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Intraarticular α_2-Macroglobulin Complexes and Proteolytic Activity in Children with Juvenile Rheumatoid Arthritis

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ABSTRACT. In juvenile rheumatoid arthritis (JRA), it is likely that the release of proteolytic enzymes from activated synovial fluid neutrophils overwhelms the major protease inhibitor, α_2-macroglobulin (α_2-MG), and leads to cartilage destruction. Due to the unique nature of the α_2-MG–protease complex, proteolytic function is maintained until the complex is cleared. In this study, we sought to determine the concentration of α_2-MG–protease complexes in synovial fluid of patients with JRA, the proteolytic activity found in their synovial fluid, and whether the α_2-MG complexes are associated with increased proteolytic activity. The JRA patients' synovial fluids had complex levels of 217.0 ± 192.2 nmol/L—significantly elevated compared with plasma values ($p < 0.001$) and with control synovial fluid ($p < 0.05$). Elastase activity (almost entirely neutrophil elastase) was detectable in all JRA synovial fluid samples (mean 2.9 ± 2.6 mg/L) and significantly correlated with α_2-MG–complex values ($r = 0.67$, $p < 0.01$). Synovial fluid tryptic activity was detectable in all JRA patients but did not significantly correlate with α_2-MG complexes ($r = 0.53$, $p > 0.05$). Seventy-four percent of total elastase activity and 41% of total tryptic activity were contained in the α_2-MG–complex fractions. We suggest that the increased concentration of synovial fluid α_2-MG complexes with retained elastase activity contributes to continued proteolysis and joint destruction and may affect the subsequent disease course through its role as a modulator of IL-6. (*Pediatr Res* 34: 204–207, 1993)

Abbreviations

α_2-MG, α_2-macroglobulin
JRA, juvenile rheumatoid arthritis

Proteolytic enzymes in plasma are derived from the breakdown of bacterial and host cells and may be generated during coagulation, fibrinolysis, and phagocytosis (1). Large numbers of phagocytic cells are present in the synovial fluid of adults with rheumatoid arthritis (2, 3). In rheumatoid arthritis, synovial fluid neutrophils may be rapidly activated by the phagocytosis of cellular debris and immune complex aggregates resulting in the release of proteolytic enzymes and oxygen metabolites (4, 5). The release of these proteases may overwhelm the natural protease inhibitors, leading to cartilage destruction (6). On clinical, serologic, and immunogenetic grounds, JRA is distinct from adult rheumatoid arthritis and consists of several distinct subtypes. However, the synovial fluid and synovial lesions are indistinguishable (7).

The major protease inhibitor found in plasma is α_2-MG (1). Binding of proteases to a bait region on α_2-MG initiates a conformational change, during which the protease becomes trapped within the α_2-MG molecule (8, 9). This change reveals a receptor-recognition site that permits rapid and selective clearance of α_2-MG complexes from the circulation by the reticuloendothelial system. Consequently, all of the α_2-MG found in the systemic circulation is believed to exist in the uncomplexed form (10, 11). Due to the unique nature of the protease–α_2-MG complex, the proteolytic function is maintained until the complex is cleared (12). The mechanism involved in the clearance of synovial fluid α_2-MG complexes is unknown, although it probably is via macrophage uptake (13).

Abbink *et al.* (14) have recently shown that synovial fluid α_2-MG is inactivated by complex formation with neutrophil proteases and reactive oxygen species. The authors hypothesize that inactivation of α_2-MG through the release of reactive oxygen species and proteases by neutrophils contributes to tissue damage by facilitating the action of uninhibited proteoglycan-degrading proteases. In this study, we sought to determine the concentration of α_2-MG complexes in synovial fluid of patients with JRA, the proteolytic activity found in their synovial fluid, and whether the α_2-MG complexes are associated with increased proteolytic activity.

