Chondromucoprotein-degrading neutral protease activity in rheumatoid synovial fluid

G. C. WOOD, R. H. PRYCE-JONES, AND D. D. WHITE

Department of Pharmaceutical Chemistry, University of Strathclyde, Glasgow,

AND

G. NUKI

Centre for Rheumatic Diseases and University Department of Medicine, Royal Infirmary, Glasgow

Erosion of articular cartilage, which is characteristic of inflammatory joint diseases, including rheumatoid arthritis (RA), is due in part to enzymic breakdown of the components of the cartilage matrix (Bollet, 1963), particularly collagen and chondromucoprotein (CMP). It has been suggested that lysosomal hydrolases, possibly derived from cells of the synovial membrane or from leucocytes present in synovial fluid, are involved in this process (Dingle, 1962; Weissmann, 1966). In this communication we report the existence, in the synovial fluids of patients with rheumatoid arthritis, of protease activity which degrades CMP optimally near neutral pH. It thus differs from the majority of lysosomal enzymes, which are optimally active at pH values lower than those (Goldie and Nachemson, 1969) likely to appertain at the cartilage surface.

Material and methods

Synovial fluids were aspirated in the course of routine diagnostic or therapeutic arthrocentesis from the knees of patients with a variety of arthritides and the few which were grossly contaminated with blood were discarded. The samples came from 74 patients with RA (68 'definite' or 'classical' and six 'probable'), six with osteoarthritis, two Charcot joints, one erythema nodosum, one Reiter's syndrome, and three ankylosing spondylitis. A portion of each fluid was transferred to a tube containing ethylene diamine tetra-acetic acid (EDTA) for total and differential cell counting (Cohen, 1967) and the cells were immediately separated from the remaining fluid by centrifuging (15 min.; 1,000, g.; 4°C.). The cells were washed three times with isotonic saline solution, resuspended in a small volume (3-6 ml.) of buffer (0.1M Tris-HCl, pH 7.5) and disrupted by ultrasonication (MSE Ultrasonic Disintegrator, 2 min.; 0-4°C.). Cell debris was removed by centrifuging and the extract tested for CMP-degrading activity. The synovial fluid from which the cells had been removed was also tested.

CMP was prepared from bovine nasal cartilage as described by Partridge, Davis, and Adair (1961) (typical

analysis: hexosamine 28.0 g./100 g.; uronic acid 25.5 g./100 g.; hydroxyproline 0.20 g./100 g.). Degradation of CMP by cell extracts was detected by measuring the fall of viscosity of solutions in 0.1M Tris-HCl buffer at 25°C. in 2-ml. Ostwald viscometers. A unit of enzyme activity was defined as that amount of enzyme which caused a 20 per cent. fall in specific viscosity in 10 min. when added in 0.2 ml. solution to 2 ml. CMP solution (0.4 per cent.) at pH 7.5; 25°C.

Disc electrophoresis of CMP, its degradation products, and chondroitin sulphate (*ex* shark cartilage) was carried out on polyacrylamide gels by the method of Davis (1964) (spacer gel 5 per cent. acrylamide; pH 8.9; running gel 8 per cent. acrylamide; pH 7.5). The gels were stained for mucopolysaccharide with 1 per cent. Alcian blue in 7 per cent. acetic acid and scanned with a Joyce-Loebl 'Chromoscan' densitometer.

Separation of CMP-degrading enzymes in cell extracts was investigated by the disc electrophoresis method of Barrett (1966). Electrophoresis conditions were as described above, except that the running gel contained CMP (0.05 per cent.) and electrophoresis was at 0-4°C. After electrophoresis the gels were incubated in 5 ml. 0.1M Tris-HCl buffer pH 7.5 for 16 hrs at 37°C. The liberated chondroitin sulphate was removed electrophoretically from the gels, which were then stained for mucopolysaccharide with 1 per cent. toluidine blue in 7 per cent. acetic acid. Zones of enzymic degradation of CMP were thus revealed as clear mucopolysaccharide-free zones against a blue background.

Proteolytic activity of cell extracts was assayed using urea-denatured haemoglobin as substrate (Laskowski, 1955). Collagenase was assayed by the viscosity method of Eisen, Jeffrey, and Gross (1968). β -galactosidase and β -xylosidase were assayed colorimetrically (Levy and Conchie, 1966) and hyaluronidase viscometrically, using chondroitin sulphate and hyaluronic acid as substrates.

Results

When cell extracts, or dilutions thereof, were added to CMP, a fall in specific viscosity of up to 80 per cent. was observed in 1 hr, the change being similar

to that observed when CMP is treated with proteolytic enzymes (Muir, 1958) or hyaluronidase (Partridge and Wood, 1963).

Sixty per cent. of the cell extracts from a total of 88 fluids tested contained high levels of viscosity-reducing activity (CMP-ase), while 30 per cent. contained little or none. All the samples from joints affected by degenerative joint disease were in the latter group. In a group of 28 fluids (of which 25 were from patients with sero-positive rheumatoid arthritis) the intracellular CMP-ase of which was assayed quantitatively, the range of activity was 0-120 units/ml. synovial fluid (specific activity 0-700 units/mg. protein). There was a weak correlation of enzyme activity (units/ml. fluid) with the total cell count ($r = 0.464$; $P < 0.05$) and with the polymorphonuclear leucocyte count ($r = 0.454$; $P < 0.05$). No better correlation was observed with lymphocyte or mononuclear cell counts.

