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OSTEOARTHRITIS and CARTILAGE

Production of cartilage oligomeric matrix protein (COMP) by cultured human dermal and synovial fibroblasts

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Summary

Objective: Cartilage oligomeric matrix protein (COMP) is a large disulfide-linked pentameric protein. Each of its five subunits is approximately 100,000 Da in molecular weight. COMP was originally identified and characterized in cartilage and it has been considered a marker of cartilage metabolism because it is currently thought not to be present in other joint tissues, except for tendon. To confirm the tissue specificity of COMP expression we examined cultured human dermal fibroblasts, human foreskin fibroblasts, and normal human synovial cells for the synthesis of COMP in culture.

Method: Normal synovial cells and normal human dermal foreskin fibroblasts were isolated from the corresponding tissues by sequential enzymatic digestions and cultured in media containing 10% fetal bovine serum until confluent. During the final 24 h of culture, the cells were labeled with ^{35}S-methionine and ^{35}S-cysteine in serum- and cysteine/methionine-free medium. The newly synthesized COMP molecules were immunoprecipitated from the culture media with a COMP-specific polyclonal antiserum, or with monoclonal antibodies or affinity-purified COMP antibodies. The immunoprecipitated COMP was analyzed by electrophoresis in 5.5% polyacrylamide gels. For other experiments, synovial cells cultured from the synovium of patients with rheumatoid arthritis(RA) and osteoarthritis(OA) were similarly examined.

Results: A comparison of the amounts of COMP produced by each cell type (corrected for the DNA content) revealed that synovial cells produced ≥ 9 times more COMP than chondrocytes or dermal fibroblasts. COMP could be easily detected by immunoprecipitation in all cell types. Electrophoretic analysis revealed a distinct band with an apparent MW of 115–120 kDa in samples from each of the three cell types, regardless of the antibody used. COMP expression in cultures of synoviocytes derived from OA and RA patients showed that OA and RA synovial cells produced similar amounts of monomeric COMP of identical size to those COMP monomers produced by normal synovial cells. The addition of TGF-β to these cultures resulted in an increase in COMP production in normal, OA and RA synovial cells (45, 116 and 115% respectively).

Conclusion: These studies demonstrate that substantial amounts of COMP are produced by several mesenchymal cells including synoviocytes and dermal fibroblasts. These findings raise important concerns regarding the utility of measurements of COMP levels in serum or in synovial fluid as markers of articular cartilage degradation because of the likelihood that a substantial proportion of COMP or COMP fragments present in serum or synovial fluid may be produced by cells other than articular chondrocytes.

Introduction

Loss of articular cartilage matrix is the hallmark of degenerative and inflammatory arthritis [1]. Recent interest has been focused on the identification of biochemical markers that would accurately follow the process of articular cartilage loss [2–6]. Cartilage oligomeric matrix protein (COMP) is a large disulfide-linked pentameric glycoprotein which was first identified as a structural component of articular cartilage extracellular matrix by Heinegard *et al.* [7, 8]. Although substantial knowledge has been recently acquired about its molecular characteristics, its precise function within articular cartilage matrix is still unknown. The COMP molecule is comprised of five disulfide bonded subunits of $\sim 100\,000$ Daltons in molecular weight [8, 9]. COMP has been shown to be expressed by chondrocytes from individuals of various ages, although temporal and spatial differences have been observed, particularly during development [10]. With the exception of cartilage [7, 8], and tendon [11], COMP has not been identified as a constitutive component of the extracellular matrix of other tissues. Based on