MATERIALS AND METHODS

Subjects. Synovial fluid was obtained from 18 patients (12 females, six males; mean age 8.4 y, range 2.0 to 18.9) who satisfied diagnostic criteria for JRA and had active disease (13 pauciarticular, five polyarticular-1 with associated psoriasis). Control synovial fluid ($n = 4$) was obtained from three adult patients with osteoarthritis and one child with reflex sympathetic dystrophy. The synovial fluid was obtained by arthrocentesis. Plasma was obtained from nine patients with active, polyarticular JRA. Control plasma was obtained from 100 consecutive children admitted for elective surgery to the Schneider Children's Hospital and from seven adult volunteers. Samples were obtained using polypropylene syringes containing 55 U heparin (Elkins-Sinn, Cherry Hill, NJ)/mL blood. An anticoagulant mixture was prepared according to the method of Cronlund *et al.* (15), and

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heparinized samples were immediately transferred to new polypropylene tubes containing 111 mL of anticoagulant/L to prevent the *in vitro* generation of α_2-MG complexes. The samples were then centrifuged at 2000 × *g* and stored at −70°C. The study protocol was approved by the Human Subjects Review Committee of Long Island Jewish Medical Center.

ELISA for α_2-MG complexes. An MAb was obtained from the fusion of spleen cells of mice immunized with methylamine-treated α_2-MG with a myeloma cell line. A competitive binding assay demonstrated that the antibody was specific for a neoantigen expressed on α_2-MG when the inhibitor reacts with proteases or with methylamine (16). In addition, we have demonstrated that the ELISA is specific for α_2-MG complexes and not for native α_2-MG (17).

An ELISA was developed by coating microtiter wells with the antibody and residual binding sites with 2% BSA. Samples were then added to the wells, incubated with rabbit IgG–anti-human α_2-MG (Boehringer Mannheim, Indianapolis, IN), and detected with an IgG–anti-rabbit alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO). A standard curve using α_2-MG–complex standards was generated on each plate. The results did not vary by more than 20%, using two dilutions within the standard curve generated on each plate. All determinations were performed in triplicate with the resulting mean ± SEM taken as the value for that patient.

Elastase activity. An assay for elastase activity was prepared by using a modification of the method of Tanaka *et al.* (18). A standard curve was prepared using porcine pancreatic elastase (Sigma) diluted 1:10 000 in Tris buffer (17.76 g of Tris HCl and 10.6 g of Tris base/L, pH 8.0) with 10 mL of 1% BSA/L. A 25-nM solution of the synthetic substrates N-succinyl-(L-ala)$_3$-*p*-nitroanilide (pancreatic elastase, Sigma) and N-methoxysuccinyl-L-ala-L-ala-L-pro-L-val-*p*-nitroanilide (most sensitive to levels of neutrophil elastase, although there was a slight response to pancreatic elastase; Sigma) in DMSO (Sigma) was prepared. Ten μL of substrate were added to 180 μL of Tris solution before incubation. Elastase levels were assessed by measuring the rate of hydrolysis of the substrates over time after the addition of a 20-μL sample and expressed as ng/mL. Neutrophil elastase activity was determined by comparing results obtained from the two different substrates. Standards were assayed in duplicate and specimens in triplicate with the resulting mean ± SEM taken as the value for that patient.

Tryptic activity. A modification of the method of Hummel (19) was used to determine the tryptic activity in plasma and synovial fluid. In this modification, 2.6 mL of a Tris-base solution (0.04 M, pH 8.1) containing 0.0115 M $CaCl_2$ was used to make a solution (0.001 M) of the low-molecular-weight synthetic substrate *p*-tosyl-L-arginine methyl ester (Sigma) that is not specific but most sensitive to overall trypsin activity. To this mixture, 50 μL of plasma were added at 30°C and the solution was immediately pipetted into a 3.0-mL quartz cuvette and placed in a Perkin-Elmer UV/VIS spectrophotometer (Oak Brook Instruments, Oak Brook, IL). The rate of hydrolysis of *p*-tosyl-L-arginine methyl ester was determined by the increase in absorbance at 247 nm; the enzyme activity was expressed as the change in OD/h/mL fluid with results given as mean ± SEM.

