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Protease inhibitors in inflammatory synovial effusions

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SUMMARY Granulocyte lysosomal enzymes can potentially participate in cartilage degradation in inflammatory arthritides. However, we have previously shown that the quantity of several such enzymes in an inflammatory synovial effusion correlates negatively with the degree of radiographic damage of the joint from which the fluid was sampled. In the current work the quantity of the following 5 protease inhibitors was determined immunochemically in the same fluids: α_1 antitrypsin, α_1-antichymotrypsin, α_2-macroglobulin, inter-α-trypsin inhibitor, and C1 esterase inhibitor. These inhibitors are generally covariate and correlate positively with the total protein in the fluid as well as the number of granulocytes and the concentration of granulocyte lysosomal enzymes in the fluid. As did the lysosomal enzymes, the protease inhibitors correlate negatively with radiographic destruction. It is likely that lysosomal enzymes in solution in inflammatory synovial effusions are rendered effete by the presence of protease inhibitors.

One theory of the pathogenesis of articular destruction in rheumatoid arthritis holds that polymorphonuclear neutrophil (PML) lysosomal enzymes in solution in the inflammatory synovial fluid are primarily responsible for cartilage degradation.[1] If this were so, one would predict that the quantity of active PML lysosomal enzymes in synovial fluid would correlate with the degree of joint destruction. In a recent study[2] we have tested this corollary hypothesis. By 9 immunochemical and enzymatic assays we quantitated 5 different granulocytic enzymes in synovial fluids from 36 patients. We are sampling a single point in time in a spectrum of disease with considerable chronicity and variability. Nonetheless a population of this size allows us to test a null hypothesis (an absence of correlation between the quantity of enzymes and the degree of radiographic destruction of the joint sampled) with some confidence. We were able to reject this null hypothesis—but the correlation we observed was negative. In other words, the greater the quantity of enzymes we detected, the less likely was the fluid sampled from a radiographically damaged joint.

This unexpected result raises 2 important questions: Since human lysosomal enzymes have the potential to participate in cartilage destruction,[3-7] why are the enzymes in the fluid effete? And granted these enzymes are effete, why are they inversely related to destruction rather than simply unrelated? This second question provides impetus to considerable speculation. However, one potential answer to the first question is quite apparent: protease inhibitors present in the same fluids render the enzymes ineffective. Testing this hypothesis is the subject of the current report.

Normal human plasma contains at least 9 inhibitors of proteolytic enzymes [8 9]. All these protease inhibitors are synthesised in the liver, and most, particularly α_1-antitrypsin and α_1-antichymotrypsin, are acute-phase reactants. Vascular, pulmonary, hepatic, and immunological diseases are associated with genetic deficiencies of several of these inhibitors. In view of the morbidity associated with their absence, a major role for protease inhibitors is postulated in normal homoeostasis. Most of the protease inhibitors combine with the target enzyme rendering the enzyme inactive or altering the substrate specificity. The latter appears to be the case for α_2-macroglobulin, where enzyme-inhibitor complexes can still hydrolyse low-molecular-weight substrates.[10] In order to function to bind and inhibit a protease, most inhibitors are first substrates of the protease they inhibit, that is, limited specific proteolysis of the inhibitor is prequisite to binding.[11 12]

In the current study we employed highly specific

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electroimmunoassays to measure 5 protease inhibitors. We assayed the same pathological synovial fluids that were studied previously for their content of lysosomal enzymes.[2]

Materials and methods

PATIENTS AND SYNOVIAL FLUID COLLECTION

The same synovial fluid samples used in the earlier study of lysosomal enzyme content[2] were used in the current study. These had been collected at the time of a clinically indicated diagnostic arthrocentesis. All fluids were obtained from the knee with the exception of one olecranon bursal aspirate. No patient had a fixed deformity of the knee that precluded ambulation. All patients were taking nonsteroidal anti-inflammatory agents, 2 prednisone in low doses, and 5 were receiving gold salt therapy. The drug regimen did not influence the results of the data analyses.

At the time of arthrocentesis an aliquot was collected in EDTA (3 mg/10 ml) and the total leucocyte count and differential recorded. The remainder was not anticoagulated but was clarified by centrifugation within hours of sampling. Another aliquot was analysed for total protein (TP) and C3.[13] The remainder was frozen in small aliquots at −20°C and thawed prior to enzyme assay. Thirty-two synovial fluids were tested from 31 patients.

