Proteoglycan-Degrading Enzymes of Rabbit Fibroblasts and Granulocytes

By ZENA WERB,* JOHN T. DINGLE, JOHN J. REYNOLDS and ALAN J. BARRETT
Strangeways Research Laboratory, Worts' Causeway, Cambridge CB1 4RN, U.K.

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A neutral proteinase secreted by rabbit synovial fibroblasts in parallel with specific collagenase was partially purified by ion-exchange chromatography. At pH 7.6 this proteinase degraded ³⁵S-labelled bovine nasal proteoglycan and azo-casein. The enzymic activity was inhibited by EDTA, 1,10-phenanthroline and serum, whereas di-isopropyl phosphorofluoridate and soya-bean trypsin inhibitor had little effect. By gel filtration the apparent mol.wt. of the enzyme was 25000. The fibroblast neutral proteinase was compared with the proteoglycan-degrading neutral proteinases of rabbit polymorphonuclear-leucocyte granules. Two distinct activities were found in the granules: one was inhibited by soya-bean trypsin inhibitor and the other by EDTA. The proteoglycan-degrading proteinases of rabbit fibroblasts and polymorphonuclear leucocytes at acid pH also were examined. Both cathepsin D and a thiol-dependent proteinase contributed to the degradation of proteoglycan at pH 4.5.

Glycosaminoglycans are found in all connective tissues, including cartilage. As a component of the basal lamina the proteoglycans play an important role in promoting and maintaining orderly tissue structure, particularly during development (Hay & Meier, 1974; Trelstad *et al.*, 1974; Kosher & Lash, 1975; Cohn *et al.*, 1977). Degradation of proteoglycans may play an important role during morphogenesis. Although the enzymes that degrade the basal lamina have not been identified, there are data suggesting that these enzymes are produced by cells of mesenchymal origin (Smith & Bernfield, 1977).

In the adult, proteoglycans are most abundant in cartilaginous tissues. Joints affected by rheumatoid arthritis or osteoarthritis show accelerated degradation of both major components of the articular-cartilage matrix, collagen and proteoglycan. Probably the tissue damage is due to the action of several proteolytic enzymes produced by several types of cell (Barrett, 1975). Much has yet to be learnt about the identity of the enzymes and the cells involved in the various processes, but there is evidence implicating the synovial fibroblasts and the polymorphonuclear leucocytes of the synovial fluid.

Because the rabbit provides the best experimental

model for rheumatoid arthritis (Page-Thomas, 1969), it is clearly appropriate to investigate the cartilage-degrading enzymes of the articular cells present in the joints of this species. Fibroblastic cells of rabbit are relatively easy to culture and are an important resource for studying functions of mesenchymal cells.

Previously it was found that fibroblastic cells grown from rabbit synovial tissue synthesize and secrete collagenase (Werb & Burleigh, 1974; Werb & Reynolds, 1974), and the enzyme was purified from the culture media of these cells (Werb & Reynolds, 1975a). It was of particular interest that the characteristics of secretion of the collagenase and a neutral endopeptidase were dissimilar from those of the lysosomal enzymes, especially in that the intracellular pool of the enzyme seemed extremely small compared with the amount of enzyme secreted (Werb & Reynolds, 1974). In the present study we used a new, sensitive assay procedure (Dingle *et al.*, 1977) to characterize a proteoglycan-degrading neutral proteinase secreted from the rabbit fibroblasts in parallel with collagenase. We also described the proteoglycan-degrading lysosomal proteinases of the fibroblasts.

Because human neutrophil leucocytes contain enzymes very effective in the degradation of cartilage matrix (Barrett & Starkey, 1977; Starkey *et al.*, 1977), it was worthwhile to compare the rabbit fibroblast enzymes with the neutral and acid proteinases of rabbit polymorphonuclear leucocytes.

Materials and Methods

Materials

Bio-Rex 70 and Chelex resins were purchased from Bio-Rad Laboratories, Bromley, Kent, U.K. Sodium or calcium derivatives of Chelex were prepared by

* Present address: Laboratory of Radiobiology, University of California, San Francisco, CA 94143, U.S.A.

Abbreviations used: Ac-(Ala)₃-Ala-CH₂Cl, *N*-acetyltri-L-alanyl-L-alanine chloromethyl ketone; Bz-DL-Arg-NNap, α -*N*-benzoyl-DL-arginine 2-naphthylamide; Bz-Phe-2-ONap, *N*-benzoyl-DL-phenylalanine 2-naphthyl ester; Dip-F, di-isopropyl phosphorofluoridate; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamido-heptan-2-one; Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamido-butan-2-one; Z-Ala-2-ONap, *N*-benzyloxycarbonyl-L-alanine 2-naphthyl ester.

suspending Chelex (H^+ form) in either 1M-NaCl or 1M- $CaCl_2$ for 30min, then washing to remove the excess salt.

