Synovial hyaluronate in rheumatoid arthritis binds C1q and is covalently bound to antibodies: a model for chronicity

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Acute inflammation of joints can lead to the development of chronic arthritis. The nature of agents that sustain the chronic inflammation remains an enigma, although infectious agents such as *Rubella* virus, Epstein-Barr virus or *Chlamydia* have been invoked. In most cases in which an inducing agent was sought, none was found.¹ However, several antigens have been identified that elicit acute arthritis in animal models. These include a 65 kDa *Mycobacterium tuberculosis* protein and isolated cell walls from streptococci. Increased titres against these antigens are also found in patients with rheumatoid arthritis. In addition, their sera cross react with cartilage specific collagen type II, link protein, proteoglycans, and heat shock proteins.² It is clear that these antigens are involved in acute arthritis in animal models, but it is not understood how they maintain the inflammatory response.

Continuous production of chemotactic factors is necessary to attract granulocytes into the inflamed area. These chemotactic factors might be complement components that are produced during inflammation. The complement cascade can be initiated by the binding of C1q to multimeric antigen-IgG complexes; such structures can be produced continuously in the synovial membrane. It is known that immune complexes in synovial fluid bind C1q and activate complement,³ resulting in decreased complement activity^{4,5} and an increased level of terminal complement complexes.⁶ However, no antigenic components are detectable in association with these immune complexes.^{7–12}

The possibility was addressed that the critical antigens in chronic arthritis are generated continuously during the course of the inflammatory reaction. It is probable that a component specifically enriched in the joints, such as hyaluronate, is the key culprit, because hyaluronate is extensively degraded in the synovial membrane by the inflammatory reaction.¹³ The synovial fluids of patients with rheumatoid arthritis were therefore analysed for C1q binding immune complexes.

Materials and methods

Synovial fluid, obtained from patients with rheumatoid arthritis, was kindly supplied by Drs R Miehke and S Schneider, Rheumaklinik Sendenhorst. Normal synovial fluid was from cadavers 24 hours postmortem, supplied by

Dr W Böcker, Institut für Pathologie, Münster. Iodine-125 was from Amersham International. All other reagents were from Sigma Chemical Co, St Louis.

C1q was labelled with iodine-125 by the method of Zubler *et al.*¹⁴ Glucuronic acid was determined by the procedure of Bitter and Muir.¹⁵ Western blotting was performed by the method of Tsang *et al.*¹⁶ Peptides were sequenced using a gas phase sequencer, Model 477A from Applied Biosystems. Amino acids were analysed by the technique of Chang *et al.*¹⁷ A synthetic peptide was kindly prepared by Dr B Schmidt, University of Göttingen. The peptide was coupled to ovalbumin by the method of Kitagawa and Aikawa¹⁸ and an antiserum against the peptide-ovalbumin complex was raised in rabbits.

SEPARATION OF SYNOVIAL FLUID COMPONENTS

Synovial fluid was clarified by centrifugation at 2000 *g* for 10 minutes. The supernatant (1 ml) was diluted with 50 ml of 10 mmol/l Tris-HCl pH 8.4, 110 mmol/l NaCl and applied to a DEAE-Sephacel column (2.6 × 30 cm). The components were eluted by a linear gradient of NaCl from 110 to 430 mmol/l in 10 mmol/l Tris-HCl pH 8.4 in a total volume of 1 litre. Fractions of 20 ml were collected and analysed for C1q binding activity and glucuronic acid content. The C1q positive fractions were pooled, layered on a 5 ml cushion of 0.5 mol/l NaCl and ultracentrifuged for 16 hours at 28 000 rpm in a Beckman SW28 rotor. The supernatant was removed and the jelly like pellet suspended in 1 ml of phosphate buffered saline (PBS).

DETECTION OF SOLUBLE IMMUNE COMPLEXES

An indirect method was used for the detection of immune complexes using a modification of the method by Zubler *et al.*¹⁴ Human IgG was dissolved in PBS at 3 mg/ml and heat aggregated at 63°C for 20 minutes. The heat aggregated IgG solution (20 µl) was mixed with 50 µl of the eluate fractions from the DEAE-Sephacel column and 140 µl of 0.2 mol/l EDTA pH 7.5, 0.4% Tween 20. The mixture was incubated at 37°C for 30 minutes and chilled on ice. A solution of 50 µl ¹²⁵I-labelled C1q (0.15 µg; 220 000 cpm) was added, followed by 1 ml of 3% polyethylene glycol in 0.1 mol/l boric acid, 25 mmol/l

sodium borate pH 8.3, and 75 mmol/l NaCl. The solution was kept at 4°C and centrifuged at 1500 *g* for 20 minutes at 4°C. The supernatant was discarded and the radioactivity of the sediment determined.

