INCREASED SERUM LEVELS OF CARTILAGE OLIGOMERIC MATRIX PROTEIN IN CHRONIC EROSIIVE ARTHRITIS IN RATS

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Objective. To investigate the utility of serum cartilage oligomeric matrix protein (COMP) for disease monitoring in rats with chronic pristane-induced arthritis, and to examine the influence of age, sex, and genes on COMP concentrations in rat serum.

Methods. Serum COMP levels were quantified by immunoassay. Sera were obtained from DA, E3, and (E3 × DA)F1 rats each week between the ages of 4 and 30 weeks. The (E3 × DA)F2 (second generation after intercrossing) rats were injected intradermally with the synthetic oil pristane. Arthritis was monitored by a macroscopic scoring system, and serum levels of COMP were measured on days 6, 35, and 49 after immunization.

Results. Serum levels of COMP decreased during growth, and reached a plateau after the age of 12 weeks. The DA rats had higher COMP levels than the E3 rats, and the (E3 × DA)F1 rats had intermediate levels. No differences were observed in these levels when the rats were grouped by sex. Arthritic (E3 × DA)F2 rats had increased serum concentrations of COMP on days 35 and 49 after pristane injection (P < 0.0001 versus the nonarthritic animals). COMP levels correlated with the severity of macroscopically detectable arthritis at both time points (r > 0.9). Rats with a chronic disease course were distinguished by higher serum concentrations of COMP during the acute stage than animals with similar clinical scores but with resolving arthritis (P < 0.01).

Conclusion. Serum analyses of COMP offer promise for monitoring tissue involvement in experimental arthritis. Such analyses should be useful for monitoring therapeutic interventions aimed at retarding tissue destruction as well as for studies of the genetically determined regulation of COMP turnover and susceptibility to arthritis. The application of molecular marker measurements to experimental arthritis offers a new possibility for verifying the utility of such measurements in human arthritis.

Rheumatoid arthritis (RA) is an inflammatory disease that primarily affects the joints and leads to various degrees of destruction of bone and cartilage. The balance between matrix synthesis and degradation, which is normally maintained during the continuous turnover of these tissues, is altered, and tissue degradation may predominate. This will eventually disrupt the tissue integrity and cause permanent damage with considerable functional consequences (1).

One way to assess turnover of the extracellular matrix of the joint may be to analyze cartilage and bone macromolecules or fragments thereof in serum or synovial fluid (2). Abnormal concentrations of such molecules may reflect pathologic processes in the joint and could be more sensitive instruments for monitoring these processes than, for example, radiography (3). Recent studies have identified several bone and cartilage molecules that have been suggested to reflect changes in joint tissue turnover, and promising results have been obtained in studies of human joint disease by quantification of tissue macromolecules in serum and synovial fluid (e.g., for diagnostic and prognostic purposes) (for review, see refs. 4 and 5).

One promising such candidate serum marker protein is cartilage oligomeric matrix protein (COMP).
This noncollagenous protein was primarily isolated from the extracellular matrix of cartilage, although the protein is not unique to this tissue (6). It has recently been found in the cartilaginous parts of bovine and equine tendon (7,8), and synovial cells can be stimulated in vitro by transforming growth factor β to produce COMP (9). COMP is a large (M, 524-kd) acidic protein consisting of 5 subunits that form a bouquet-like structure (10). The protein is structurally related to the thrombospondins (11). In adult cartilage, COMP is preferentially located in the superficial territorial matrix (6). The function of COMP is not known, but it has been suggested that it binds cells (refs. 12 and 13, and Heinegard D, Halasz K: personal communication). Thus, COMP may have both structural and metabolic functions in the tissue and could play a role in the interaction between the chondrocyte and the extracellular matrix. COMP is released during the turnover of cartilage and has been quantified by enzyme-linked immunosorbent assay (ELISA) in both serum and synovial fluid from patients with various joint diseases (14). In RA, high serum levels of COMP in the early stages of the disease seem to indicate rapid progression of large joint destruction (15). High serum levels are also observed during progression of osteoarthritis (OA) of the knee (16,17).