Tos-Phe- CH_2Cl , soya-bean trypsin inhibitor, shell-fish glycogen and thermolysin were purchased from Sigma (London) Chemical Co., Kingston on Thames, Surrey KT2 2BH, U.K., and Azocoll was obtained from Calbiochem, London W1H 1AS, U.K. Leupeptin-Pr (propionyl form) and pepstatin were gifts of Professor H. Umezawa, Institute of Microbial Chemistry, Kamiosaki, Tokyo, Japan.

Azo-casein was prepared essentially as described by Charney & Tomarelli (1947), except that sodium sulphanilate replaced sulphanilamide. The preparation had an $A_{280}^{1\%}$ of 40.2. Dulbecco's modification of Eagle's medium was purchased from Flow Laboratories, Irvine KA12 8NB, Renfrewshire, U.K.

Human cathepsin D was prepared as described previously (Barrett, 1970, 1973), and the preparation of bovine nasal proteoglycan has also been described (Dingle *et al.*, 1977). Sheep antiserum against rabbit cathepsin D was prepared in this laboratory as described previously (Dingle *et al.*, 1971). α_2 -Macroglobulin was prepared as described by Starkey & Barrett (1973).

Tos-Lys- CH_2Cl (Bachem Feinkemikalein, Liestal, Switzerland), Z-Phe- CH_2Br (a gift of Dr. E. Shaw, Brookhaven National Laboratory, NY 11973, U.S.A.) and Ac-(Ala) $_3$ -Ala- CH_2Cl (a gift of Dr. J. C. Powers, Department of Chemistry, Georgia Institute of Technology, Atlanta, GA 30332, U.S.A.) were dissolved in propan-2-ol before use.

[3H]Acetyl-haemoglobin was prepared as described by Barrett & Heath (1977) and [3H]acetyl-casein was prepared essentially in the same way, by reaction of the proteins with [^{14}C]acetic anhydride.

Preparation of culture medium containing neutral proteinase

Rabbit fibroblasts were grown from rabbit synovium and cultured as described previously (Werb & Burleigh, 1974). The used culture medium was concentrated by ultrafiltration over a type 12133 membrane (Sartorius-Membranfilter G.m.b.H., Göttingen, Germany), dialysed overnight against 100 vol. of 0.05M-Tris/HCl buffer, pH 7.6, containing 0.2M-NaCl and 5mM- $CaCl_2$, and stored at $-20^\circ C$ or at $4^\circ C$. Cell lysates were prepared in the same buffer, containing 0.1% Triton X-100.

Partial purification of neutral proteinase

The neutral proteinase was separated from collagenase and cathepsin D by cation-exchange chromatography. Concentrated culture medium was dialysed against 40mM-Tris/HCl buffer, pH 7.2, containing 5mM- $CaCl_2$. The sample was applied to a

column (1.5cm $\times$ 15cm) of Bio-Rex 70 resin equilibrated with the same buffer. The neutral proteinase active against azo-casein was not retained under these conditions, whereas the collagenase and cathepsin D were eluted with a linear gradient of 0–0.5M-NaCl in the same buffer (Werb & Reynolds, 1975a). The enzyme was further purified by gel chromatography on Sephadex G-100 as described previously (Werb & Burleigh, 1974).

Assays of proteoglycan-degrading activity

The rate of decrease in viscosity of solutions of proteoglycans from bovine nasal cartilage was determined essentially as described by Morrison *et al.* (1973). Assays were made at pH 7.6 in 100mM-Tris/HCl buffer containing 5mM- $CaCl_2$ and 150mM-NaCl, in miniature Ostwald viscometers (type UM.4; A. Gallenkamp) having water flow times of about 20s. The total volume in each viscometer (2.0ml) included 1.0ml of proteoglycan solution (5.0mg/ml).

Degradation of proteoglycan was also measured with ^{35}S -labelled proteoglycan entrapped in polyacrylamide beads (Dingle *et al.*, 1977). Usually, assays were made in 0.1M-Tris/HCl buffer, pH 7.6, containing 10mM- $CaCl_2$, or in 0.1M-sodium acetate buffer, pH 4.5. All assays were in duplicate or triplicate and enzymic activities are stated as percentage release of radioactive material, or as units of activity based on standard curves for human cathepsin D at pH 4.5 measured in each assay. In the latter case, one unit of proteoglycan-degrading activity was equivalent to one unit of human cathepsin D activity determined in the haemoglobin assay (Barrett, 1970). In experiments with potential inhibitors, enzyme preparations were mixed with the inhibitors 6min before assay, unless otherwise stated in the text.

Assays for collagenase activity were made with reconstituted [^{14}C]glycine-labelled collagen fibrils (Werb & Reynolds, 1974; Werb & Burleigh, 1974). One unit of collagenase activity represented the hydrolysis of 1 μg of collagen/min under the experimental conditions.

Assays of azo-casein-degrading activity

These assays were made as described previously (Werb *et al.*, 1974), at pH 7.6 in 100mM-Tris/HCl/10mM- $CaCl_2$, but the reaction volume was 0.5ml and the final volume after addition of trichloroacetic acid was 3.0ml.