There was a close parallel (the results in Fig. 1 are typical) between the levels of protease and CMP-ase at pH 7.5 in cell-extracts from a number of fluids which show a wide range of activity and also in concentrates of CMP-ase obtained by ammonium sulphate precipitation.

FIG. 1 Relationship between viscosity-reducing activity (units/ml. cell extract) and protease activity (equivalent μg . chymotrypsin/ml. cell extract) for (a) five unfractionated cell extracts and (b) the 20 to 70 per cent. ammonium sulphate fractions of the same extracts.

On electrophoresis the major part of untreated CMP failed to penetrate the running gel (Fig. 2, opposite). CMP degraded by active cell extract gave two intensely staining bands. One was well defined

and had the mobility of chondroitin sulphate while the other had lower mobility and was more diffuse. A similar pattern was obtained when CMP was treated with the protease chymotrypsin.

A further parallel between CMP-ase and protease activities is observed in their pH-dependence (Fig. 3a). Both have an optimum between pH 6 and pH 7.5 with possibly a further increase in activity above pH 8. There was little or no proteolytic activity between pH 3 and pH 4.5 where many lysosomal enzymes are optimally active. Viscometric assay of CMP-ase was impossible at pH 4 because of precipitation on adding cell extract to CMP. The electrophoretic technique, however, showed that treatment of CMP at pH 3 or pH 4 did not produce a significant amount of chondroitin-sulphate-like material or other CMP degradation products able to enter the running gel. Above pH 5, on the other hand, both these features of degradation were observed. The intensity of the chondroitin sulphate band varied with pH of treatment (Fig. 3b) in a similar manner to protease and viscosity-reducing activities.

FIG. 3 Variation of enzyme activities with pH (above) Viscosity-reducing activity (buffers: pH 4.4, formate; pH 5.2, citrate; pH 5.9, 6.6, phosphate; pH 7.9, 8.7, Tris-HCl; all at ionic strength 0.1) and protease (buffers: pH 3.0, formate; pH 4.6, citrate; pH 7-10, Tris-HCl);

(below) Area of 'chondroitin sulphate' peak in densitometer scans of electrophoretograms (buffers as for protease).

Protease activity and CMP-ase activity (detected viscometrically and electrophoretically) were both enhanced by cysteine ($2.5 \times 10^{-2}\text{M}$) and partially

FIG. 2 Disc electrophoresis of
(a) CMP treated with synovial fluid cell extract
(1 ml. CMP, 0.4 per cent., + 0.1 ml. enzyme,
20 units/ml.; 37°C.; pH 7.5; 16 hrs),
(b) Untreated CMP.
(c) Chondroitin sulphate.

Sample solution, 0.1 ml., applied to 5 × 50 mm. gels and run at room temperature for 70 min., 20 mA/tube.

Diagrams of stained gels are accompanied in (a) and (b) by densitometer scans. Shading of diagrams indicates intensity of colour and not the presence of discrete bands.

inhibited by EDTA ($2.5 \times 10^{-2}M$) and by normal human serum (10^3 -fold dilution). These results are consistent with the view that CMP-ase and protease activities are closely related. On the other hand, hyaluronidase, β -galactosidase, and β -xylosidase, which might also reduce the viscosity of CMP, could not be detected. Although traces of collagenase were detected in some cell extracts, others, in which collagenase could not be detected, were highly active against CMP.

Fractionation of cell extracts by Barrett's electrophoretic method revealed the presence of at least two enzymes able to liberate chondroitin sulphate from CMP. In some extracts (e.g. Fig. 4) three enzymic components were observed, but in others the two rapidly moving components were not resolved.

FIG. 4 Disc electrophoresis of cell extracts on polyacrylamide gels impregnated with CMP. Electrophoresis conditions as for Fig. 2, except running gel contained CMP, 0.05 per cent., and current passed for 50 min. at 0.4°C.

Attempts to demonstrate the presence of extracellular CMP-ase, similar to the intracellular activity described above, in synovial fluids from which the cells had been removed were complicated by two phenomena:

(1) The addition of such cell-free synovial fluids, or dilutions thereof, to CMP solutions at pH 7.5 resulted in an immediate increase of specific viscosity to a value greater than the sum of the viscosities of CMP and synovial fluid. This is probably due to the interaction of the hyaluronate of synovial fluid with CMP, since the effect could be reproduced with purified hyaluronic acid in place of synovial fluid. With hyaluronic acid and with most of the synovial fluids we have examined, the enhanced viscosity of CMP remained constant with time, suggesting that extracellular CMP-ase was absent. With cell-free fluids from a few patients, however, the initial increase in viscosity was followed by a slow decrease. Since a similar change was observed when intracellular enzyme was added to mixtures of hyaluronic acid and CMP, this fall in viscosity may have been due to extracellular CMP-ase in these fluids.