An attractive way of elucidating the relevance of quantifying serum COMP levels for evaluating cartilage involvement in arthritis is to apply serum measurements in an experimental arthritis system, where the course of arthritis can be closely monitored. There are several arthritis models that are commonly used, both in the mouse and in the rat. In a majority of the models, arthritis is triggered by immunization with cartilage protein (18–21), directing the specificity of the immune response to the joint cartilage and perhaps also shifting the destructive process in a predetermined way. In rats, arthritis can also be induced with a single injection of the synthetic oil pristane (22). Two weeks after such injection, arthritis appears in the peripheral joints. The joint is infiltrated with inflammatory cells that form a pannus tissue, and the inflammation leads to erosions of the bone and cartilage. As in RA, the joint inflammation is chronic. Four months after induction, massive cellular infiltration is still present, and cartilage and bone are severely eroded. It has previously been shown that elevated serum levels of COMP are seen during the early erosive stage of pristane-induced arthritis (PIA) in the DA rat (22).

In the present study, we investigated the utility of disease monitoring by serum measurements of COMP during the development of arthritis. The variable progression of RA in humans was mimicked by intercrossing the PIA-susceptible DA strain with the PIA-resistant E3 strain. In the (E3 × DA)F2 offspring, which show variable susceptibility and a varying disease course, we investigated the relationship between arthritis and circulating levels of COMP. We also examined the influence of age, sex, and genes on the concentration of COMP in rat serum.

**MATERIALS AND METHODS**

**Animals.** Specific pathogen–free DA and E3 rats originating from the Zentralinstitut für Versuchstierzucht (Hanover, Germany) were kept in conventional animal facilities in a climate-controlled environment with 12-hour light/dark cycles, housed in polystyrene cages containing wood shavings, and fed standard rodent chow and water ad libitum. The rats were found to be free from common pathogens including Sendai virus, Hantaan virus, coronavirus, reovirus, cytomegalovirus, and Mycoplasma pulmonis. The DA and E3 rats were intercrossed to obtain the (E3 × DA)F1, and (E3 × DA)F2 rats. F1 denotes the first generation rats after intercrossing and F2 denotes the second generation. In the F2 generation, theoretically, 50% of the genome will be heterozygous, 25% will be homozygous for DA, and 25% will be homozygous for E3.

**Induction and evaluation of arthritis.** Arthritis was induced in the rats by an intradermal injection at the base of the tail with 150 μl of pristane (2,6,10,14-tetramethylpentadecane; Aldrich, Milwaukee, WI). Arthritis development in all 4 paws was monitored by a macroscopic scoring system, ranging from 0 to 4 (1 = swelling and redness of 1 joint, 2 = 2 joints involved, 3 = ≥2 joints involved, and 4 = severe arthritis in the entire paw). The scores in each of the 4 paws were added, yielding a maximum total score of 16 for each rat.

**Sample collection.** Blood was obtained by cutting the tip of the tail. The samples were kept at room temperature for ~3 hours, and the sera were separated by centrifugation. Sera were stored at −20°C until assayed if assayed within 1 month, otherwise they were stored at −70°C. Normal DA, E3, and (E3 × DA)F1 rats were bled at ages 28, 35, 42, 49, 57, 64, 74, 88, 103, 124, and 154 days. The (E3 × DA)F2 rats injected with pristane were bled on days 6, 35, and 49 after pristane injection regardless of age.

**Preparation of COMP.** Two hundred grams of rat chondrosarcoma tissue was finely minced, mixed with 10 volumes of 20 mM Tris HCl, 0.15M NaCl, 100 mM e-aminocaproic acid, 5 mM benzamidine, 10 mM N-ethylmaleimide (NEM), pH 7.4 (TBS) and homogenized on ice with a Poltron homogenizer operated at full speed. The homogenate was immediately centrifuged for 30 minutes at 10,000 revolutions per minute (Beckman JA14 rotor; Beckman Instruments, Munich, Germany) at 4°C. The tissue pellet was washed once in 10 volumes of TBS by resuspension followed by centrifugation as above.