Column fractionation. Synovial fluid from seven patients who had elevated concentrations of α_2-MG complexes was separated using a Sephacryl 300 (Pharmacia, Piscataway, NJ) gel filtration column (60 × 1.6 cm). The gel was equilibrated and elution performed with 50 mM Tris-HCl (pH 7.6) containing 0.14 M NaCl. One-mL samples were applied to the column, and 2-mL fractions of the eluant were collected. The column was calibrated with blue dextran (2000 kD), α_2-MG (720 kD), BSA (66 kD), and vitamin B_{12} (1 kD) (Sigma). Samples beginning with the void volume were pooled in groups of three (6 mL) and concentrated to 1 mL using a Minicon Macrosolute concentrator (Amicon, Danvers, MA). Fractions corresponding to α_2-MG complexes and uninhibited proteases were collected separately and analyzed to determine whether increased proteolytic activity was associated with the complexes or with free proteases.

Statistics. Results from patient groups were compared by the Wilcoxon rank sum test and corrected for multiple-group comparisons, where applicable. The significance of an association between α_2-MG complexes and proteolytic activity was determined by Spearman rank correlation.

RESULTS

α_2-MG complexes. The mean childhood control plasma level (mean ± SEM) was 10.1 ± 8.7 nmol/L of plasma. This value is significantly elevated ($p < 0.01$) compared with adult levels (4.7 ± 1.9 nmol/L). The mean plasma complex level from JRA patients was 8.7 ± 5.1 nmol/L, not significantly different from controls (Fig. 1). The mean concentration of α_2-MG complexes in the synovial fluid of the four control patients was 17.4 ± 4.8 nmol/L. In contrast, the mean concentration of α_2-MG complexes in synovial fluid of the patients with JRA was 217.0 ± 192.2 nmol/L (Fig. 1). These values were significantly elevated when compared with the plasma results from controls and JRA patients ($p < 0.001$) and with the concentration of complexes in control synovial fluid ($p < 0.05$). Two patients with pauciarticular disease and one with polyarticular disease had values in the range of the control patients (mean 11.6 ± 3.8 nmol/L), whereas 15 patients (11 with pauciarticular and four with polyarticular disease) had markedly elevated values (mean 258.1 ± 184.9 nmol/L). The three patients whose synovial fluid α_2-MG–complex values were not significantly elevated subsequently went into remission, whereas 11 of 15 children with elevated complex values continued with active disease (average follow-up 1.7 y, range 0.8–2.5 y, Table 1). There was no significant correlation between the levels obtained from the four patients who went into remission compared with the levels seen in the patients who remained with active disease ($p > 0.05$).

Synovial fluid proteolytic activity. Elastase activity (almost entirely consisting of neutrophil elastase rather than pancreatic elastase) was detectable in all JRA synovial fluid samples (mean 2.9 ± 2.6 mg/L). The three patients with complex concentrations in the control range had neutrophil elastase values of 0.2 ± 0.1 mg/L compared with 3.4 ± 2.5 mg/L for the 15 patients with elevated levels of α_2-MG–protease complexes. The elastase activity significantly correlated with the concentration of α_2-MG complexes ($r = 0.67$, $p < 0.01$).

Synovial fluid tryptic activity was detectable in all patients with JRA, corresponding to a concentration of 20 to 30 mg of trypsin/mL. The three patients with complex concentrations in the range of the control synovial fluid had detectable but de-

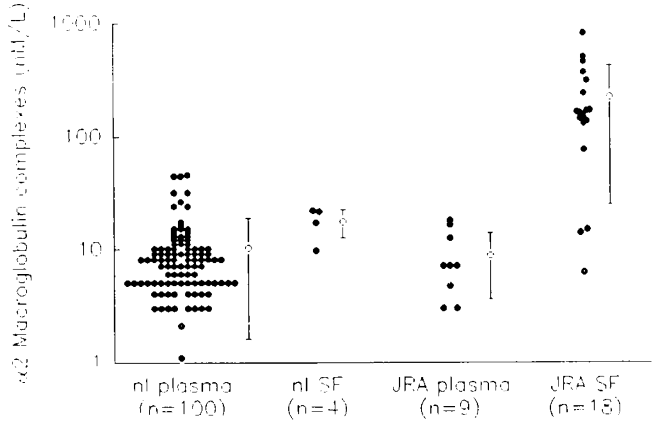

Fig. 1. Concentration of α_2-MG complexes (nmol/L) in control plasma (*nl plasma*, $n = 100$), control synovial fluid (*nl SF*, $n = 4$), plasma from patients with JRA (*JRA plasma*, $n = 9$), and synovial fluid from patients with JRA (*JRA SF*, $n = 18$). *Black circles* represent individual values; *white circles* and *error bars* represent means ± SEM.