X-rays were available of 27 of the aspirated knees. These were taken in close temporal proximity to the arthrocentesis. The films were scored from 0 to 4 in accordance with the degree of destruction as in previous studies.[2] This scoring was performed without access to diagnosis or results of the synovial fluid analysis.

ASSESSMENT OF PROTEASE INHIBITORS AND LYSOSOMAL ENZYMES

The 5 protease inhibitors measured by electroimmunoassay are listed in Table 1. Different aliquots of 10 of the fluids were thawed and analysed by all assays on 2 occasions separated by 6 months. Differences were less than 10%.

The methods and results of the assays for lysosomal enzymes in these same fluids are detailed in the earlier publication.[2] Using 2 categories of assay, enzymatic and immunochemical, we detected the moieties listed in Table 2. Elastase was not detectable if elastin was used as a substrate (elast–1). Cationic protein was not detectable by a radial immunodiffusion assay (CP–2).

Table 1 *Assays used to measure the protease inhibitors**

Protease inhibitor	*Abbreviation used*	*Lower limit of detectability*	*Units†*	*Examples of enzymes inhibited*
α_1-antitrypsin	α_1AT	3·8	%NPS	Serine endopeptidases (trypsin, elastase, collagenase)
α_1-antichymotrypsin	α_1ACT	10·0	%NPS	Cationic protein‡, mast cell, and basophil chymotrypsin
α_2-macroglobulin	α_2M	10·0	mg/dl	Most endopeptidases
Inter-α-trypsin inhibitor	IαTI	10·0	%NPS	Trypsin
C1 esterase inhibitor	C1Inh	7·7	%NPS	C1 (C1 esterase), plasmin, activated Hageman factor

*All assays are electroimmunoassays as described by Laurell.[14]
†%NPS is the percent of the concentration in pooled normal adult serum. Valid weight/volume standards are available for all except α_1ACT and IαTI. For α_1AT, 100%NPS = 134 mg/dl. For C1Inh, 100%NPS = 13 mg/dl. The lower limit of detectability is given for the assay as employed.
‡See Table 2.

Table 2 *Assays used to measure lysosomal enzymes*

*Enzyme**	*Abbreviation used*	*Type of assay (substrate)*	*Lower limit of detectability*	*Units*	*Reference*
Lactoferrin	LF	Immunochemical	0·0156	µg/ml	16 17
Myeloperoxidase	MPO	Immunochemical	0·0186	µg/ml	16 17
Elastase	Elast-2	Enzymatic (N-benzyloxycarbonyl-L alanine 2 naphthyl ester)	0·005	nk/ml†	15
	Elast-3	Immunochemical	0·0041	µg/ml	18
Cationic protein	CP–1	Enzymatic (2–benzyl–DL phenylalanine 2–naphthyl ester)	0·005	nk/ml	15
Lysozyme	LYZ–1	Enzymatic (M. lysodeikticus)	0·087	g/ml	19

*These enzymes were readily detected in our earlier study of inflammatory synovial effusions[2] using the assays listed. Several synonyms are commonly used. CP is also referred to as the chymotryptic-like cationic protein or cathepsin G. Lysozyme is also known as muramidase.
†Nanokatals/ml.

DATA ANALYSIS

Because the intention of this series of investigations is to understand the role of PML lysosomal enzymes in joint destruction, the synovial fluids were divided into inflammatory and noninflammatory fluids based solely on the absolute number of polymorphonuclear leucocytes (PML)/mm^3. Inflammatory fluids have ≥ 500 PML/mm^3, noninflammatory <500. There were 26 of the former and 6 of the latter.

The noninflammatory group includes fluids from 4 patients with early degenerative joint disease, 1 with systemic lupus erythematosus, and 1 with rheumatoid arthritis. The inflammatory group included 17 fluids from rheumatoid arthritis and the variants, and 7 from crystal deposition disease (5 gout, 2 CPPD). The remaining 2 inflammatory fluids were from necrotising vasculitis and systemic lupus erythematosus.

