for fibroblasts. These results were coherent with the RPA by which COMP mRNA could only be detected in synovial fibroblast cultures, but not in the myelomonocytic cell line U937. By 3′-RACE, we verified homology along the coding sequence (bp 30–2299) of COMP mRNA in fibroblasts with COMP mRNA in chondrocytes. These data confirm that COMP mRNA is expressed in synovium and indicate that expression in synovium is largely restricted to fibroblasts.

The comparative analyses of COMP protein in fibroblast cell lysates, culture supernatants, synovial fluids, cartilage and isolated chondrocytes, applying different antibodies, revealed, as expected, a 450–500 kDa pentameric COMP form in cartilage and chondrocytes. However, using polyclonal antibodies, fibroblast COMP, as well as COMP in culture supernatants, were not only detected as high-molecular-weight (pentameric) bands, but mainly as LMW bands. The same bands were described in synovial fluids, but not in cartilage, by our group [9]. In synovial fluids, these so-called α1 and β1 fragments (80 and 90 kDa, respectively) had previously been considered as possible products of intra-articular enzymatic degradation of COMP released from articular cartilage, although we had not been able to obtain these fragments by *in vitro* degradation of purified cartilage COMP by co-incubation with synovial fluids of RA patients. The detection of the α1 and β1 fragments in fibroblast extracts and culture supernatants was not due to *ex vivo* degradation of COMP during preparation because the methods used for protein isolation (1) included proteinase inhibitors and/or (2) were the same as used for chondrocytes. Differences between chondrocyte COMP and fibroblast COMP were only observed under non-reducing conditions, suggesting an alternative process impairing pentamer formation in fibroblasts.

Interestingly, the α1 and β1 bands of COMP in fibroblasts and synovial fluids were not reacting with a monoclonal antibody to human cartilage COMP. These findings suggest that there are certain forms of

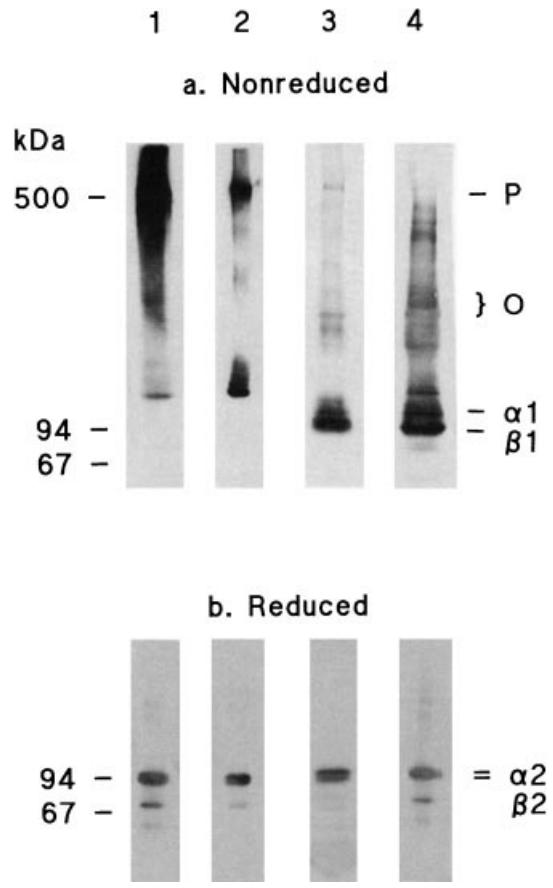

FIG. 4.—Representative immunoblots of COMP extracted from normal human femoral cartilage (lane 1), isolated chondrocytes (lane 2), synovial fibroblasts (lane 3) and synovial fluid (lane 4) using polyclonal antibodies to COMP. Under non-reducing conditions (a), articular cartilage and chondrocytes show bands for the pentameric (P) and monomeric-like forms, while immunoreactive COMP in both fibroblasts and synovial fluid shows a high proportion of LMW α1 and β1 bands (90 and 80 kDa, respectively). The synovial fluid also shows significant amounts of oligomers and oligomeric fragments (O). Under reducing conditions (b), the α2 and β2 bands (90 and 80 kDa, respectively) are detected in all four samples.