Other proteinase assays

Neutral proteinase assays with Azocoll as substrate were made as described by Werb & Reynolds (1974). Proteolytic activity against fibrinogen (native or denatured) or elastin was assayed by use of

substrate-agarose plates, essentially as described by Werb & Gordon (1975). Assays with [^3H]acetyl-haemoglobin or [^3H]acetyl-casein at pH 7.6 were made essentially as for azo-casein, except that portions of the trichloroacetic acid-soluble material were taken for measurement of radioactivity by liquid-scintillation spectrometry (Hall & Cocking, 1965). Cathepsin B was assayed as described by Barrett (1976). Assays with Z-Ala-2-ONap and Bz-Phe-2-ONap were as described previously by Starkey & Barrett (1976).

Preparation of fibroblast mitochondrial-lysosomal fraction

Fibroblast monolayers grown in 500-ml prescription bottles were rinsed three times with 0.9% NaCl, then scraped with a rubber policeman to resuspend in 0.25M-sucrose at about 10^6 cells/ml. Homogenization with 20 passes of a tight-fitting Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.) was followed by centrifugation for 8000g-min to remove nuclei and debris. The mitochondrial-lysosomal fraction was collected from the supernatant by further centrifugation for 300000g-min.

Preparation of granule fractions from rabbit heterophil granulocytes

Polymorphonuclear leucocytes were elicited by injecting shellfish glycogen (0.1% in 0.9% NaCl) (300ml) into the peritoneal cavity of adult New Zealand white rabbits from the Strangeways Research Laboratory stock: 4h later the fluid was tapped, and the total granule fraction was isolated by the method of Cohn & Hirsch (1960). The cell preparations contained over 95% heterophil leucocytes.

Results

Rabbit fibroblast enzymes

pH-dependence of the proteoglycan-degrading activities of the culture medium. The activity of the culture medium was assayed in the 'bead assay' in the range pH 3.0-9.5. A broad maximum of activity was detected in the range pH 7.5-9.0, and a second peak at pH 4.0 showed the presence of acid proteoglycan-degrading activity. Further experiments were made to characterize the enzymes acting at neutral to alkaline and at acid pH values.

Neutral proteinase activities. Used culture medium (concentrated 50-fold) was tested for activity against native and denatured fibrinogen, gelatin, casein, azo-casein, [^3H]acetyl-haemoglobin and Azocoll at pH 7.5. No digestion of haemoglobin was apparent, but plasminogen activation was detected in the assays with plates containing native fibrinogen and plas-

minogen; it was blocked by EDTA, dithiothreitol and Dip-F. Denatured fibrinogen, gelatin, casein, azo-casein and Azocoll were hydrolysed, and all these activities shared the same inhibitor profile as the partially purified proteoglycan-degrading proteinase (see below), being inhibited by chelators and unaffected by Dip-F and soya-bean trypsin inhibitor. However, further work is needed to establish whether all these activities are due to a single proteinase or a family of (apparently metallo-) proteinases.

Behaviour of neutral proteinase in cation-exchange chromatography. Azo-casein-degrading activity and proteoglycan-degrading activity were unretarded in ion-exchange chromatography on Bio-Rex 70 resin (see the Materials and Methods section). Thiol proteinase activity (see below) also was not separated from the neutral proteinase, although contaminating cathepsin D was removed.

Gel filtration of neutral proteinase. When chromatographed on Sephadex G-100 the neutral proteinase (determined with the 'bead assay') had an elution volume greater than those of cathepsin D and collagenase, but similar to that of cathepsin B, indicating a mol.wt. of about 25000. Some neutral proteolytic activity appeared with an elution volume corresponding to a mol.wt. of about 40000, but it is possible that this was due to association of the enzyme with other proteins. The partially purified enzyme characterized in this study was that of the lower molecular weight.

Fig. 1. Dose-response curves in the bead assay for proteoglycan-degrading activity

Results are shown for human cathepsin D (○) and two preparations of partially purified rabbit fibroblast neutral proteinase (●, ■). Activity is expressed as the amount of ^{35}S released from the beads, as a percentage of the total present; incubations were 4h at 37°C.

Fig. 2. Viscometric assay for proteoglycan degradation by rabbit fibroblast neutral proteinase

The results plotted directly (—), or in the second-order form (see the Materials and Methods section for details) in which η_{∞} was taken as 27.6% of η_0 (----), show the decrease in viscosity of cartilage proteoglycan in solution at pH 7.6 in the presence of no enzyme (▲), rabbit fibroblast neutral proteinase (○) and the enzyme with 1 mM-EDTA (●).

Degradation of ^{35}S -labelled proteoglycan by partially purified neutral proteinase. Two preparations of neutral proteinase partially purified by Bio-Rex 70 chromatography of culture medium were compared for their proteoglycan-degrading activity in the bead assay at pH 7.6 with human cathepsin D at pH 4.5. As shown in Fig. 1, logarithmic dose-response relationships were found for all three preparations, and the slopes of the curves for the neutral proteinase and cathepsin D were not significantly different. One of the preparations was assayed for azo-casein-degrading activity; activity was clearly demonstrable, and increasing amounts of enzyme gave a linear increase in ΔA_{366} up to 0.4 at 300 μl /tube.