To extract COMP, the tissue was resuspended in 10 volumes of TBS which also contained 10 mM EDTA and was incubated overnight at 4°C, with constant stirring. After sedimentation of the tissue residue, the EDTA extract was diluted...
with an equal volume of 10 mM Tris HCl, pH 7.4 and applied to a 100-ml DEAE-Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Tris HCl, 10 mM EDTA, 2 mM NEM, pH 7.4. After washing with equilibration buffer, adsorbed material was eluted with a 1,000-ml linear 0–0.5M NaCl gradient in the buffer. COMP-containing factions were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), were combined and dialyzed against 20 mM Tris HCl, 10 mM EDTA, 2 mM NEM, pH 7.4. The COMP pool was concentrated by adsorption to a 5-ml HiTrap Q column (Pharmacia) equilibrated with 20 mM Tris HCl, 10 mM EDTA, 2 mM NEM, pH 7.4, followed by elution with a 10-ml pulse of 0.5M NaCl in the buffer. The protein was recovered in a volume of 4 ml.

Further purification was done by gel filtration on a Superdex 200 HR 10/30 column (Pharmacia) equilibrated and eluted with 20 mM Tris HCl, 10 mM EDTA, 0.15M NaCl, 2 mM NEM, pH 7.4, at a flow rate of 0.5 ml/minute. COMP-containing factions were identified by SDS-PAGE. Because the resulting preparation still contained contaminating thrombospondin 1, the material was passed over a 5-ml HiTrap heparin column (Pharmacia) equilibrated with 20 mM Tris HCl, 10 mM EDTA, 2 mM NEM, pH 8. After this step, COMP that had been recovered in the flow-through was purified to apparent homogeneity, as judged by SDS-PAGE and Coomassie staining (results not shown). The recovery was ~15 mg, starting with 200 gm of chondrosarcoma tissue.

Preparation of COMP antibody. Antibodies against purified native rat COMP were raised in rabbits. Each rabbit was immunized with 100 μg of purified COMP in Freund’s complete adjuvant and, after 2 weeks, was given a booster injection of 100 μg of COMP in Freund’s incomplete adjuvant.

Immunoassay of COMP. Serum concentrations of COMP were determined by ELISA, using conditions similar to those described for the assay for human COMP (14). The assay was modified by using rat COMP to coat the microtiter plates and to prepare the standard curve included in each plate and by using the polyclonal antisera raised against rat COMP as the capture antibody. To facilitate solubilization of COMP and to dissociate it from any interaction with other proteins in the fluid analyzed (14), the assay was performed with the protein solubilized as an SDS complex.

Assay of dilutions of rat sera (n = 3) showed parallelism with the standard curve, indicating the absence of interfering substances in the serum both at low and high concentrations, as well as showing that the epitopes present in the serum are similar to those present in the reference standard (data not shown). The interassay percentage coefficient of variation (CV) for the serum samples was 8.3 ± 4.2 (mean ± SD; n = 20) and the intrassay CV was 5.2 ± 4.3 (n = 20). All samples from an individual animal were assayed on the same plate, and the maximum acceptable CV between triplicate samples was 10%. To investigate the recovery of COMP from serum, rat COMP in the range of 10–250 μg was added to serum and assayed. The percentage of recovery was 95.5 ± 3.5 (mean ± SD; n = 5).

Statistical analysis. Serum concentrations of COMP were compared using Student’s 2-tailed t-test, and correlations were calculated by regression analysis. P values less than 0.05 were considered significant.

RESULTS

Serum concentrations of COMP in normal rats. The serum concentrations of COMP were studied in DA, E3, and (E3 × DA)F1 rats (n = 6 per group) and b, individual (E3 × DA)F2 rats. Bars in a show the mean ± SD; each point in b represents 1 rat on day 6 after pristane injection, before the expected onset of pristane-induced arthritis.

Figure 1. Age dependence of serum levels of cartilage oligomeric matrix protein (COMP) in a, groups of DA, E3, and (E3 × DA)F1 rats (n = 6 per group) and b, individual (E3 × DA)F2 rats. Bars in a show the mean ± SD; each point in b represents 1 rat on day 6 after pristane injection, before the expected onset of pristane-induced arthritis.
strains, but the variability was, as expected, greater in the genetically heterogeneous F2 population.

Pristane-induced arthritis in (E3 × DA)F2 rats. The (E3 × DA)F2 rats were injected with pristane, and the subsequent disease course was monitored by macroscopic scoring 1–3 times a week for 125 days. Ninety-one (59%) of the 156 (E3 × DA)F2 rats developed arthritis. Different disease patterns were observed. Most commonly, arthritis appeared between days 14 and 35 after immunization, but the first signs of arthritis developed throughout the observation period. In 50% of the arthritic animals, the disease course was chronic and arthritis persisted during the whole scoring period. In 30% of the animals, complete recovery was seen after a few days to several weeks of disease. Fifteen rats had a relapsing disease course, with symptoms in previously clinically unaffected paws appearing as late as day 120 after immunization. The severity of arthritis also varied. Some rats had scores >10 for a long period, whereas others never had scores higher than 1 or 2.