Comparison of the 2 groups (Table 3) shows the relative absence of radiographic destruction in the noninflammatory group. Unlike the results for the lysosomal enzymes,[2] the inflammatory and noninflammatory groups are similar in the quantity of protease inhibitor. However, most of the clinical states represented in the noninflammatory group do not cause erosive synovitis, nor are they likely to have as many PML in synovial effusions. Since our intent is to gain some insight into the reason PML lysosomal enzymes correlate negatively with joint destruction, the noninflammatory fluids were excluded from the correlation analyses. The 7 crystal-induced inflammatory effusions did not differ significantly by any of the analyses performed on the remainder of the inflammatory fluids. The analyses presented consider all the inflammatory fluids together.

The data for the inflammatory fluids were subjected to a correlation analysis utilising a program written for the Hawlett-Packard 9830 computer equipped with a 9862 plotter. The program takes a set of data points (x,y) and calculates the coefficients of a polynomial (in this case, first degree) using a least-squares fit. Correlation coefficients (R) and R^2 measure of fit are computed.

Results

Unlike the finding with lysosomal enzymes[2] the quantity of several protease inhibitors reflects the total quantity of protein in the fluid (Table 4). Since the inhibitors are synthesised principally in the liver and all except α_1AT and α_1ACT exceed albumin in molecular weight,[9] their presence in the synovial fluid reflects the exudative nature of the effusion.[20] The inhibitors similarly correlate with the number of PML, though not as strongly as did the lysosomal enzymes.[2]

However, both the lysosomal enzymes[2] and the protease inhibitors (Table 4) generally correlate negatively with the degree of joint destruction observed radiographically. In spite of the fact that the protease inhibitors and lysosomal enzymes are synthesised in different sites by different cells, they are covariate in the inflammatory synovial effusions (Table 5). This is striking for the lysosomal enzymes LF and MPO, which were determined immunochemically and are highly covariate with the PML/mm^3.[2]

Table 4 *Correlation coefficients† for each protease inhibitor with joint destruction and synovial fluid characteristics*

Protease inhibitor	*X-ray score*	*PML/ mm^3*	*Total protein*	*C3*
α_1-antitrypsin	0·040	0·321	0·415*	0·130
α_1-antichymotrypsin	−0·328	0·097	0·011	0·237
α_2-macroglobulin	−0·346	0·346	0·488*	0·073
Inter-α-trypsin inhibitor	−0·569**	−0·157	0·448*	0·616**
C1 esterase inhibitor	−0·399	0·182	0·086	0·155

†The level of significance of the correlation coefficients (R) is indicated by asterisks: *=0·01<P<0·05; **=0·001<P<0·01; ***=P<0·001.

Table 3 *Comparison of the mean (± SEM) inhibitor assay values between the inflammatory and noninflammatory fluids**

		Inflammatory		
Values†	*Noninflammatory*	*Total*	*Crystal induced*	*Noncrystal induced*
Number of fluids	6	26	7	19
PML/mm^3	136±136	11 648±11 594	6514±3814	13 540±12 956
Total protein (g/dl)	3·9±0·67	4·89±1·47	4·7±1·28	4·96±1·56
X-ray score	0·20±0·45	1·91±1·57	2·20±1·79	1·82±1·55
α_1-antitrypsin	56±57	100±55	123±68	87±51
α_1-antichymotrypsin	89±76	112±89	154±121	95±71
α_2-macroglobulin	79±50	128±62	119±48	131±67
Inter-α-trypsin inhibitor	39±11	52±26	65±40	49±21
C1 esterase inhibitor	186±159	246±209	269±263	238±193

*An inflammatory synovial fluid is defined as having >500 PML/mm^3. The noninflammatory fluid has <500. †The notations, units, and methods for the inhibitor assays are given in Table 1.

Table 5 *Correlation coefficients (R) between the protease inhibitors and the lysosomal enzymes*†

Protease inhibitors	*Lysosomal enzymes*					
	LF	*MPO*	*Elast–2*	*Elast–3*	*CP–1*	*LYZ–1*
α_1-antitrypsin	0·561**	0·511**	0·007	0·276	0·182	0·276
α_1-antichymotrypsin	0·413*	0·487*	−0·016	0·160	0·199	0·332
α_2-macroglobulin	0·218	0·160	0·501*	0·054	0·105	0·368
Inter-α-trypsin inhibitor	0·216	0·216	0·518*	0·121	0·132	0·067
C1 esterase inhibitor	0·656***	0·663***	0·069	0·309	−0·106	0·490*

†Notations, units, and methods of determination are given for the protease inhibitors in Table 1 and lysosomal enzymes in Table 2. See Table 4 for notations of significance.