Effect of rabbit fibroblast neutral proteinase on the viscosity of bovine nasal-cartilage proteoglycan in solution. The neutral proteinase (800 μl of the 10-fold-concentrated culture medium) decreased the viscosity of the proteoglycan solution as shown in Fig. 2. Since the viscosity change followed a second-order function, $\eta_0 - \eta / \eta - \eta_{\infty}$ (where η_0 , η and η_{∞} represent viscosity at zero-time, time t and time sufficiently long that no further decrease in viscosity is measurable respectively) was plotted as described by Woessner (1973) to determine the rate of degradation and the extent of inhibition. The addition of EDTA (1 mM in the presence of 5 mM- CaCl_2) abolished the activity completely and 1,10-phenanthroline (1 mM) (results not shown) decreased the enzymic activity to 18% of that of the uninhibited enzyme.

Effect of inhibitors on the proteoglycan-degrading neutral proteinase. The effects of a variety of potential

inhibitors on the activity of the enzyme partially purified by chromatography on Bio-Rex 70 and Sephadex G-100 were tested in the assay with entrapped proteoglycan. As shown in Table 1, inhibition was achieved with chelating agents such as EDTA at moderately high concentrations. There was very little inhibition of the partially purified enzyme by Dip-F. With crude culture medium Dip-F produced up to 40% inhibition (on the basis of cathepsin D activity) and this presumably indicated the presence of other proteinases.

The highly reactive bromomethyl ketone, Z-Phe- CH_2Br , was an effective inhibitor, but the chloromethyl ketone inhibitors of chymotrypsin and trypsin showed little activity. The complete inhibition of enzymic activity by α_2 -macroglobulin indicates that the enzyme degrading the proteoglycan was an endopeptidase (Barrett & Starkey, 1973; Werb *et al.*, 1974) rather than a polysaccharidase.

In view of the inhibition of the proteoglycan-degrading neutral proteinase by Z-Phe- CH_2Br and EDTA, but only partial inhibition by the Ca^{2+} -equilibrated Chelex, it was difficult to identify the enzyme as either a serine proteinase or a metallo-proteinase. An experiment was therefore made to test the effect of these inhibitors on a well-characterized metallo-proteinase, thermolysin (Colman *et al.*, 1972). Thermolysin (10 $\mu\text{g}/\text{ml}$ in 50 mM-Tris/HCl, pH 7.6) was incubated with Chelex (Ca^{2+} or Na^{+} forms) for up to 1 h. The Chelex was removed by centrifugation and 200 μl samples were assayed for activity against azo-casein in the presence of 5 mM- CaCl_2 . There was a decrease in activity with time of incubation, but at 1 h, 46% of the control activity had survived treatment with Chelex (Ca^{2+} form) and 38% with Chelex (Na^{+} form). In contrast, the addition of EDTA (15 mM, in the presence of 5 mM- Ca^{2+}) completely inhibited the activity of thermolysin. It was concluded that under these conditions, Chelex was unable to remove completely the tightly bound metals of thermolysin. In a similar experiment, thermolysin (2 μg) was preincubated for 1 h with Z-Phe- CH_2Br (0.75 mM) at 22 $^{\circ}\text{C}$, and assayed for activity against azo-casein (the final concentration of Z-Phe- CH_2Br in the reaction mixture being 0.375 mM). Under these conditions only 28% of the control thermolysin activity remained. Thus the reagent can inhibit metallo-proteinases, and we think it probable that the fibroblast neutral proteinase studied is a metallo-proteinase.

Comparison of collagenolytic and proteoglycan-degrading activities of rabbit collagenase and neutral proteinase. In view of a report that purified human granulocyte collagenases degrade proteoglycans (Ohlsson & Olsson, 1973) the contribution of rabbit fibroblast collagenase to the degradation of ^{35}S -labelled cartilage proteoglycan was examined. Sufficient quantities of the granulocyte proteinase and

Table 1. *Effect of potential inhibitors on the proteoglycan-degrading activity of partially purified rabbit fibroblast neutral proteinase*

Partially purified rabbit fibroblast neutral proteinase was used in assays with ^{35}S -labelled proteoglycan beads at pH 7.6 in 0.1 M-Tris/HCl buffer containing 10 mM- CaCl_2 . The inhibitors were added to the enzyme preparation for the preincubation times indicated, before the addition of substrate. The results were obtained with several different batches of beads, for which the slopes of standard curves were different. Activities are shown as (a) percentage of ^{35}S release by uninhibited control and (b) activity as a percentage of the control after conversion into equivalent units of cathepsin D (see the Materials and Methods section).