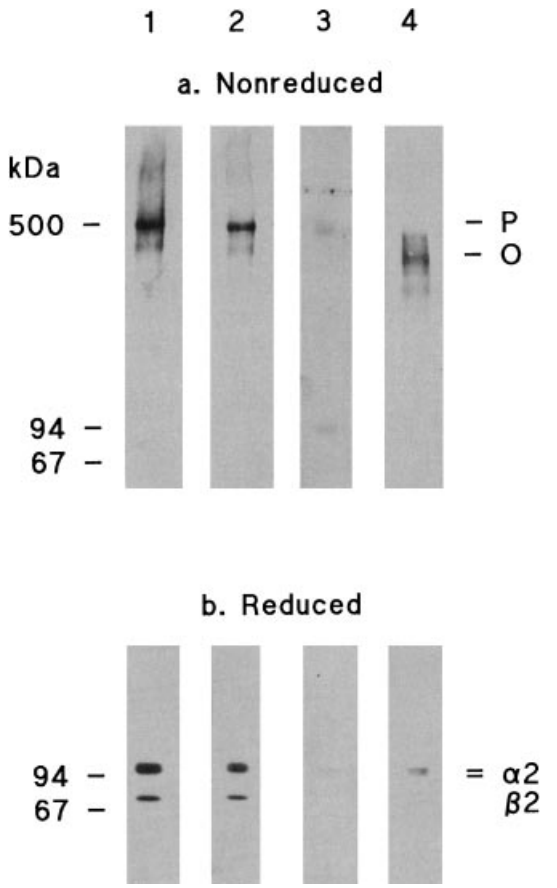

FIG. 5.—Representative immunoblots, using monoclonal antibodies to COMP, of extracts of human femoral cartilage (lane 1), isolated chondrocytes (lane 2), synovial fibroblasts (lane 3) and synovial fluid (lane 4). Under non-reducing conditions (a), articular cartilage and chondrocytes show exclusively COMP pentamers (P). The synovial fluid shows some oligomeric fragments (O). In synovial fibroblasts and synovial fluid, the LMW fragments (α1 and β1), which represent the majority of COMP detected with the polyclonal antiserum, are only found at very discrete levels. Under reducing conditions (b), the α2 and β2 bands are not recognized in synovial fibroblasts, but they are distinctly found in cartilage and chondrocytes, and faintly in synovial fluid after overexposure.

COMP in synovial fluids that are only produced by synovial fibroblasts. It may well be speculated that these forms of COMP differ in the extent of glycosylation, in the polypeptide conformation and/or length. For example, removal of the N-terminal region that mediates pentameric arrangement of COMP monomers would result in a small shift in the molecular weight of the monomers and impairment of pentamer arrangement. Such changes could occur at the post-translational level.

In summary, we conclude that certain forms of immunoreactive COMP in synovial fluids originate specifically from synovial fibroblasts. It is evident that the results presented here, although not quantitative, have to be considered for future evaluations of COMP as a marker for cartilage degeneration and destruction, as well as joint inflammation. Thus, the synovium is a further non-cartilage source of COMP that has significant implications for evaluating the level of COMP in synovial fluid and/or serum. Increased amounts of COMP reported in synovial fluids in various joint diseases, as determined with polyclonal antisera, also include fibroblast COMP of affected synovium and not only COMP released by degenerated cartilage. This may explain the high level of COMP in synovial fluid and/or serum found in patients with RA [25] and early-stage OA [6] when radiological changes have not yet occurred. Specific antibodies to fibroblast COMP and distinct antibodies to cartilage COMP are required to differentiate cartilage degeneration from disease activity in the synovium.

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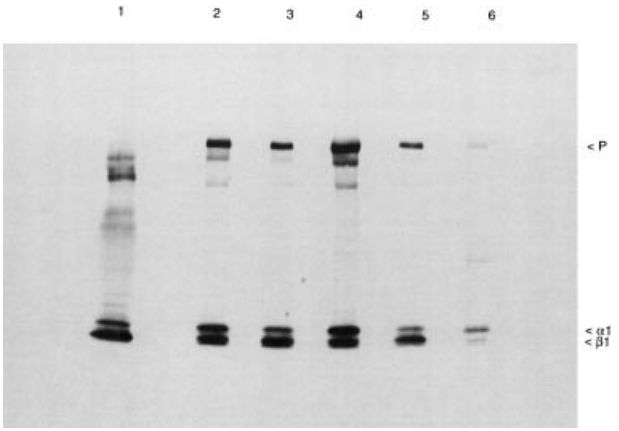

FIG. 6.—Non-reduced immunoblots of COMP in RA synovial fluid (lane 1), culture supernatants of synovial fibroblasts (lanes 2–5) and in protein extract of synovial fibroblasts (lane 6) using polyclonal antibodies. Pentameric forms of COMP (P) and LMW bands (α1 and β1) were detected in synovial fibroblasts and culture supernatants. The α1 and β1 bands were similar to those found in synovial fluid. Specimens were obtained from patients with rheumatoid arthritis.

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