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Metalloproteinases from rabbit bone culture medium degrade types IV and V collagens, laminin and fibronectin

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Gel-filtration chromatography of culture medium from rabbit bone explants separates three latent metalloproteinases with activities against collagen, proteoglycan and gelatin respectively. The fractions degrading proteoglycan also degrade laminin, fibronectin and the polymeric products of pepsin-solubilized type IV collagen and can also solubilize insoluble type IV collagen. The fractions degrading gelatin are capable of degrading solubilized type V and 1α,2α,3α (cartilage) collagens, as well as the lower-molecular-weight products of pepsin-solubilized type IV collagen. All activities can be inhibited by 1,10-phenanthroline and occur in either partially or totally latent forms that can be activated by 4-aminophenylmercuric acetate.

Collagens designated types IV and V (AB) and the glycoproteins laminin and fibronectin, which are associated with basement membranes and cellular exoskeletal structures, have been studied in detail, and much information concerning their molecular nature, organization and location is available (Yamada & Olden, 1978; Timpl *et al.*, 1979; Bornstein & Sage, 1980). Proteinases from human polymorphonuclear leucocytes (Mainardi *et al.*, 1980*a*; Uitto *et al.*, 1980), a proteinase from mast cells (Sage *et al.*, 1979) and a metalloproteinase from the culture fluid of a highly metastatic mouse tumour (Liotta *et al.*, 1981*a*) can all cleave type IV collagen. Type V collagen is degraded by a metalloproteinase secreted by activated macrophages (Mainardi *et al.*, 1980*b*) as well as by enzymes from mast cells (Sage & Bornstein, 1979) and some tumour cells (Liotta *et al.*, 1981*b*). Little is known, however, of physiological mechanisms that may be involved in the turnover of types IV and V collagens, fibronectin and laminin by connective-tissue cells.

Several groups have reported the inability of specific mammalian collagenases (which cleave the interstitial collagens, types I, II and III) to degrade types IV and V collagens (Sage *et al.*, 1979; Liotta *et al.*, 1981*a*; Sage & Bornstein, 1979; Woolley *et al.*, 1978). In addition to collagenase, rabbit bone and other connective tissues in culture are known to synthesize and secrete two other metalloproteinases that can degrade proteoglycan (and casein) and gelatin respectively, and the three are separable by gel-filtration chromatography (Sellers *et al.*, 1978; Cambray *et al.*, 1981; Murphy *et al.*, 1981). Here we describe the ability of connective-tissue metalloproteinases to degrade various connective-tissue components and attempt to relate these data to our previous observations and those of others (Liotta *et al.*, 1981*a,b*) working with latent metalloproteinases from tumours.

Materials and methods

Materials

Most materials were described previously (Sellers *et al.*, 1978). Soluble type V collagen was prepared either from human chorioamniotic membrane (Burgeson *et al.*, 1976) or from human aorta prepared by modification of the method of Rhodes & Miller (1978). 1α,2α,3α collagen was prepared from human neonatal hyaline cartilage (Burgeson & Hollister, 1979). Laminin from mouse EHS (Engelbreth-Holm and Swarm) sarcoma was generously given by Dr. R. Timpl, Max-Planck-Institut für Biochemie, Martinsried, Federal Republic of Germany. Rabbit plasma fibronectin was prepared by the gelatin–Sepharose method of Vuento & Vaheri (1978). Soluble type IV collagen was prepared from bovine anterior lens capsules by the method of Schwartz & Veis (1978) and the resulting collagen was similar to that described by these workers, although more bands corresponding to intermediate molecular weight were detected on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 2 below). Material in all of the bands was totally

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degraded by bacterial collagenase. Insoluble type IV collagen was prepared from powdered bovine anterior lens capsules by extraction of soluble materials with 0.1 M-acetic acid containing 5 mM-EDTA, 10 mM-*N*-ethylmaleimide and 1 mM-phenylmethanesulphonyl fluoride.

Fractionation of bone culture medium

Calvariae from newborn rabbits were cultured to produce latent metalloproteinases as described previously (Sellers *et al.*, 1978). Medium was concentrated 100-fold by using an Amicon ultrafiltration cell, fitted with a PM 10 membrane, before chromatography on Ultrogel AcA 44. The eluate fractions were assayed after activation with 4-aminophenylmercuric acetate for collagen- and gelatin-degrading activities (Sellers *et al.*, 1978), casein-degrading activity (Murphy *et al.*, 1981) and proteoglycan degradation (Nagase & Woessner, 1980). One unit of activity is 1 μg of substrate degraded/min.