PURIFICATION OF HYALURONATE FROM SYNOVIAL FLUID

Hyaluronate was purified from synovial fluid under dissociative conditions by a modification of the method of Sher and Hamerman.¹⁹ Synovial fluid (5 ml) was clarified by centrifugation for five minutes at 10 000 *g* and dialysed overnight against 1 litre of 20 mmol/l Tris-malonate pH 7.0. Guanidinium chloride (7.7 g) was added to the dialysate and the solution diluted to 20 ml with water. Caesium chloride (CsCl) (12 g) was dissolved in this solution and the mixture centrifuged in a Beckman Ti70 rotor at 40 000 rpm for 44 hours at 20°C. The self forming gradient was eluted from the bottom in 3 ml fractions (fig 1A). Aliquots of 200 µl were dialysed against 20 mmol/l Tris-HCl pH 7.0 and their glucuronic acid content determined. To dissociate the hyaluronate from all non-covalently bound proteins,²⁰ the fractions containing glucuronic acid were pooled, diluted with CsCl (0.6 g/ml) in 4 mol/l guanidinium chloride and subjected to the same centrifugation. Fractions containing glucuronic acid were pooled and dialysed against water (fig 1B). Hyaluronate was precipitated by addition of four volumes of ethanol and dissolved in 2 ml of water.

Figure 1 Purification of hyaluronate from synovial fluid. A: Three millilitre fractions were collected from ultracentrifugation of synovial fluid on a dissociative CsCl gradient. B: Fractions 5 to 7 collected from A were subjected to this second ultracentrifugation, from which fractions 5 and 6 were collected and subsequently analysed by gel electrophoresis.

IODINATION AND ANALYSIS OF C1q PROTEINS

Hyaluronate samples containing about 10 µg of protein in 30 µl were mixed with 30 µl 0.25 mol/l sodium phosphate pH 7.5, 10 µl of ¹²⁵I-labelled sodium iodide (1 mCi), 30 µl chloramine T (5 mg/ml) in 50 mmol/l sodium phosphate pH 7.5, 200 µl sodium thiosulphate (1.2 mg/ml) and 400 µl NaI (2 mg/ml). Samples were boiled in a disintegration buffer containing sodium dodecylsulphate and 2-mercaptoethanol to disperse non-covalently linked molecules.²¹ Half of the samples were digested with 0.5 mg of testicular hyaluronidase at 37°C for one hour. Aliquots of the hyaluronidase treated and untreated samples were adsorbed to 70 µg of Protein A-Sepharose in 50 µl of PBS for one hour at 4°C, washed three times with cold PBS and subjected to gel electrophoresis on 10% polyacrylamide.

ISOLATION AND SEPARATION OF HYALURONATE PEPTIDES

Synovial fluid samples were obtained from two patients with rheumatoid arthritis and 0.5 ml was diluted with 45 ml of 10 mmol/l phosphate buffer pH 7.2, 100 mmol/l NaCl. A solution of 10% cetylpyridinium chloride (0.5 ml) was added and incubated at 37°C for 30 minutes. The precipitate was removed by centrifugation for five minutes at 10 000 *g* and dissolved in 2 ml of 0.5 mol/l NaCl by ultrasonication. Undissolved material was sedimented at 10 000 *g* for five minutes and 8 ml of ethanol added to the supernatant. The solution was kept at -20°C overnight and centrifuged at 1500 *g* for five minutes. The precipitate was dissolved in 1 ml of 10 mmol/l cysteine, 1 mmol/l EDTA pH 7.6, 50 µg pronase and digested overnight at 37°C. The hyaluronate was precipitated by addition of 8 ml of ethanol and standing overnight at -20°C. After centrifugation for five minutes at 1000 *g*, the sediment was dissolved in 1 ml of 10 mmol/l cysteine, 1 mmol/l EDTA pH 5.5 and residual pronase was inactivated at 100°C for five minutes. Testicular hyaluronidase (0.1 mg, 72 U), β-N-acetyl glucosaminidase (5 µg, 0.05 U) and β-glucuronidase (5 µg, 50 U) were added and the solution incubated overnight at 37°C. The solution was acidified with 10 µl of trifluoroacetic acid, centrifuged at 10 000 *g* for three minutes and the supernatant applied to a C₁₈-reverse phase HPLC column (VYDAC 218 TP54). Peptides were eluted by a linear gradient of 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 70% acetonitrile over 30 minutes with a flow rate of 1 ml/min. The elution was monitored at 220 nm.