Compound	Preincubation time (h)	Final concn.	Enzymic activity	
			(a) ^{35}S release (% of control)	(b) Human cathepsin D equivalents (% of control)
None	—	—	100	100
EDTA	0.1	20 mM	1	*
1,10-Phenanthroline	0.1	5 mM	1	*
		1 mM	36	23
Chelex (Ca^{2+} form)	0.1	—	75	75
Z-Phe- CH_2Br	0.5	1 mM	20	*
Tos-Phe- CH_2Cl	0.5	1 mM	65	67
		0.1 mM	92	91
Tos-Lys- CH_2Cl	0.5	1 mM	64	67
		0.1 mM	93	92
Dip-F	20	1 mM	82	76
4-Nitro-4'-phenylguanidinobenzoate	0.1	0.25 mM	82	76
Soya-bean trypsin inhibitor	0.1	1 mg/ml	95	87
Leupeptin (propionyl form)	0.1	100 $\mu\text{g}/\text{ml}$	91	74
Pepstatin	0.1	1 $\mu\text{g}/\text{ml}$	100	100
α_2 -Macroglobulin	0.1	100 $\mu\text{g}/\text{ml}$	0	0

* Release below linear part of standard curve.

Table 2. *Proteoglycan-degrading activity of purified rabbit collagenase and partially purified rabbit neutral proteinase*
Neutral proteinase and collagenase separated and purified by chromatography on Bio-Rex 70 (Werb & Reynolds, 1975a) (two preparations of each) were assayed for activity on reconstituted collagen fibrils, Azocoll and proteoglycan beads.

Enzyme	Collagenase activity		Neutral proteinase activity	
	(units/ml)	(units/mg of protein)	Azocoll (mg/h per ml)	^{35}S release (% of total/24 h per 0.35 ml)
Neutral proteinase (1)	0.3	10	0.12	45.3
Neutral proteinase (2)	0.5	6	0.32	34.9
Collagenase (1)	2	1800	0.015	2.5
Collagenase (2)	11	1300	0.053	13.9

the fibroblast collagenase were used to give measurable activities of each in the assays for the other (Table 2). The ratios between the activities against collagen, Azocoll and proteoglycan were very different for the two enzyme preparations. It was also shown that the neutral proteinase activity was unaffected by immunoglobulins isolated from an inhibitory antiserum raised against rabbit fibroblast collagenase (Werb & Reynolds, 1975b).

Proteoglycan-degrading proteinases of rabbit fibroblasts active at acid pH. Since a large portion of the proteoglycan-degrading capacity of rabbit fibro-

blasts was seen at acid pH values, the enzymes responsible were characterized further (Table 3). Part of the activity had the characteristics of cathepsin D, including sensitivity to pepstatin (Barrett & Dingle, 1972), whereas the remainder was attributable to a thiol proteinase activated by EDTA and cysteine, and inhibited by leupeptin and chloromethyl ketones, and thus indistinguishable from cathepsin B (Barrett, 1973) and related lysosomal thiol proteinases.

The fibroblast lysates were rich in cathepsin D as measured by activity on haemoglobin at pH 3. Lysates also contained enzymic activity against

Bz-DL-Arg-NNap at pH6 in the assay described by Barrett (1973) for cathepsin B. Both activities were enriched in lysosomal fractions of the fibroblasts. Hence, it is probable that the proteoglycan-hydrolysing activity at acid pH values was due to the combined action of these two cathepsins.

Distribution of proteoglycan-degrading proteinases between rabbit fibroblasts and their culture media. Previous studies (Werb & Burleigh, 1974; Werb & Reynolds, 1974) have indicated that both collagenase and Azocoll-degrading neutral proteinases of rabbit synovial fibroblasts are primarily secreted enzymes and have very small intracellular pools. This contrasted with a lysosomal proteinase, cathepsin D, which is mostly cell-bound.

Experiments were made to examine the distribution of proteoglycan-degrading activity between cells and medium in cultures of rabbit fibroblasts (Table 4). Enzymes were assayed at pH 7.6 for neutral proteinase and at pH 4.5 for the lysosomal proteinase. Assays at pH 4.5 were made in the presence of EDTA (1 mM) and cysteine (5 mM) to activate thiol proteinases. It was found that proteoglycan-degrading proteinases active at pH 7.6 were primarily secreted, accumulating with time in the culture medium (results not shown), with small intracellular activities, similar to the distribution of collagenase. In contrast, acid proteinase activity against either proteoglycan or haemoglobin was mostly cell-bound.

Rabbit polymorphonuclear-leucocyte enzymes

Proteoglycan-degrading proteinases present in the granules of rabbit polymorphonuclear leucocytes. Granule fractions prepared from about 10^{10} peritoneal leucocytes and suspended in 10 ml of 7.5% (w/v) sucrose containing 0.1% Brij-35 were frozen and thawed four times before use. The preparations

were very rich in neutral proteoglycan-degrading activity and were used at dilutions of 1:3000 (in 50 mM-Tris/HCl containing 5 mM-CaCl₂ and 0.1% Brij-35) (300 μ l per tube) to achieve the release of about 30% of the ³⁵S in 4 h at pH 7.6 in the bead assay. For assays at pH 4.5 the extracts were diluted 1:200. At each pH value a linear relationship was obtained in the semilogarithmic plot of enzyme concentration against release of radioactivity from the beads.