^{125}I-labelling of connective-tissue macromolecules

Types IV, V and 1α,2α,3α collagens and laminin (about 100 μg; 1 mg/ml) were labelled by using *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)-propionate (Bolton & Hunter, 1973). The labelled material was diluted 10-fold and dialysed against the incubation buffer described below.

Analysis of the degradation of collagen types IV and V, laminin and fibronectin

The individual purified connective-tissue macromolecules were incubated in 50 mM-Tris/HCl (pH 7.6) / 200 mM-NaCl / 5 mM-$CaCl_2$ / 0.5 mM-4-aminophenylmercuric acetate with fractions from Ultrogel AcA 44 filtration of concentrated bone medium in the absence and presence of 2 mM-1,10-phenanthroline for 20 h at various temperatures. The assays were terminated with 1,10-phenanthroline, if not already present, and the total incubation mixture treated with 1% sodium dodecyl sulphate and 2.5% (v/v) 2-mercaptoethanol at 100°C for 1 min before electrophoresis on sodium dodecyl sulphate/polyacrylamide gels (Laemmli & Favre, 1973). Gels were fixed and stained with Coomassie Brilliant Blue before being dried. Where ^{125}I-labelled substrates were used, the gel was exposed to X-ray film for 24–48 h.

Degradation of insoluble type IV collagen

Powdered collagen (2 mg) was incubated with column fractions in a final volume of 1 ml of the buffer described in the above paragraph. Incubations were carried out in 1.5 ml tubes, which were revolved at 37°C. Control incubations included: (i) 1,10-phenanthroline (2 mM) to check that metal-dependent activities were involved; (ii) buffer alone; (iii) 20 μg of trypsin to determine the amount of material susceptible to general proteolytic activity (usually less than 5% of the total hydroxyproline content); and (iv) 50 μg of bacterial collagenase to determine the total solubilizable hydroxyproline content of the powdered collagen (98–99%). At the end of the incubation undigested collagen was removed by centrifugation. Both supernatant and the pellet were analysed for hydroxyproline content (Burleigh *et al.*, 1974). In some cases samples of the supernatant were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the method described above being used. One unit of activity represents 1 μg of collagen solubilized/h.

Results and discussion

Concentrated culture medium from rabbit calvariae cultures was fractionated on Ultrogel AcA 44 as described previously (Sellers *et al.*, 1978) and the elution profile of three metalloproteinase activities against gelatin (M_r 70000), cartilage proteoglycan (also casein; M_r 50000) and type I collagen (M_r 45000) substrates respectively, is shown in Fig. 1. These activities were eluted in either a wholly or partially latent form and could be detected by the inclusion of 4-aminophenylmercuric acetate in the assays. In other work we have been able to establish that three separate enzymes are responsible for these activities. Collagenase has been purified by the method of Cawston & Tyler (1979) and shown to have low gelatin- and casein-degrading ability. The separation and purification of gelatin- and proteoglycan (casein)-degrading enzymes (W. A. Galloway & G. Murphy, unpublished work) indicate that there is some cross-over in the specificity of the enzymes for gelatin and casein substrates. As described by Sellers *et al.* (1978), the proteoglycan-degrading enzyme is an endopeptidase and also degrades casein. All the activities were inhibited by 1,10-phenanthroline.

Concentrated bone medium was found to contain latent metalloproteinase activities against type IV collagen solubilized from anterior lens capsule, type V collagen from either human aorta or chorioamniotic membrane, 1α,2α,3α collagen from human neonatal cartilage, laminin from mouse EHS sarcoma and fibronectin from rabbit plasma. These activities were also activated by the presence of 4-aminophenylmercuric acetate and could be inhibited by 1,10-phenanthroline.