DERIVATION OF HYALURONATE PEPTIDES

The analysis described above yielded one predominant peptide in each patient synovial sample. Peptide 1 (from patient 1) was subjected to sequence analysis and subsequently synthesised with an additional cysteine at the N-terminal site. The peptide was coupled to

ovalbumin through the free sulphhydryl group of cysteine, and antibodies were raised in rabbits. The antiserum was used to identify the origin of the peptide. Proteins from 2 μ l of human serum were separated on sodium dodecyl sulphate-polyacrylamide gels, transferred to nitrocellulose and stained in Western blots with antibodies against the synthetic peptide. Additional protein samples were first absorbed to a 1:1 suspension of 200 μ l Protein A-Sepharose in PBS for one hour at room temperature, then the Protein A-Sepharose was removed by centrifugation and washing with PBS; adsorbed proteins were solubilised by boiling in disintegration buffer before being applied to the electrophoretic gel. The supernatant from this Protein A-Sepharose step was also subjected to electrophoresis.

Results

ISOLATION OF THE C1q BINDING COMPONENT

Preliminary experiments indicated that a C1q binding component could bind to DEAE-Sephacel at low ionic strength; a high ratio of DEAE-Sephacel to synovial fluid was required to adsorb the activity. Separation of the components of the synovial fluid on a DEAE-Sephacel column with a linear salt gradient revealed two C1q binding fractions (fig 2): the first coeluted with hyaluronate; the second coincided with unknown components adsorbing at 280 nm, and was not analysed further. When the hyaluronate fractions were collected and treated with hyaluronidase, pronase or DNase, C1q binding was abolished by hyaluronidase and pronase, but not by DNase treatment.

Ultracentrifugation of pooled hyaluronate fractions under conditions which cleared the solution of aggregates sedimenting faster than 15 s produced a jelly like precipitate which contained the total soluble C1q binding activity, 17% of total hyaluronate and 21% of total proteins. Normal synovial fluid subjected to the same separation contained no C1q binding activity in the ion exchange eluate or after ultracentrifugation.

Figure 2 Components of synovial fluid separated on a DEAE-Sephacel column by a linear salt gradient (NaCl). C1q = C1q binding fraction; OD₂₈₀ = unknown components absorbing at 280 nm.

ANALYSIS OF C1q PROTEINS

Figure 3 shows the results of the electrophoretic separation of the C1q proteins. Lane A shows a protein of molecular mass 67 kDa that may be albumin and with radioactivity on top of the concentrating gel. After hyaluronidase digestion, additional proteins appeared which migrated at 51 kDa, 29 kDa, and 23 kDa (lane B), suggesting that these proteins were covalently bound to hyaluronate. Gel electrophoresis of aliquots of samples adsorbed to Protein A-Sepharose again revealed the 67 kDa protein in the undigested sample (lane C); in the hyaluronidase treated sample, only the proteins of 51 kDa, 29 kDa and 23 kDa molecular mass were adsorbed (lane D). The 51 kDa and 23 kDa proteins comigrated with the heavy and light chains of human IgG. When normal synovial fluid was similarly processed (lanes G and H), only the 67 kDa protein was labelled and the labelling did not alter after hyaluronidase digestion.

PURIFICATION OF HYALURONATE FROM SYNOVIAL FLUID

In the above experiment the hyaluronate-IgG complex withstood boiling in sodium dodecylsulphate and mercaptoethanol—conditions sufficient to disrupt most, but not all covalent bonds. The hyaluronate preparation purified by two successive centrifugations to dissociate it from all non-covalently bound proteins²⁰ (fig 1) contained 15% protein in the second gradient. In gel electrophoresis, radioactive material appeared on top of the concentrating gel and at 67 kDa (fig 3, lane E), and after hyaluronidase treatment, additional proteins migrated at 51 kDa, 40 kDa, 29 kDa and 21 kDa (fig 3, lane F), indicating again that the latter proteins were covalently bound to hyaluronate.

