CATHEPSIN D IN CARTILAGE: THE IMMUNOHISTOCHEMICAL DEMONSTRATION OF EXTRACELLULAR ENZYME IN NORMAL AND PATHOLOGICAL CONDITIONS

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SUMMARY
Extracellular cathepsin D has been demonstrated immunohistochemically in cultured chicken and rabbit cartilage. Enzyme released from viable cells was captured by specific anti-cathepsin D antibody to form extracellular immunoprecipitates which were demonstrated with fluorescein-labelled antibodies. In unfixed frozen sections these immunoprecipitates could be dissolved in antigen excess, and closely resembled those observed in immunoprecipitin lines. Extracellular cathepsin D was detected in the perichondria of normal chick cartilaginous limb-bones and, to a lesser degree, beneath them in the epiphyseal cartilage. In cultured rabbit ear cartilage, cathepsin D was detected around chondroblasts and some peripherally situated chondrocytes. In chicken and rabbit cartilage exposed to supra-physiological levels of retinol, much extracellular enzyme was detected which had been released from chondrocytes of hypertrophic and epiphyseal cartilage and rabbit ear cartilage.

INTRODUCTION
The substantial degradation of cartilage matrix in embryonic chick-limb bones (Fell & Dingle, 1963) and immature rabbit ear cartilage (J. T. Dingle, R. M. Hembry & A. R. Poole, unpublished results) induced by culture with added retinol has been shown to be dependent upon the retention of cell viability. The action of the vitamin results in the secretion into the culture medium of increased amounts of cathepsin D and proteoglycan. This is associated in both species with a loss of matrix proteoglycan staining with toluidine blue (Fell & Mellanby, 1952; Lucy, Dingle & Fell, 1961; and see below).

Pepstatin (Dingle, Barrett, Poole & Stovin, 1972), which is a potent inhibitor of cathepsin D (Barrett & Dingle, 1972), and species-specific inhibitory antisera to cathepsin D (Weston, Barrett & Dingle, 1969; Dingle, Barrett & Weston, 1971) both arrest almost completely the autolytic degradation of cartilage matrix induced when ruptured cells release lysosomal enzymes. Excessive matrix degradation can also be partially inhibited in cultured cartilage by specific antisera to cathepsin D (J. T. Dingle, R. M. Hembry, A. R. Poole & A. J. Barrett, unpublished results). Together these observations provide evidence for a role for cathepsin D in the pathological destruction of cartilage matrix. It has been suggested that such lysosomal enzymes may be important in the erosion of cartilage matrix in rheumatoid arthritis (see Dingle, 1962).
It is likely that cathepsin D may act synergistically with other acid and neutral tissue proteinases, including collagenase; the relative importance of this enzyme may depend upon local microenvironmental conditions (Dingle, 1973). Thus it is important to be able to localize these enzymes at intracellular and extracellular sites and to correlate such distributions with the local erosion of cartilage matrix, under both normal and pathological conditions. Antibodies which react specifically with cathepsin D have already been used immunohistochemically to localize this enzyme in intracellular sites in fixed tissue sections and in cell monolayers (Poole, Dingle & Barrett, 1972; Weston & Poole, 1973). Attempts in this laboratory at the direct localization of extracellular cathepsin D using similar methods have usually proved unsuccessful, owing to a lack of sensitivity in the staining methods employed. This difficulty has now been overcome and in this paper we describe the use of antibodies to cathepsin D to capture this enzyme in extracellular sites in living tissues. The immunoprecipitates formed in cultured chick limb-bone rudiments and in adult rabbit ear cartilage were demonstrated immunohistochemically. Similar methods have been used to study the relationship of extracellular cathepsin D to periosteal ossification in growing chick limb-bones (Poole, Hembry & Dingle, 1973).

MATERIALS AND METHODS

Calf serum (no. 2) (Cs) was obtained from Wellcome Reagents Ltd., Beckenham, Kent. Species-specific ovine antisera reacting only with rabbit (As-RD) or chicken (As-CD) cathepsin D were raised in this laboratory and characterized as described previously (Weston, 1969; Dingle et al. 1971). Non-immune sheep serum (Ns) was a pool of samples taken from 12 different animals. Sera were heated at 56 °C for 30 min to inactivate complement.

The precipitating activities of these antisera were estimated by reversed radial immunodiffusion using a method based on that described by Mancini, Carbonara & Heremans (1965). Gels of uniform thickness (2 mm) were freshly prepared from 1 % agarose ('Meath', Paines and Byrne Ltd., Greenford, Middlesex) in phosphate-buffered saline pH 7.4 (PBS) and contained purified rabbit or chicken cathepsin D. PBS contains 145 mM NaCl, 9 mM Na₂HPO₄ and 1 mM NaH₂PO₄. The mean diameters of duplicate precipitation rings were measured after 24-h diffusion of 5 μl of antiserum (total well capacity) at room temperature and were plotted against the concentration of the antisera as shown in Fig. 1.

Purified rabbit IgG was labelled with fluorescein isothiocyanate (FITC) by the method of The & Feltkamp (1970) after isolation from a rabbit anti-(sheep IgG) serum raised in this laboratory (see Poole et al. 1972). This conjugate (RaS-F) is the same as that described by Poole et al. (1972). It exhibited a weak cross-reaction with calf serum IgG in Ouchterlony gel diffusion.

Cathepsin D was purified from rabbit and chicken liver and assayed for protein content and enzyme activity by the method of Barrett (1970).

Ouchterlony immunodiffusion gels were prepared with 1 % agarose in PBS.