To investigate the relationship between known metalloproteinases and the activities just described, column fractions (after Ultrogel AcA 44 chromatography) were incubated with the various substrates and the products analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. To facilitate interpretation of gel patterns, the substrates

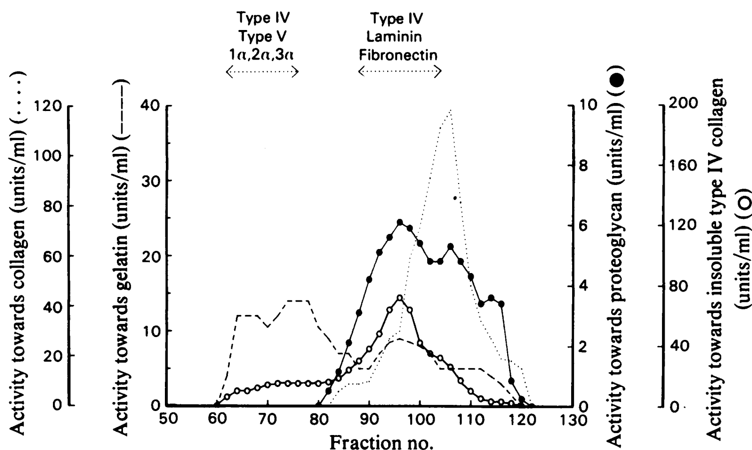

Fig. 1. *Fractionation of bone medium by filtration on Ultrogel AcA 44*
Concentrated rabbit bone culture medium was chromatographed on Ultrogel AcA 44 (column size 4.4 cm × 115 cm) and fractions (12 ml) were assayed for activity towards gelatin (----), proteoglycans (●), insoluble type IV collagen (O) and type I collagen (····) substrates in the presence of 4-aminophenylmercuric acetate (0.7 mM). Fractions were also tested for their activity against solubilized types IV, V and 1α,2α,3α collagen, laminin and fibronectin, and the products were analysed as described in the Materials and methods section. The arrows indicate fractions with detectable activity on these substrates.

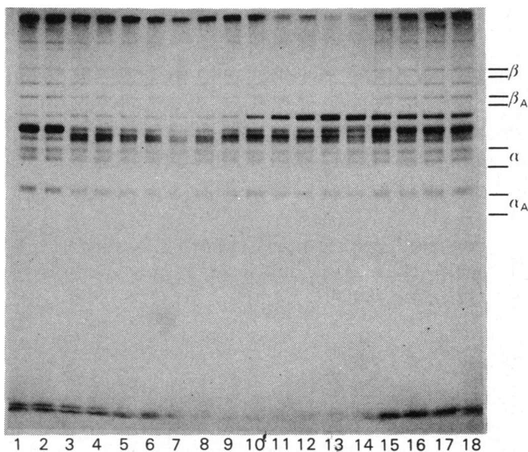

Fig. 2. *Autoradiograph of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the products of the soluble type IV collagen degradation by bone medium fractions at 25°C*
^{125}I-labelled type IV collagen (4 μg), solubilized by pepsin digestion of bovine anterior lens capsule, was incubated for 20 h with fractions (10 μl) from the Ultrogel AcA 44 filtration of bone medium shown in Fig. 1. Incubation were analysed on 6.5% (w/v) polyacrylamide gels under reducing conditions. Lane 1, non-incubated collagen; 2, incubated collagen; 3, with fraction 68; 4, 70; 5, 72; 6, 74; 7, 76; 8, 80; 9, 84; 10, 88; 11, 90; 12, 92; 13, 94; 14, 96; 15, 100; 16, 104; 17, 108; 18, 110. The migration positions of type I collagen before and after the action of specific collagenase are given as molecular-weight markers (β, 200000; β_A, 150000; α, 100000; α_A, 75000).

were generally labelled with ^{125}I so that the products could be detected by autoradiography free from protein derived from the crude enzyme fractions. No differences in substrate susceptibility to degradation were detectable after radiolabelling. Fig. 2 shows the products of incubation with type IV collagen at 25°C. Fractions corresponding to the gelatinase activity (65–80; Fig. 1) degraded predominantly material in the band corresponding to 140000 M_r (M_r assessed by comparison with the mobility of type I collagen) to yield a product of slightly reduced size. Fractions corresponding to the proteoglycan- and collagen-degrading activities, which overlap significantly (90–110; Fig. 1), appeared to cleave the more highly cross-linked components of M_r 200000 and above to yield components of M_r about 160000, as well as showing limited activity on lower-M_r components. It is known that purified mammalian tissue collagenases do not substantially degrade soluble type IV collagen (Sage *et al.*, 1979; Liotta *et al.*, 1981*a*), and our studies with purified rabbit bone collagenase confirmed these observations. The Ultrogel AcA 44 fractions corresponding to the proteoglycanase also solubilized insoluble type IV preparations from the anterior lens capsule at 37°C (Fig. 1). Analysis of the products on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that they covered a large range of sizes, from highly polymeric material that did not enter the gel to small fragments. Prominent bands of M_r about 100000 and 75000 were detectable. Activity was similar, but much decreased, at 25°C: studies at lower temperatures are required to establish whether limited