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these observations it has been suggested that COMP measurements in serum or in synovial fluid may serve as a valid indicator of articular cartilage degradation [2, 4, 12]. The utilization of COMP as a reliable marker of articular cartilage breakdown requires that its expression be either confined to this tissue or, alternatively, that a clear understanding of other cells and tissues capable of its production is available. To confirm the tissue specificity of COMP expression, we examined whether COMP synthesis can be detected in non-chondrocytic cells such as cultured human dermal fibroblasts and normal human synovial cells. For these studies, the cells were isolated from the corresponding tissues by sequential enzymatic digestions and cultured in media containing 10% fetal bovine serum until confluent. During the final 24 h of culture, the cells were labeled with ^{35}S-methionine and ^{35}S-cysteine in serum-free and cysteine/methionine-free medium. Immunoprecipitation of media employing three different COMP-specific antibodies showed that the three types of cells produced relatively large amounts of COMP. A comparison of the amounts produced by each cell type, corrected for the DNA content of the cultures, revealed that synovial cells produced ≥ 9 times more COMP than chondrocytes or dermal fibroblasts. Electrophoretic analysis revealed a distinct band with an apparent MW of 115–120 kDa in samples from the three cell types, regardless of the antibody used. These results were similar to those obtained with COMP from culture media of articular chondrocytes from other species [7, 8]. The unexpected demonstration of COMP production by synovial cells prompted the investigation of whether there were differences in COMP expression in cultured synoviocytes from osteoarthritis (OA) and rheumatoid arthritis (RA) patients. The results of these studies showed that OA and RA synovial cells produced COMP polypeptides of identical size to those produced by normal synovial cells. However, there were no significant differences in the amounts of COMP produced in unstimulated cultures of the three types of synovial cells tested ($N = 8$). When the effects of TGF-β on the expression of COMP in synovial cells were examined it was found that TGF-β caused an increase in COMP production in normal, OA and RA synovial cells (45, 116 and 115% respectively).

The studies described here demonstrate that COMP is produced by various mesenchymal cells, including synoviocytes and dermal fibroblasts. These findings raise important concerns regarding the utility of measurements of COMP levels in serum or in synovial fluid as markers of cartilage degradation because of the likelihood that a substantial proportion of COMP or COMP fragments present in serum or synovial fluid may be produced by cells other than articular chondrocytes and, particularly, by cells from synovium, a tissue critically involved in joint diseases. Further characterization of the expression and regulation COMP in synovium and dermis will help to elucidate the role of this homopolymeric matrix molecule in tissue homeostasis and inflammatory connective tissue diseases.

Methods

Isolation and culture of cells

Normal articular cartilage was obtained from the knees from healthy adults through the organ donor network (NDRI, Philadelphia, PA). Chondrocytes were isolated from the tibial plateau and femoral chondyles of samples procured less than 18 h *post-mortem*, employing sequential enzymatic digestions (trypsin/collagenase, Worthington Biochemicals, Freehold, NJ) as described [13]. Single cell suspensions of isolated chondrocytes were washed three times in Dulbecco's modified Eagle's medium (DMEM), counted, and cultured in suspension at a density of $5–10 \times 10^6$ chondrocytes/60 mm dish. The culture dishes were pre-coated with poly–(2–hydroxyethyl methacrylate (poly-HEMA) as described [13]. Chondrocyte cultures were routinely maintained in DMEM supplemented with 10% fetal bovine serum (Whittaker, Walkersfield, MD) containing 1% vitamin solution (GIBCO, Gaithersburg, MD), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (Boehringer Mannheim, Indianapolis, IN) and 50 μg/ml ascorbic acid. We have previously shown that under these culture conditions the chondrocytes maintain a cartilage-specific phenotype for at least 8 months [13]. For metabolic labeling and subsequent immunoprecipitation the cells were cultured in serum-free and methionine/cysteine-free medium.

Synovial cells from normal, OA, and RA synovium were established employing an explant outgrowth technique where explants of synovial tissue are grown in cultures until synovial fibroblasts migrate out and can be propagated. The synovial tissues were removed at the time of arthroplasty or amputation. Cells were stored in the TJU/Rheumatology Division's Tissue/Cell Bank under cryogenic conditions and were used at a passage ≥ 4. Normal human foreskin fibroblasts (ATCC # 2208) and synovial fibroblasts were

cultured in minimum essential medium (MEM) (Mediatech, Herndon, VA) supplemented as described above for DMEM. In experiments in which the effects of TGF-β on COMP production were examined, cultures of either chondrocytes or synovial cells were treated for the last 24 h with 5 ng/ml recombinant human TGF-β (Boehringer Mannheim).