Figure 3 Protein pattern of the C1q binding material. Separation of radio-iodinated C1q binding material by gel electrophoresis on 10% polyacrylamide before (lane A) and after (lane B) digestion with hyaluronidase. Separation of samples adsorbed to Protein A-Sepharose directly (lane C) or after hyaluronidase digestion (lane D). Gel electrophoresis separation of hyaluronate dissociated from non-covalently bound protein (fractions 5 and 6 from figure 1B), without (lane E) or with (lane F) hyaluronidase digestion. Analysis of normal synovial fluid without (lane G) and with (lane H) hyaluronidase treatment.

DERIVATION OF HYALURONATE PEPTIDES

A major peptide was isolated from the synovium samples of each of the two patients. The sequence analyses were: peptide 1 (patient 1): WPGPPKYEIM; peptide 2 (patient 2): GPGGP. Figure 4 shows the Western blot analysis of the synthesised peptide 1. A 51 kDa protein (lane A) was recognised, which was removed from the serum by Protein A-Sepharose (lane B).

Discussion

In conventional C1q binding assay procedures, immune complexes are precipitated with polyethylene glycol, and as hyaluronidase-containing complexes are likely to remain in the soluble fraction under these conditions, they would escape detection. A modified assay was therefore developed which depended on competitive binding of C1q to a mixture of these soluble complexes with heat aggregated IgG, and subsequent detection using precipitation by polyethylene glycol.

Johnston²² observed that hyaluronate from rheumatoid synovial fluid consisted of fast and slow sedimenting fractions. Normal hyaluronate had a sedimentation rate of about 5 s. A fast sedimenting fraction was not found in normal synovial fluid in either the present study or that of Johnston. In the present study, the pellet produced by an ultracentrifugation which cleared aggregates larger than 15 s was found to contain all the C1q binding activity, 21% of total proteins, and 17% of total hyaluronate. This suggested that the hyaluronate was present in the pellet as part of a large aggregate.

The aggregate contained one non-covalently bound protein of 67 kDa, and three firmly bound proteins of 51, 29, and 21 kDa. The complex was not disrupted by conditions known to dissociate most covalent bonds.²¹ Isolation of hyaluronate by a method shown to dissociate all non-covalently bound proteins,²⁰ and analysis of the proteins after digestion with hyaluronidase again demonstrated 51 and 21 kDa proteins. As these proteins were liberated under treatment with hyaluronidase, they thus appeared to have been bound to hyaluronate by some covalent linkage. They also bound to Protein A, which suggested that they may represent the heavy and light chains of human IgG.

It was possible to isolate a hyaluronate bound peptide fragment, analyse its amino acid sequence, synthesise it, and identify its origin by raising antibodies to the synthetic form. A 51 kDa protein was recognised in human sera that could be adsorbed to Protein A-Sepharose, indicating that the peptide was derived from the heavy chain of IgG.

It has already been documented that hyaluronate from pathological synovial fluid is firmly bound to proteins.^{19 23-25} These proteins have been identified as IgG,^{23 26} acute phase proteins such as α_1 -protease inhibitor, inter- α 1-trypsin inhibitor, and haptoglobin.^{27 28} Recent studies also suggested that hyaluronate can be bound to proteins^{29 30} or to a serum

protein.³¹ Detailed analysis of a similar serum derived hyaluronate associated protein revealed that the heavy chain of inter- α -trypsin inhibitor can form stable complexes with hyaluronate.^{32 33} In addition, chondroitin 4 sulphate is able to bind covalently to inter- α -trypsin inhibitor by a covalent linkage between C-6 of an internal N-acetylgalactosamine and the α -carbon of its C-terminal asparagine.³⁴

It is thus possible that synovial antibodies are covalently coupled to hyaluronate by an unknown inflammatory reaction. The linkages of IgG to hyaluronate may create new antigenic sites and elicit antibodies. When these antibodies in turn recognise their target antigen on hyaluronate, they are likely to be coupled covalently to hyaluronate and become antigenic themselves. Thus an acute inflammation in the joint could lead into a chronic phase during which antibodies are formed against the hyaluronate-IgG complex which binds C1q to initiate the complement cascade. It is therefore necessary to analyse in detail the immunological reactions of the hyaluronate-containing immune complexes.

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Figure 4 Recognition of IgG by antibodies against the synthetic peptide: Western blot analysis of proteins, stained with antiserum against the synthetic peptide. Lane A: Detection of a 51 kDa protein, which was removed from the serum by Protein A-Sepharose treatment (lane B). Lane C = Supernatant from the Protein A-Sepharose procedure.

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