Table 3. *Effect of inhibitors and activators on proteoglycan-degrading proteinases of rabbit synovial fibroblasts active at pH 4.5*

Concentrated medium from rabbit fibroblast cultures was used as a source of proteinase activity. Assays with ³⁵S-labelled proteoglycan in polyacrylamide beads were made in 0.1 M-sodium acetate buffer, pH 4.5, for 4 h at 37°C. Inhibitors and activators were added to enzyme preparations 6 min before adding the substrate. 'Activator' indicates the addition of 1 mM-EDTA/5 mM-cysteine to the assay mixture.

Compound	Final concn.	Enzymic activity: ³⁵ S release (% of total)
None	—	32
Activator	—	72
Pepstatin	1 μ g/ml	15
Pepstatin+activator	1 μ g/ml	52
Leupeptin	10 μ g/ml	14
Leupeptin+activator	10 μ g/ml	13
Pepstatin+leupeptin+activator	1 μ g/ml+ 10 μ g/ml	0
Tos-Phe-CH ₂ Cl+activator	0.1 mM	17
Ac-(Ala) ₃ -Ala-CH ₂ Cl+activator	0.1 mM	9

Table 4. *Distribution of proteoglycan-degrading proteinase between fibroblasts and their culture medium*

Rabbit fibroblasts (3×10^6 /bottle) were cultured in Dulbecco-Eagle's medium (25 ml) for 96 h. The medium was removed and the cells were lysed in 0.1% Triton X-100 (5 ml). Assays of proteoglycan-degrading activity were made at pH 7.6 as described in the Materials and Methods section, and at pH 4.5 in the presence of EDTA (1 mM) and cysteine (5 mM). Results (the average of two experiments) are stated as human cathepsin-D-unit equivalents. Cathepsin D and collagenase were determined as described by Barrett (1970) and Werb & Reynolds (1975a) respectively.

Enzyme	Activity		Ratio (medium/cells)
	Cell lysate (total munits)	Medium (munits secreted/ 24 h)	
Proteoglycan-degrading activity			
pH 7.6	<9*	87	>10
pH 4.5	69	27	0.39
Cathepsin D	1700	50	0.03
Collagenase	<100	2100	>21

* Below linear part of standard curve.

Table 5. *Inhibition characteristics of proteoglycan-degrading activity of rabbit polymorphonuclear leucocyte granules*
Results of an experiment in which two granule preparations (a and b) were assayed; all assays were made in the presence of 10 mM-CaCl₂ for 4 h. See legends to Tables 1 and 3 for additional details. Abbreviation: n.d., not determined.

Compound	Final concn.	Enzymic activity (³⁵ S release as % of total)			
		pH 4.5		pH 7.6	
		(a)	(b)	(a)	(b)
None	—	12.4	7.5	12.6	18.8
Activator	—	25.2	14.7	7.3	9.4
Pepstatin	1 µg/ml	2.5	2.4	13.3	17.0
Leupeptin	10 µg/ml	11.6	6.8	12.1	16.7
Leupeptin+activator	10 µg/ml	10.6	7.3	n.d.	n.d.
4-Chloromercuribenzoate	1 mM	n.d.	n.d.	10.3	14.5
Dip-F	1 mM	11.2	6.5	1.7	5.5
Soya-bean trypsin inhibitor	100 µg/ml	13.7	8.4	2.6	6.1
Tos-Phe-CH ₂ Cl	0.1 mM	n.d.	n.d.	11.2	n.d.
Ac-(Ala) ₃ -Ala-CH ₂ Cl	0.1 mM	n.d.	n.d.	6.1	n.d.
Ac-(Ala) ₃ -Ala-CH ₂ Cl+activator	0.1 mM	n.d.	6.7	n.d.	n.d.
Ac-(Ala) ₃ -Ala-CH ₂ Cl+EDTA	0.1 mM; 10 mM	n.d.	n.d.	0	n.d.
EDTA	10 mM	9.7	5.0	6.6	12
EDTA+soya-bean trypsin inhibitor	10 mM; 100 µg/ml	n.d.	n.d.	0	n.d.
1,10-Phenanthroline	1 mM	11.6	8.2	10.7	14.6
α ₂ -Macroglobulin	200 µg/ml	n.d.	n.d.	0	n.d.

The inhibition characteristics of the granulocyte enzymes are shown in Table 5. The results suggest that at least two distinct enzymes were active at pH 7.6. One part of the enzymic activity was inhibited by soya-bean trypsin inhibitor, Dip-F and Ac-(Ala)₃-Ala-CH₂Cl, and the remainder by chelators such as EDTA and 1,10-phenanthroline. The activities were unaffected by pepstatin and leupeptin, which inhibited the activities at pH 4.5. At pH 4.5, two distinct enzyme activities were again detectable. One was inhibited by pepstatin; the other, a thiol proteinase activated by EDTA (1 mM) and cysteine (5 mM), was inhibited by leupeptin and Ac-(Ala)₃-Ala-CH₂Cl. It is probable that the granule preparations, having been diluted in 50 mM-Tris/HCl buffer, pH 7.6, containing 5 mM-CaCl₂, did not retain the full activity of the thiol proteinases active at acid pH values (Barrett, 1973).