(2) Further attempts to investigate this revealed another complicating factor. When 0.2 ml. of an active cell extract were added to 2 ml. CMP solution to which had been added 0.2 ml. hyaluronic acid (0.2 g./100 ml.), the extent of the fall in viscosity (48.4 per cent.) was approximately the same as that

(52 per cent.) observed without added hyaluronic acid; when the experiment was repeated, using a cell-free synovial fluid instead of hyaluronic acid, no fall in viscosity was observed. This suggested the presence of an extracellular CMP-ase inhibitor in the synovial fluid.

It was found that not only did most synovial fluids inhibit the fall in viscosity induced by cell extracts but that they also inhibited the electrophoretic changes and the protease activity. The inhibitor was active even at high dilutions of some synovial fluids (Fig. 5). This means that failure to detect extracellular CMP-ase in a fluid did not necessarily imply that this enzyme was absent—it might merely have been inhibited by the potent extracellular inhibitor. Its detection in a few fluids may have been facilitated by unusually low levels of inhibitor.

FIG. 5 Inhibition of viscosity-reducing activity of cell extracts at pH 7.5 by cell-free synovial fluid. Per cent. inhibition = $100(A_1 - A_2)/A_1$, where A_1 = activity (approx. 5 units/ml.) of cell extract observed with 2 ml. CMP + 0.2 ml. extract + 0.2 ml. buffer under standard conditions (see text) and A_2 = activity when 0.2 ml. synovial fluid dilution is substituted for buffer.

Concentration of inhibitor is expressed as a fraction of its concentration in undiluted synovial fluid.

Discussion

The results indicate that degradation of CMP by the cellular extracts is primarily due to neutral protease activity. This does not imply that only one such enzyme is present. Nor does it exclude the possibility that other types of enzyme may also be involved, possibly by mechanisms which result in a comparatively small fall in CMP viscosity. The electrophoretic experiments indeed indicate the presence of at least two enzymes which degrade CMP at pH 7.5. However, we have not detected significant amounts of hyaluronidase, β -galactosidase, or β -xylosidase and, although traces of collagenase were detected in some cell extracts, others in which collagenase could not

be detected were equally active against CMP. The protease activity extracted from synovial cells has somewhat similar properties to neutral proteases, sometimes said to be lysosomal, extracted by various workers from leucocytes from other sources (Ziff, Gribetz, and Lospalluto, 1960; Weissmann and Spilberg, 1968; Mounter and Atiyeh, 1960; Janoff and Zeligs, 1968; Wasi, Murray, MacMorine, and Movat, 1966). Ziff and others (1960) and Weissmann and Spilberg (1968) have shown that neutral protease, extracted from polymorphonuclear leucocytes from human peripheral blood and rabbit peritoneal exudates respectively, can degrade CMP. Leucocyte neutral protease preparations have, however, almost invariably contained acid protease activity, which was almost completely absent from our extracts.

The shapes of the pH-activity curves which we have observed are remarkably similar above pH 5 to those observed by Mounter and Atiyeh (1960) for the degradation of several proteins by extracts of leucocytes from peripheral blood. Our further observation of at least two enzymes which degrade CMP at pH 7.5 is also in line with their findings. They concluded from inhibition studies that their leucocyte extracts contained two proteases active above pH 5, and suggested that one of these derived from lymphocytes, the other from polymorphonuclear leucocytes. Although the latter is the predominant cell type in rheumatoid synovial fluids, other types of cell, including synovial membrane cells, are undoubtedly present in varying proportions. It is perhaps not surprising, therefore, that we did not observe a strong correlation of enzyme activity with cell count. Further work is in progress to define the cellular and subcellular origin of the enzymes we have observed and to characterize them more precisely.

Clearly, intracellular CMP-degrading enzymes will be of pathological significance only if they can escape from the cells into the extracellular fluid where they may attack cartilage. Our attempts to demonstrate the presence of such an enzyme in synovial fluids from which the cells have been removed were complicated by the presence of the potent extracellular inhibitor. In order to investigate this further, methods are being developed for separating enzymes and inhibitor.

The results suggest that, under certain conditions, intracellular neutral proteases able to degrade CMP may be released from PMN leucocytes or other cells of the synovial milieu into the extracellular fluid. These enzymes may be significant in the pathogenesis of cartilage erosion in inflammatory joint diseases. Their ability to degrade articular cartilage would depend on a number of factors, including the concentration of cells, the ease with which the enzymes are released from the cells, and the concentration of inhibitor. The relationship of these factors

to each other and to the clinical features of various forms of arthritis and the effects of anti-inflammatory drugs are currently being investigated.

Summary

Evidence is presented that chondromucoprotein-degrading enzyme activity, observed in extracts of cells from the synovial fluids of patients with inflammatory joint disease, is primarily due to neutral

protease activity. Some properties of this enzyme activity are described, including its inhibition by an extracellular synovial fluid inhibitor. It is suggested that the enzyme activity and the inhibitor may be significant factors in the pathogenesis of cartilage erosion in inflammatory joint disease.

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