Metabolic labeling and immunoprecipitation

To perform a quantitative comparison of the amounts of COMP synthesized by dermal and synovial fibroblasts with that synthesized by chondrocytes, each culture was washed twice with 10 ml DMEM to remove the serum and then with methionine/cysteine-free-DMEM containing 0.1% bovine serum albumin. ^{35}S-labeled cysteine and methionine (Expre^{35}S^{35}S, Amersham, Arlington Heights, IL) was added to the cultures for the last 24 h. Following metabolic labeling, the media were removed and stored, and the cell layers (for DNA assay) were harvested in cold PBS containing 1% Nonidet, 0.5% sodium deoxycholate and 0.1% SDS. An aliquot of a proteinase inhibitor mixture (Complete, Boehringer Mannheim) was added to the medium and cell layer lysate from each sample prior to freezing. The immunoprecipitation was performed using three different antibodies: LS25, which is a polyclonal antiserum (raised against human COMP from cultures of ligament cells) that reacts with COMP monomers [14]; 6F/12A which is a pool of two monoclonal antibodies which were raised against purified human COMP (generously provided by D. Heinegard); and DH, an affinity-purified polyclonal anti-human COMP antibody (generously provided by D. Heinegard). For these experiments, 100 µl of medium or cell layer lysate samples were mixed with 150 µl PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 2 mg/ml BSA, and the protease inhibitors described above. Each sample was pre-incubated with normal rabbit IgG (when polyclonal antibodies were employed for immunoprecipitation) or mouse IgG (when monoclonal antibodies were used) and subsequently with an affinity purified polyclonal antiserum to fibronectin (Cappel Organon Teknika, Durham, NC). The samples were then precleared by precipitation with 10 µl protein A/G PLUS-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and washed repeatedly as outlined by the manufacturer. Following the last washing, the pellets were collected by centrifugation for 5 min at 12 000 × ***g*** and washed once in 100 µl of ethanol and held at −80°C for ≥ 15 min. The supernatants were decanted and the pellets were air-dried and then resuspended in 20 µl SDS-PAGE sample buffer containing 1% β-mecaptoethanol (ME) (unless noted otherwise), heated for 10 min at 100°C and centrifuged for 5 min. When COMP was evaluated under non-reducing conditions, the samples were resuspended in 40 µl of SDS-PAGE sample buffer without ME and each sample was divided into two aliquots one of which was subsequently reduced with 1% ME. A 15 µl aliquot of the supernatants was loaded onto 5.5% polyacrylamide gels (SDS-PAGE) and electrophoresed at 100 V for ∼ 1 h. The gels were fixed in a solution of 45% methanol and 15% acetic acid, dried, and analyzed using a PhosphorImaging System (Molecular Dynamics, Sunnyvale, CA).

Results and Discussion

To determine whether COMP was constitutively synthesized by fibroblasts and synovial cells, confluent cultures were metabolically labeled for 24 h and the amounts of COMP produced by each cell type were compared with the amounts of COMP produced by human articular chondrocytes. We have previously shown that under these culture conditions the chondrocytes maintain a cartilage-specific phenotype for at least 8 months [13]. For purposes of comparison, media were collected from 2–4 day-old primary suspension cultures of adult human chondrocytes and from confluent monolayer cultures of fibroblasts and synovial cells. Immunoprecipitation assays were performed employing equal concentrations of total protein (as determined by incorporated ^{35}S-methionine and cysteine). In the experiment shown in Figure 1, COMP was precipitated using a monoclonal antibody (6F/12A) to COMP and electrophoresed under reducing conditions. Figure 1A shows the COMP monomers detected in chondrocytes (lane 1), human foreskin fibroblasts (lane 2), and normal synovial cells (lane 3). The COMP monomers produced by fibroblasts and synovial cells examined under reducing conditions were of identical size (∼ 115 kDa) to COMP monomers synthesized by human articular chondrocytes. To exclude any cross-reactivity with fibronectin, aliquots of media from chondrocytes, fibroblasts and synovial cell cultures were precleared with an affinity-purified antibody to fibronectin. Several bands corresponding to fibronectin and related polypeptides were partially removed by the initial pre-clearing precipitation (Figure 1A, lanes 4–6). A comparison of the levels of COMP synthesized by each cell type corrected for the DNA content of each culture showed that the relative amount of COMP/ng DNA was ≥ 9

times higher in synovial cells than in chondrocytes (Figure 1B). The amounts of COMP synthesized by foreskin fibroblasts was approximately equal to that synthesized by human articular chondrocytes. Although other studies have indicated that synovial cells produced COMP under stimulation