Proteolytic activities of rabbit leucocyte granules. In a further attempt to characterize the acid proteinases of rabbit leucocyte granules, assays for cathepsin B and cathepsin D were made at acid pH. The granules contained Bz-DL-Arg-NNap-hydrolysing activity at pH 6, and this activity was stimulated by the addition of cysteine to reaction mixtures. The granules also contained haemoglobin-degrading activity at pH 3, and an extract of the granules gave a line of immunological identity with rabbit liver cathepsin D against specific sheep anti-(rabbit liver cathepsin D) serum (Dingle *et al.*, 1971).

The presence of various neutral proteinase and esterase activities in the leucocyte granules was also examined. The azo-casein-degrading activity at pH 7.6 had an inhibition profile similar to that of the proteoglycan-degrading activities. About 5% of the activity was inhibited by soya-bean trypsin inhibitor (1 mg/ml), the remainder being sensitive to EDTA (10 mM).

Since in human spleen (Starkey & Barrett, 1976) the azo-casein-degrading activity at neutral pH is due to lysosomal elastase and cathepsin G, an attempt to characterize further the azo-casein-degrading activity of rabbit leucocyte granules was made. Elastase activity was detected both with elastin-agarose plates and with the synthetic substrate Z-Ala-2-ONap (Starkey & Barrett, 1976). Although this activity was minute compared with the elastase activity of human spleen and polymorphonuclear leucocytes (less than 0.1% of the activity/mg of protein), it could account for part (5–30%) of the azo-caseinase activity, if the relative activities of the human and rabbit enzymes are the same for the various substrates.

Only a very small amount of cathepsin G-like activity (against Bz-DL-Phe-2-ONap) was found in the rabbit granules. Thus it is likely that part of the proteoglycan-degrading activity of rabbit granules was due to lysosomal elastase, but the rest of the activity cannot be attributed to other recognized proteinases.

Discussion

In this study we have described the proteoglycan-degrading proteins of rabbit fibroblasts and leucocytes. The 'bead assay' for proteoglycan-degrading enzymes proved valuable in this study, both for its sensitivity and for its applicability over a wide range of pH values (in contrast with most methods for detecting such enzymes: Dingle *et al.*, 1977).

The rabbit synovial fibroblasts released into their culture medium proteoglycan-degrading proteinases active in the slightly alkaline and acid pH ranges. There have been few previous reports of neutral-to-alkaline proteolytic activity associated with fibroblasts, although Werb & Reynolds (1974) reported that the culture medium of rabbit cells contains neutral proteinase activity against Azocoll, gelatin and azo-casein, and Werb & Aggeler (1978) have demonstrated plasminogen activator. In the present study the enzyme active against proteoglycan at pH 8 was strongly inhibited by chelating agents, and only slightly by inhibitors of serine proteinases. The enzyme is therefore tentatively concluded to be a metallo-proteinase and may account for the general proteolytic activity detected with other substrates; however, the possibility that several metallo-proteinases exist cannot be excluded. It was shown that inhibition of proteoglycan-degrading activity by Z-Phe-CH₂Br, a subtilisin inhibitor, was not inconsistent with the enzyme being a metallo-proteinase.

Apart from the specific collagenases (Harris & Cartwright, 1977) and the rabbit kidney brush-border enzyme (Kerr & Kenny, 1974*a,b*), the metallo-endopeptidases of mammalian tissues are not well characterized. An enzyme requiring Ca²⁺ or Mg²⁺ was detected in bovine and human lens (Blow *et al.*, 1975) and there have been several reports of a metal-dependent 'gelatinase' in human neutrophil granulocytes (Harris & Krane, 1972; Sopata & Danciewicz, 1974; Murphy *et al.*, 1977). Sapolsky *et al.* (1976) and Nagase & Woessner (1977) have reported the detection of metallo-proteinases in human and bovine cartilage respectively.

Werb & Reynolds (1974) noted that very little collagenase was detectable in the rabbit fibroblasts, although they were secreting appreciable amounts of the enzyme into the medium. In contrast, the amounts of lysosomal acid hydrolases in the cells were appreciable compared with the amounts secreted. It now appears that the proteoglycan-degrading metal-dependent proteinase resembles the collagenase (also a metallo-enzyme) in having a very small intracellular pool. It is possible that these two 'exo-enzymes' share a secretory organelle.