Serum concentrations of COMP in (E3 × DA)F2 rats with PIA. The serum concentrations of COMP were determined in 156 (E3 × DA)F2 rats on days 6, 35, and 49 after pristane injection. Increased levels of COMP on day 35 compared with day 6 were found only in rats with arthritis. In the rats that did not develop arthritis, the concentration of COMP decreased. In arthritic rats with a score ≥4 on day 35 (n = 39), the serum concentration increased by 1.1 ± 1.4 μg/ml (mean ± SD) from day 6 to day 35 after pristane injection, whereas in rats that remained healthy throughout the entire scoring period (n = 65), the concentration decreased by 1.0 ± 0.6 μg/ml (P < 0.0001). The variation in COMP levels is largely dependent on the genetic heterogeneity and on the different ages of the rats at the time of arthritis induction. Young healthy rats showed a more pronounced decrease than did old rats, whereas the increase in COMP levels was most pronounced in the oldest arthritic rats (Figure 2). The arthritis score was similar for the different age groups.

The time course of COMP elevation with respect to arthritis onset was studied in arthritic rats on days 35 and 49. The rats were grouped according to the duration of clinically apparent joint swelling, which was then related to the concentration of COMP on days 35 and 49, respectively. Figure 3a indicates that the serum levels of COMP start to increase ~12 days after the onset of arthritis. The corresponding study performed on day 49 (Figure 3b) shows a similar time course. It is also apparent from Figure 3 that the concentrations of COMP reached a maximum after ~3 weeks and did not continue to increase thereafter.

To investigate whether serum COMP may give a quantitative estimate of the severity of arthritis, COMP concentrations on days 35 and 49 were related to the arthritis score on the corresponding days. The mean
concentration of COMP was calculated for rats with the same score. Figure 4 shows that the concentration of COMP correlated strongly with the severity of arthritis on both day 35 and day 49 (r = 0.95 and 0.90, respectively) (P < 0.0001).

Since the disease course varied among the individual animals, we examined whether serum concentrations of COMP differed between rats that recovered completely and rats that developed a chronic disease. Three rats had severe arthritis, with joint scores of 10–14 on day 21 and scores of 6–8 on day 35. The disease activity in these 3 rats subsequently declined, and the animals had completely recovered on days 50–70. The serum concentrations of COMP on day 35 in these rats were compared with those of 5 rats with a similar disease course and severity up to day 35, but with a subsequent chronic arthritis that persisted throughout the entire scoring period. On day 35, all animals that were examined had a similar duration of clinical arthritis and similar joint scores. Figure 5 shows that the 5 animals with a chronic disease course had higher serum levels of COMP than did the animals with a more benign arthritis (P < 0.01). This finding was further supported by the observation that all rats with elevated levels of COMP both on day 35 and day 49 (n = 13) had severe arthritis (joint score >10) at both time points, and the disease in these animals was still active on day 100 (joint score >10).

**DISCUSSION**

Joint cartilage, like other connective tissues, undergoes continuous remodeling throughout life. As a consequence, in the joint, cartilage matrix macromolecules are released into the synovial fluid and may subsequently reach the bloodstream. This scenario forms the rationale for efforts to identify changes in cartilage turnover by quantifying cartilage-derived tissue components in serum.

In the present study, we used a novel immunoassay for quantification of a primarily cartilage-derived matrix macromolecule, COMP, in the sera of healthy rats and rats with PIA. In healthy young rats, we found high serum concentrations of COMP, which rapidly decreased during growth. This finding is consistent with observations in growing children. It was found that in infants, there were high serum concentrations of aggrecan core protein epitopes and COMP (23), which decreased gradually, but most markedly, after puberty. For the epitope recognized by the monoclonal antibody 5-D-4 in keratan sulfate, a glycosaminoglycan side chain of aggrecan, a somewhat similar pattern is seen; however, the initial levels are low, which reflects the delayed expression of this aggrecan epitope (ref. 24 and Saxne T: unpublished observations). The high release of COMP during growth most probably reflects a physiologically high tissue turnover in growing individuals.