Elast–2 and CP–1 are measured enzymatically on small synthetic substrates (Table 2) which sacrifice specificity for high degrees of sensitivity. We previously showed that elast–2 and CP–1 values are independent of the PML/mm^3, raising the possibility that they derive from a source other than the PML. These enzymatic activities are not only detectable but even correlate with the quantity of some of the inhibitors (Table 5). Elast–2 is detectable in the presence of an inhibitor such as α_2M because the latter does not block access to the enzymes' active site by low molecular weight substrates.[10] No elastase is measured if elastin is the test substrate.[2]

The data in Tables 4–6 are presented as univariate correlation coefficients. This allows the reader the option of choosing his own level of confidence in suggesting the possibility that particular protease inhibitors are independent. Any such inference is testable with a larger population followed longitudinally. We believe the data do not allow one to single out any protease inhibitor as an independent variable either by inspection of the univariate correlation coefficients (Table 6) or by calculation of partial correlation coefficients. Therefore the data allows us to make the following general assertions. Protease inhibitors in inflammatory synovial effusions correlate with the total protein and PML/mm^3 (Table 4) and with the lysosomal enzymes (Table 5). As we previously observed for the lysosomal enzymes,[2] the quantity of these protease inhibitors is inversely related to the degree of articular destruction (Table 4).

Table 6 *Correlation coefficients (R) between protease inhibitors*†

	α_1AT	*α_1ACT*	*α_2M*	*IαTI*	*C1INH*
α_1AT	—	0·603**	0·307	0·063	0·375
α_1ACT		—	0·402*	0·385	0·587**
α_2M			—	0·572**	0·163
IαTI				—	0·276
C1INH					—

†See Table 1 for notations, units and methods for protease inhibitors and Table 4 for notations of significance.

Discussion

An extensive body of data suggests that lysosomal enzymes play a major role in articular destruction in the erosive synovitides,[21] in gout,[22] and in degenerative joint disease.[23] It is equally clear that the cellular and tissue localisation of lysosomal catabolism differs in each of these states.[21] The current studies were designed to test one long-held postulate regarding cellular and tissue localisation of lysosomal degradation in rheumatoid arthritis,[1] namely, that lysosomal enzymes released from PML into synovial fluid are primarily involved in articular destruction. At the very least this postulate has been a landmark in stimulating productive investigation. Certainly such enzymes have the potential to participate in cartilage destruction.[3–7] However, if it were true, one would expect that the cumulative quantity of active enzyme in the fluid would correlate with the degree of destruction. To test such a hypothesis in a disease with the chronicity and variability of rheumatoid arthritis would require longitudinal observation or single sampling of a reasonably large population. We chose the latter design realising that the absence of a correlation between lysosomal enzymes and joint destruction would be subject to considerable type 2 statistical error.[24] Our observation of a negative correlation between the PML lysosomal enzymes and joint destruction,[2] however, casts considerable doubt on the hypothesis.

The data reported herein provide a reasonable explanation for the ineffectiveness of PML lysosomal enzymes in solution in cartilage destruction. Inflammatory synovial fluids are replete with protease inhibitors; the inhibitors are covariate with the lysosomal enzymes and correlate negatively as well with joint destruction. The experiments of Oronsky and Perper[7] demonstrate in vitro the considerable capacity of rheumatoid synovial fluid to inhibit relevant human PML lysosomal hydrolases.

These observations render tenuous the hypothesis that PML lysosomal hydrolases in solution in

inflammatory synovial fluids participate in cartilage degradation. They do not address the possibility of the effectiveness of PML lysosomal hydrolases released directly on the cartilage[25 26] or of the participation of lysosomal hydrolases with another cellular or tissue distribution.[27]

There is no a-priori reason for the *negative* correlation between joint destruction and protease inhibitors (Table 4) and between joint destruction and PML lysosomal enzymes[2] in the synovial fluid. This implies more than the inference that these moieties are irrelevant to joint destruction—they are relevant but not in the way one might predict. We have observed a negative correlation between C3 determined immunochemically in the effusions and joint destruction[2] but a positive correlation with some protease inhibitors (Table 4). It appears that there are control mechanisms modulating the several inflammatory processes in the joint in rheumatoid arthritis yet to be elucidated.

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