The acid-acting proteoglycan-degrading enzymes released by the fibroblasts were completely inhibitable by the combination of pepstatin and leupeptin, powerful inhibitors of the carboxy and thiol protein-

ases respectively (Barrett & Dingle, 1972; Barrett, 1977). It is very probable that cathepsin D is the carboxy proteinase involved, although cathepsin E cannot be excluded on the present evidence. Cathepsin B is likely to contribute the thiol-dependent component of the activity, although, again, other thiol proteinases are not excluded. Dingle *et al.* (1977) detected in rabbit ear cartilage an enzyme, cathepsin F, that degraded proteoglycan at acid pH values, but was resistant to inhibition by pepstatin or leupeptin. Our failure to detect this enzyme in the culture medium of rabbit synovial fibroblasts does not rule out the presence of cathepsin F, since it was apparently associated with a membrane system of the chondrocytes, and not easily solubilized. Hyaluronidase, an enzyme with a pH optimum of 3.5, may degrade proteoglycans, releasing ³⁵S-labelled fragments. We were unable to detect hyaluronidase activity in the rabbit fibroblast cultures (Z. Werb, unpublished work) and this activity has not been found in human fibroblast cultures (Abogast *et al.*, 1975). However, primary cultures of fibroblasts from embryonic-chick skin contained hyaluronidase activity, both in the cells and secreted into the medium (Orkin *et al.*, 1977). The possible secretion of hyaluronidase must be taken into consideration in all studies of proteoglycan degradation by mesenchymal cells.

The proteoglycan-degrading and proteolytic activities of human neutrophil leucocytes are predominantly active at neutral to alkaline pH values, being due to the serine proteinases lysosomal elastase and cathepsin G (Barrett & Starkey, 1977; Roughley & Barrett, 1977). By contrast, the proteolytic activity of rabbit polymorphonuclear leucocytes is mainly at acid pH values (Davies *et al.*, 1971; Ignarro *et al.*, 1973) and the proteoglycan-degrading activity has been found to show a similar characteristic (Weissmann & Spilberg, 1968; Oronsky *et al.*, 1973). It was therefore surprising that we found greater activity at pH 7.6 than at pH 4.5 in the bead assay for proteoglycan-degrading activity. These differences may be due to differences in presence of activators of enzymic activity (e.g. Ca²⁺) present in reaction mixtures.

The activity of the rabbit leucocyte granules assayed against proteoglycan at pH 7.6 could be attributed to two enzymes in approximately equal activities. One enzyme was a serine proteinase inhibited by Ac-(Ala)₃-Ala-CH₂Cl, a specific inhibitor of the elastases (Powers, 1976). It has been suggested that the serine proteinase of the azurophil granules of rabbit polymorphs, characterized by Davies *et al.* (1971), is an elastase (Starkey, 1977). The second neutral proteinase was metal-dependent, and has not been reported for rabbit leucocytes; it might be the counterpart of the gelatin-degrading metal-dependent enzyme of human neutrophils (Harris & Krane, 1972; Sopata & Danciewicz, 1974),

but equally could be related to the fibroblast metalloproteinase. Neutral collagenolytic activity has also been found in the specific granules of rabbit leucocytes (Robertson *et al.*, 1972).

The proteoglycan-degrading activity of the leucocyte-granule preparations at pH4.5 showed components sensitive to pepstatin and leupeptin. Of the carboxy proteinases sensitive to pepstatin (Barrett & Dingle, 1972), cathepsin D certainly was present (detected by immunoprecipitation), and cathepsin E may also have contributed, since it is reported to be present in these cells (Stefanovič *et al.*, 1962). Leupeptin inhibits the thiol proteinases, of which the best known is cathepsin B (Barrett, 1977); there is very little doubt that the hydrolysis of Bz-DL-Arg-NNap was due to cathepsin B, but we cannot say whether other thiol proteinases also contributed to the leupeptin-sensitive activity. Most acid hydrolases are found in the azurophil granules of polymorphonuclear leucocytes (Baggiolini *et al.*, 1969); however, the localization of the proteoglycan-degrading proteinases has yet to be established.

The catabolism of the macromolecules of connective tissues is largely mediated by enzymes produced by cells within the matrix. Although roles for the proteoglycan-degrading proteinases in physiological and pathological conditions have not been determined, several intriguing possibilities exist. These enzymes may have an important regulatory function in the alteration of the basal lamina separating epithelium and mesenchyme during morphogenesis and growth (Smith & Bernfield, 1977); they may degrade proteoglycans during bone growth at the epiphyseal plate and during resorption. In conjunction with proteinases from leucocytes the fibroblast proteinases may facilitate erosion of joints in arthritis. Further work defining the biochemical characteristics of these enzymes and studies on the factors regulating the expression of proteoglycan-degrading activity may help elucidate roles for these interesting proteinases.

Note Added in Proof (Received 9 March 1978)

Since completion of the work described in the present paper, new data have been obtained that strongly suggest that rabbit connective tissues secrete at least three distinct neutral metallo-proteinases. These have been separated chromatographically in latent forms (Sellers *et al.*, 1978), and when activated degrade collagen, gelatin and proteoglycans respectively. Consequently, it seems likely that the neutral proteinase activity secreted from rabbit synovial fibroblasts, as described in the present paper, would have been due to several enzymes.

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