It might be hypothesized that the nature of the...
SERUM COMP LEVELS IN CHRONIC EROSIVE ARTHRITIS IN RATS

fragments released during growth differs from that of fragments released during arthritis; that is, in growing individuals, the release of newly synthesized molecules that are not incorporated in the matrix may be expected, whereas in RA or PIA, the release of structural molecules is more likely. However, at the current state of the technology, where a polyclonal antiserum is used, only quantitative changes can be detected. Since COMP is not exclusively found in articular cartilage, the influence of extraarticular sources—both cartilage and tendons as well as synovial tissue—on serum levels of COMP should be borne in mind when interpreting changes in concentrations. However, the synovial capsule of RA and OA patients contains COMP concentrations that are at least 100 times lower than the concentration in articular cartilage (Saxne T et al: unpublished observations), and increased serum concentrations are not seen during tendinitis in horses (25).

Interestingly, we found different levels of COMP in the different strains of rats tested, which indicates that the metabolism of COMP is genetically determined. This observation should be further explored. It might be one reason for the interindividual variation in serum levels in apparently healthy people as well as for the remarkably low intraindividual variation of serum COMP levels found in healthy persons monitored over several years as well as in RA patients with a stable disease course monitored over time (26).

The serum concentrations of COMP increased in rats that developed PIA. In contrast, the serum levels decreased in the pristane-injected rats that did not develop arthritis, corroborating the influence of growth on serum levels. Thus, the increase in serum COMP caused by arthritis is markedly underestimated in rats that are still growing. In future experiments, it would be advisable to select somewhat older rats to minimize the confounding effect of growth on serum levels of COMP.

Increased COMP levels were not observed until ~1 week after arthritis began, which suggests that the elevated COMP levels do not reflect the initial inflammatory process, but rather, the results of the subsequent involvement of cartilage. Indeed, histopathologic studies of PIA show that the first days of clinically apparent arthritis involve only pannus formation, without visible changes in cartilage and bone (22). Erosions of bone and cartilage are not detectable until a few days later, coinciding with the rise in serum levels of COMP. In humans with arthritis, serum COMP seems not to be closely associated with the degree of generalized inflammation, which corroborates the idea that serum COMP levels may reflect the destructive component of the disease (27).

The variable disease course in the (E3 × DA)F2 rats provided an ideal experimental setting for investigating the properties of arthritis that affect the serum concentration of COMP. We found high serum levels of COMP only in rats with chronic PIA, whereas in rats with a remitting disease course, the levels remained low. This shows that experimentally induced, well-defined arthritis developing initially with a similar clinical appearance may involve different pathologic mechanisms in the joint, and that increased serum levels of COMP are not merely a result of joint inflammation, but mirror a specific pathologic process in the cartilage involved in chronic erosive arthritis. This is consistent with observations in RA, in which high serum levels of COMP have been found in patients who subsequently developed severe damage of the large joints that required arthroplastic surgery (15). The consistency of the COMP increase in RA and PIA may indicate that similar pathogenic processes in the cartilage are taking place in RA and PIA.

Serum COMP concentrations correlated strongly with the severity of arthritis, as measured by the clinical score. This indicates that serum COMP may be a useful means of monitoring the arthritis as well as the tissue effects of therapeutic interventions. However, it also underlines the need for studies aimed at verifying the link between the pathologic process in the tissue and the elevated COMP levels in the serum. The observations of increased serum levels of COMP in PIA corroborate the findings in type II collagen–induced arthritis in rats, where increased serum concentrations of COMP, which correlated with the clinical joint score, were also found only at a time when histopathologic signs of cartilage erosion were present (28). The antibody used in that study apparently recognizes the same or closely related epitopes as the antibody used in the present study, since there was a close correlation between the results obtained with the two assays (results not shown).

This study shows that serum concentrations of COMP can be quantified in rat serum by immunoassay. Serum analyses of COMP offer promise as a novel, noninvasive tool for monitoring tissue involvement in experimental arthritis and should be useful for monitoring therapeutic interventions aimed at reducing tissue destruction. The application of molecular marker measurements in experimental arthritis represents a new possibility for verifying the utility of such measurements in the monitoring of human arthritis.
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