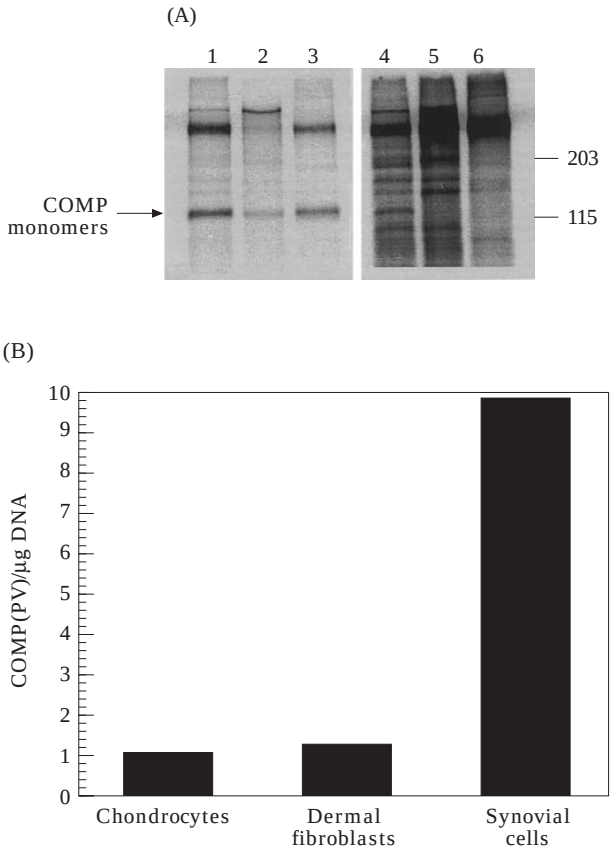

FIG. 1. Comparison of COMP expression in human dermal and synovial fibroblasts, and normal human adult articular chondrocytes. Chondrocytes were cultured under conditions that allow the preservation of their cartilage-specific phenotype [13]. Chondrocytes and dermal and synovial fibroblasts were labeled with ^{35}S methionine/cysteine as described in Methods. An aliquot of medium from each cell culture was immunoprecipitated and electrophoresed under reducing conditions as described in Methods. *A*. Immunoprecipitation of aliquots of culture media from each cell type containing equal quantities of total protein. Figure 1A shows the COMP monomers detected using the monoclonal 6F/12A in chondrocytes (lane 1), human foreskin fibroblasts (lane 2), and normal synovial cells (lane 3). The bands immunoprecipitated by preclearing with fibronectin antibodies are shown in chondrocytes, fibroblasts and synovial cells (lanes 4, 5, and 6, respectively). *B*. Graphic representation of the relative COMP amounts produced by dermal and synovial fibroblasts, and by normal adult articular chondrocytes corrected for the concentration of DNA of each culture. The data were obtained using a PhosphorImager (Applied Biosystems) and are expressed as pixel volume (PV).

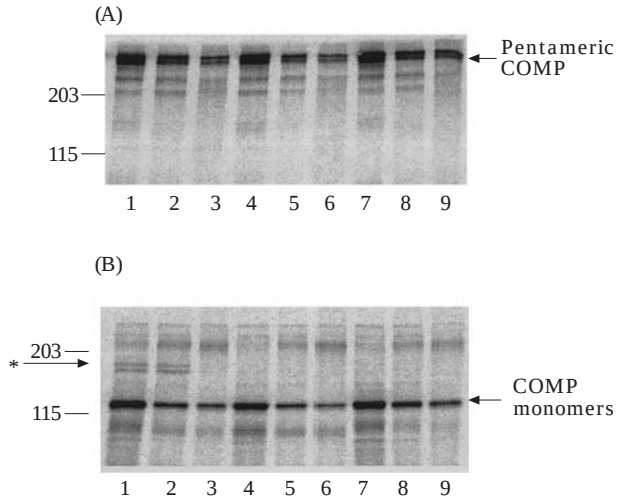

FIG. 2. Characterization of COMP immunoprecipitated by three different anti-COMP antibodies and the effects of disulfide bond reduction on the electrophoretic mobility of COMP from cultured synovial cells and chondrocytes. Samples of media from cultures of normal synovial cells (lanes 1,4,7), synovial cells from RA synovium (lanes 2,5,8), and normal adult chondrocytes (lanes 3,6,9), labeled with ^{35}S-methionine/^{35}S-cysteine were immunoprecipitated and electrophoresed under non-reducing (A) and reducing conditions (B) as described in Methods. Lanes 1-3 samples immunoprecipitated with LS25 polyclonal antisera [14]. Lanes 4–6 samples immunoprecipitated with affinity purified DH polyclonal antibodies. Lanes 7–9 samples immunoprecipitated with 6F/12A monoclonal antibodies. The position of migration of pentameric COMP is indicated in panel A as a doublet of ~500 kDa and that of COMP monomers is indicated in panel B at ~120 kDa. The *→ in panel B indicates the two additional bands recognized by the 6F/12A anti-COMP antibody that are found to be present only in samples from normal and RA synovial cells.

with TGF-β [15], our data show conclusively that COMP was constitutively produced by cultures of dermal fibroblasts and synovial cells grown in the absence of serum or exogenous stimuli. In the number of cultures examined thus far there were no significant differences in the amounts of COMP produced in unstimulated cultures of the three types of synovial cells tested ($N = 8$). When the effects of 5 ng/ml of TGF-β on the synthesis of COMP in normal, OA and RA synovial cells were studied, it was found that the growth factor caused a 45, 116 and 115% increase respectively, in COMP biosynthesis ($N = 2$) corrected for DNA content and any differences in media volumes in the three types of synovial cells analyzed (Figure 3).