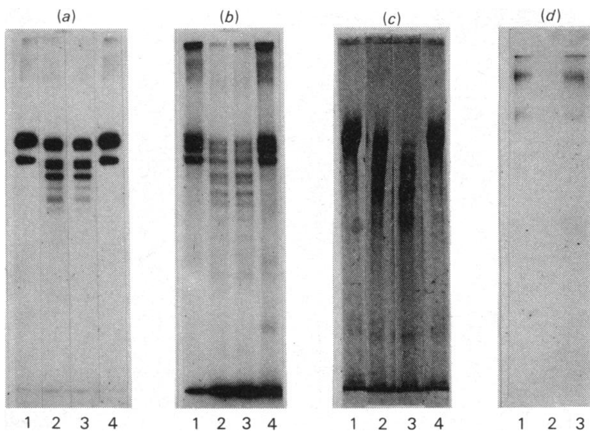

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the products of digestion of soluble type V and 1α,2α,3α collagens, fibronectin and laminin by bone medium fractions*

(*a*) ^{125}I-labelled type V collagen (4 μg) incubated at 27°C: 1, alone; 2, with fraction 66 (20 μl); 3, with fraction 72 (20 μl); 4, as for 3, but with 2 mM-1,10-phenanthroline (autoradiograph). (*b*) ^{125}I-labelled 1α,2α,3α collagen (4 μg) incubated at 27°C: 1–4, as for (*a*) (autoradiograph). (*c*) Rabbit plasma fibronectin (8 μg) incubated at 37°C: 1, alone; 2, with fraction 88 (1 μl); 3, with fraction 96 (1 μl); 4, as for 3, but with 2 mM-1,10-phenanthroline. (*d*) ^{125}I-labelled laminin (0.9 μg) incubated at 37°C: 1, alone; 2, with fraction 96 (30 μl); 3, as for 2, but with 2 mM-1,10-phenanthroline (autoradiograph).

cleavages occur under less denaturing conditions. Additionally the proteoglycanase fractions from the gel-filtration column degraded laminin and fibronectin (Figs. 3*c* and 3*d*). Purified collagenase had no activity towards these two materials. It was concluded that the major activity degrading all the preparations of type IV collagens and laminin and fibronectin was due to the proteoglycanase and not to collagenase; this conclusion can only be confirmed by the complete purification of the proteoglycan/casein-degrading enzyme. Liotta *et al.* (1981*a*) have described a latent metalloproteinase of M_r 70000–80000 from cultured metastatic-tumour cells which specifically cleaves mouse sarcoma type IV collagen, isolated in a soluble form without pepsin digestion, but this activity did not degrade either fibronectin or other collagens. This activity may be similar to the gelatinase activity of rabbit bone cultures.

By using either type V or the 1α,2α,3α cartilage collagen as substrate, analysis of the column fractions indicated that those corresponding to the gelatinase could degrade both substrates. Figs. 3(*a*) and 3(*b*) show the products of cleavage by two representative column fractions at 27°C and the inhibition of activity by 1,10-phenanthroline. Increased activity was observed at higher temperatures, when the fractions caused extensive degradation (results not shown). We found that purified tissue collagenases were able to degrade type V collagen at 37°C, but activity was very limited at temperatures below 30°C. 'Melting' curves of the soluble type V collagens show that they start to assume a random-coil conformation at temperatures greater than 30°C (Bentz *et al.*, 1978). Liotta *et al.* (1981*b*) have described a latent metalloproteinase of M_r 80000 secreted by malignant macrophages in culture which gives limited cleavage products with soluble type V collagen at 25°C. This activity also degraded solubilized type IV collagen to a limited extent, although there was negligible degradation of insoluble type IV preparation.

Our observations suggest that normal tissues in culture produce a family of metalloproteinases which have the combined ability to digest most extracellular matrix components. Liotta *et al.* (1981*a*) proposed the existence of metalloproteinases from tumours with different substrate specificities with respect to collagen type, but there seems no need at present to infer that such activities are specifically related to tumour cells. The precise number of metalloproteinases and their exact specificities remain to be determined, as does a precise understanding of their role both physiologically and pathologically.

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