Table 1. *JRA patients, synovial fluid α_2-MG complexes, and clinical outcome**

Patient	Diagnosis	α_2-MG Complex (nmol/L)	Outcome
1	Pauci	156.2	Remission
2	Pauci	302.4	Active
3	Poly	441.0	Active
4	Pauci	353.2	Active
5	Pauci	162.5	Active
6	Pauci	792.7	Remission
7	Poly-psoriatic	484.3	Active
8	Poly	232.9	Active
9	Pauci	6.2	Remission
10	Pauci	13.8	Remission
11	Pauci	159.3	Active
12	Pauci	127.9	Remission
13	Poly	165.5	Active
14	Pauci	75.1	Active
15	Poly	14.8	Remission
16	Pauci	140.1	Active
17	Pauci	132.5	Remission
18	Pauci	145.4	Active

* Pauci, pauciarticular disease; poly, polyarticular disease.

Table 2. *Total elastase activity in synovial fluid and in fractions corresponding to α_2-MG complexes and to uninhibited proteases, after molecular weight sieve chromatography**

Patient (diagnosis)	Total elastase (mg/L)	α_2-MG fraction activity (% total)	Free protease activity (% total)
1 (pauci)	6.2	70.9	15.7
2 (pauci)	3.1	69.5	14.2
3 (poly)	1.5	79.7	2.9
4 (pauci)	1.9	69.8	11.6
5 (pauci)	1.3	75.6	1.7
7 (poly)	3.8	91.1	1.1
8 (poly)	1.0	62.2	15.5
Mean ± SEM	2.7 ± 0.7	73.5 ± 3.5	8.4 ± 2.6

* Abbreviations are the same as those in Table 1.

creased proteolytic activity compared with those patients with higher concentrations of α_2-MG complexes (5.1 ± 1.9 ΔOD/h/mL *versus* 20.7 ± 19.3, $p < 0.05$). Tryptic activity (change in OD) correlated with the concentration of α_2-MG complexes with a coefficient of 0.53, although it did not reach statistical significance ($p < 0.1$).

Proteolytic activity associated with α_2-MG complexes. ELISA determination of all fractions after column fractionation demonstrated a recovery of 85.4% of the total α_2-MG complexes. Elastase activity was determined from all fractions separately to determine the percentage of total synovial fluid activity associated with the α_2-MG complex fraction. In the patients with elevated α_2-MG complex levels (and increased elastase activity), 73.6% of total elastase activity was contained in the α_2-MG–complex fractions, whereas only 8.4% of elastase activity was associated with fractions that contained free proteases (Table 2). The percentage of elastase that was found in the α_2-MG–complex fraction was significantly correlated with the total elastase activity ($r = 0.86$, $p < 0.05$). Tryptic activity was also analyzed from the different fractions, with 41% of total activity associated with the α_2-MG–complex fraction. However, the change in OD determined from the α_2-MG complexes did not significantly correlate with total tryptic activity ($r = 0.4$, $p > 0.1$, data not shown).

DISCUSSION

Progressive joint destruction in rheumatoid arthritis may be related to proteolytic enzyme release and damage to articular cartilage. As the major protease inhibitor, α_2-MG is postulated to play a pivotal role in the balance between active protease release and clearance. The formation of α_2-MG complexes leads to a conformational change in α_2-MG, facilitating rapid and selective clearance by the reticuloendothelial system (10, 11). Due to the unique nature of the α_2-MG–protease interaction, complexes retain enzymatic activity against low-molecular-weight substances (12). Therefore, effective inhibition requires both rapid binding and rapid clearance of the protease complexes. In rheumatoid arthritis, α_2-MG, as a major inhibitor of elastase and cathepsin G (20, 21), can function in joint protection. In addition, α_2-MG has been shown to function as a modulator of IL-6, which is present in high levels in synovial fluid from patients with rheumatoid arthritis (22, 23).