The data presented here demonstrate conclusively that synovial and dermal fibroblasts constitutively produce substantial levels of COMP and that the unstimulated COMP production in

normal human synovial cells is much higher than in human articular chondrocytes. In addition, it was shown that COMP biosynthesis can be stimulated by TGF-β in all the synovial cell cultures examined. These findings expand the current understanding of the tissue distribution of COMP production and raise concerns regarding the utility of determination of COMP or COMP fragments in serum or synovial fluids as markers of cartilage pathology.

To further characterize the newly synthesized COMP in the three cell types, immunoprecipitation with three different anti-COMP antibodies followed by gel electrophoretic analysis under reducing and non-reducing conditions was performed. Figure 2A shows that under non-reducing conditions, the newly synthesized COMP immunoprecipitated with the three antibodies migrated as a doublet with an apparent ~500 kDa MW (left arrow). A similar pattern of migration was observed in samples from normal synovial cells (lanes 1,4,7), synovial cells from RA synovium (lanes 2,5,8), and normal adult chondrocytes (lanes 3,6,9), regardless of the antibody used for immunoprecipitation. When identical samples were examined following reduction, it was found that COMP migrated as monomers with an estimated MW of ~115 kDa and its migration was the same in all the cell cultures tested (Figure 2B). Two unique bands were identified using the LS25 polyclonal antisera in the samples from the synovial cells in Figure 2B, lanes 1 and 2 (*→). It is not apparent at this time whether these bands represent some unique feature of synovial cell COMP or are the result of cross-reaction of the antibodies with an unidentified protein(s).

The data presented here demonstrate that COMP, previously considered to be a cartilage-specific extracellular matrix molecule [7, 8], is ubiquitously expressed by cells from various tissues throughout the body, including mesenchymal cells such as dermal and synovial fibroblasts. Although these data do not preclude the utilization of COMP or COMP fragments as markers of connective tissue damage occurring in various forms of arthritis [14], they raise concerns regarding their specificity as indication of changes in articular cartilage matrix. In light of these data COMP fragments may indeed be the result of active synovitis. However, it is very likely that COMP found in synovial fluid and blood arises from a variety of cellular sources in addition to articular cartilage. Our data would lead us to suggest that while a non-invasive marker of cartilage pathology is paramount to early detection of diseases affecting articular cartilage, COMP does not fulfill that role.

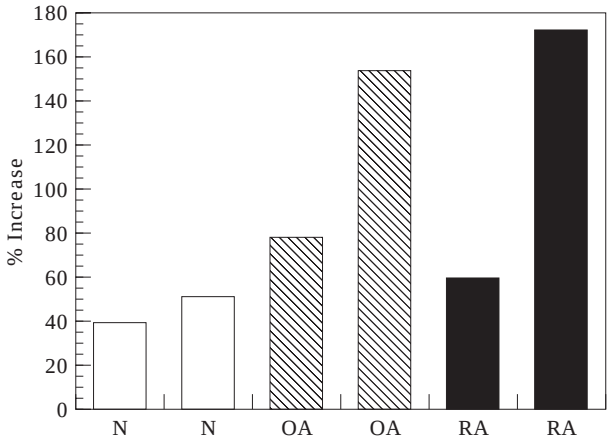

FIG. 3. Effects of TGF-β on the levels of COMP synthesized in cultures of normal (□), OA (▨), RA (■) synovial cells. Cultures of synovial cells (isolated from two separate individuals) were treated with 5 ng/ml of TGF-β and the levels of COMP synthesized in the cultures were examined by immunoprecipitation using the monoclonal antibodies, 6F/12A. A comparison was made with the corresponding untreated cultures and the percent increase calculated following correction with the differences in cell numbers (DNA assays). The average increase in the TGF-β treated cultures was 45, 116 and 115%, respectively.

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