We have demonstrated that synovial fluid from patients with JRA have significantly increased concentrations of α_2-MG complexes. Our results are similar to the findings of Abbink *et al.* (14), who demonstrated increased concentrations of α_2-MG complexes in the synovial fluid from adult patients with rheumatoid arthritis. In addition, similar to findings in adult rheumatoid arthritis (24), we have demonstrated that synovial fluids from patients with JRA have increased proteolytic activity in large part due to neutrophil elastase. Finally, we have shown that a major part of this increased synovial fluid proteolytic activity (especially that due to neutrophil elastase) is due to the α_2-MG–protease complexes rather than to free, uninhibited proteases. The increased elastase activity associated with the complexes occurred both in patients with pauciarticular as well as polyarticular disease and did not correlate with the actual α_2-MG complex level or with the clinical outcome (Tables 1 and 2). This suggests that tissue damage in JRA may be due to the continued high synovial fluid concentrations of α_2-MG complexes rather than uninhibited proteolysis after α_2-MG inactivation as suggested by Abbink *et al.* (14), although other factors may be involved to explain the lack of association with disease outcome. The possible mechanisms involved in joint destruction from α_2-MG complexes may be through the retained enzymatic activity of the complexes to small-molecular-weight substances (12), leading to direct joint damage, or by changes in the balance between proteases and other smaller-molecular-weight antiproteases. A second possibility is that because α_2-MG has been shown to function as a modulator of IL-6 (22), increased α_2-MG bound to proteases may affect that role, leading to changes in activity of IL-6 and thereby affecting joint inflammation.

Clotting of samples leads to the activation of several proteolytic enzymes that might serve as a source of α_2-MG complexes (25). To prevent *in vitro* protease generation and complex formation, we collected our samples with a method previously tested and found capable of preventing clot formation (15). Blood collected by this method did not contain detectable levels of fibrinopeptide A, suggesting that proteolytic cleavage does not occur. In addition, we have used this method previously (26) and demonstrated that this method of collection did not lead to the generation of proteases that stabilize α_2-MG. We have also previously demonstrated (17) that the method used, including sodium citrate, prevented the development of complexes from blood coagulation. Finally, the same method of blood sampling was used in all groups. Based on these considerations, we believe that the method of sample collection could not explain the results and the differences noted in this study.

It is interesting to note that two of three JRA synovial fluids with low concentrations of α_2-MG complexes and low proteolytic enzyme activity were from patients with pauciarticular disease. Neutrophil and β_2-microglobulin levels have been found to be lower in pauciarticular patients compared with those with polyarticular disease and has been postulated to identify patients with decreased risk of severe erosive joint destruction (27). It should also be noted that all three patients with synovial fluid α_2-MG complex values in the control range achieved remission over the follow-up period. The concentration of α_2-MG complexes may

similarly be useful in prognostication regarding the risk of erosive disease. Long-term follow-up of our patients and further study is necessary to determine if this is the case.

In this study, we have also demonstrated that normal control children have significantly increased plasma concentrations of α_2-MG complexes compared with adults. Plasma levels of α_2-MG complexes reflect a balance between complex formation and the rapid reticuloendothelial clearance of these complexes (10, 11). These results confirm our previous findings (using an indirect method to assess plasma levels of α_2-MG complexes) that healthy 3-d-old infants have elevated levels of complexes compared with adults (26). We suggest that increased plasma concentration in newborns may be a response to excessive protease uptake across an immature intestinal barrier (28) or may reflect delayed clearance of these complexes by the reticuloendothelial system. Future studies will be needed to assess α_2-MG–complex clearance in children and to determine whether the increased plasma levels of complexes lead to increased plasma proteolytic activity.

These data suggest that neutrophil activation in JRA leads to release of proteolytic enzymes including elastase into the synovial fluid. Proteases are rapidly bound to α_2-MG, but α_2-MG complexes accumulate due to poor clearance from the joint. The increased concentration of synovial fluid α_2-MG complexes with retained proteolytic activity contributes to continued proteolysis and joint destruction and may affect the subsequent disease course through its role as a modulator of IL-6 (22). Future studies, including serial measurements of synovial fluid α_2-MG complexes and proteolytic activity and the correlation between those results and disease activity, will be important in determining the value of an individual determination in a child with JRA. Therapeutic interventions that increase clearance of α_2-MG complexes or provide other intraarticular protease inhibitors that function without retaining proteolytic activity (α_1-protease inhibitor) may prove useful in JRA.

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