Organ culture

Cartilaginous tibiae and femora of 8-5-day-old chick embryos were dissected as free as possible from muscle and connective tissue in BGJw holding medium prior to culture, BGJw has the same composition as BGJ (Dingle, Fell & Coombs, 1967) except that it contains 6 mM sodium bicarbonate and 10 times the normal antibiotic concentrations. Ears of 6- to 8-week-old rabbits were stripped of skin exposing the cartilage plate and, by use of a leather punch, discs 5 mm in diameter were prepared for culture. Tissues were explanted on to stainless steel grids (Fell & Dingle, 1969). The tibia and femur of one chick embryo limb or 2-3 discs of rabbit ear cartilage were cultured on each grid at the interface between culture medium and a gaseous phase of...
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Fig. 1. Radial immunodiffusion estimations of the precipitating activities of antisera to cathepsin D. The relationship between the concentration of antiserum to cathepsin D and the square of the diameter (in mm) of the precipitation ring is shown. O—O, antiserum to chicken cathepsin D; the gel contains 5.2 μg/ml or 1.65 units/ml of chicken cathepsin D. ●—●, antiserum to rabbit cathepsin D; the gel contains 13.2 μg/ml or 5.4 units/ml of purified rabbit cathepsin D.

5% CO₂, 75% nitrogen and 20% oxygen. The culture medium was BGJ6, to which was added, in the case of rabbit cultures only, 100 units/ml of mycostatin (Squibb), and the appropriate serum. Retinol (Roche) was used at a final concentration of 3 μg/ml. It was dissolved and stored in ethanol, under nitrogen, in sealed ampoules, and was added to serum-containing medium immediately before use. The final ethanol concentration was 0.1% (v/v). Cultures were incubated at 37°C, and media were changed every 2 days.

Histochemistry

For immunohistochemical studies, tissues were removed from culture vessels and immediately frozen for 30 s at approximately −68°C in a bath of n-hexane and solid CO₂. They were mounted frozen on cryostat specimen holders with distilled water and sectioned within 48 h with a knife cooled with solid CO₂. Frozen sections (6 and 10 μm thick) were fixed and stained with Ras-F as described previously (Poole et al. 1972). The specificity of the immunoreaction was controlled by pretreating fixed sections with rabbit anti-(sheep IgG) serum or non-immune rabbit serum diluted 1:4 with PBS. Only in sections pretreated with rabbit anti-(sheep IgG) serum was subsequent attachment of Ras-F abolished.

Some sections of chick tissues were counterstained for 1 min with eriochrome black, 3-hydroxy-4-(1-hydroxy-2-naphthylazo)-7-nitro-1-naphthalene sulphonic acid, sodium salt (Difco, EB) diluted 1:10 with PBS. EB emits a red fluorescence and provides good cytoplasmic staining. Although its use can sometimes make the detection of green fluorescein fluorescence at the cell surface somewhat difficult, it can greatly clarify the demonstration of intercellular fluorescein fluorescence. Sections were finally rinsed with distilled water, mounted, examined immediately by darkground fluorescence microscopy as previously reported (Poole et al. 1972), and photographed as described by Poole et al. (1973b).

Alternate sections were stained for 10 min with 0.5% toluidine blue in 5% (v/v) ethanol in distilled water, washed, and permanently mounted. Sometimes tissues were fixed for 24 h, embedded in wax, sectioned, stained with toluidine blue and permanently mounted.
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Table 1. The sizes of micro-immunoprecipitates observed in and at the edges of the precipitin line shown in Fig. 3A

<table>
<thead>
<tr>
<th>Region</th>
<th>Mean ± S.E.M., μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Edge closest to antigen</td>
<td>0.37 ± 0.02 (45)</td>
</tr>
<tr>
<td>2. Mid-precipitin line</td>
<td>1.20 ± 0.05 (52)</td>
</tr>
<tr>
<td>3. Edge closest to antibody</td>
<td>0.45 ± 0.02 (52)</td>
</tr>
</tbody>
</table>

Measurements were made from enlargements of photomicrographs of the kind shown in Fig. 3B. Each aggregate was measured in μm in 3 different directions and a mean was determined. Means ± S.E.M. of the number of micro-immunoprecipitates indicated in parentheses are given. Student’s t test analysis: 1 and 3 are significantly less (P < 0.001) than 2.

RESULTS

Interaction of antibody with antigen in an agarose gel

The microscopic appearance of the product resulting from the interaction of a precipitating antibody with an antigen was determined by examining the composition of precipitin lines formed in agarose gels. Fig. 3A shows a precipitin line formed from the interaction of a fluorescein-labelled antibody with an unlabelled antigen. This line was composed of many fluorescent particulate aggregates often of near-spherical shape (Fig. 3B). The sizes of these micro-immunoprecipitates are shown in Table 1. It can be seen that at the edges of the precipitin line these immunoprecipitates are significantly smaller, presumably in the presence of an excess of either antigen or antibody. Micro-immunoprecipitates were completely absent from areas remote from the precipitin line and were never seen in control gels in which immuno-precipitation had not taken place.

These observations form the basis of the present study: extracellular cathepsin D was trapped by antibody in living tissues. The resultant extracellular micro-immunoprecipitates were then detected in fixed tissue sections with a fluorescein-labelled rabbit antibody to the anti-cathepsin D immunoglobulin (sheep IgG).

The extracellular distribution of cathepsin D in chick limb-bone rudiments

The general organization of these limb-bones has been described by Fell & Robison (1929) and Glauert, Fell & Dingle (1969). It is shown in Fig. 2.

Cathepsin D released from cells was trapped in the extracellular matrix by culturing explants in As-CD. Controls were cultured with Ns or As-RD.

The results of this study (9 separate experiments) are summarized in Table 2. In each experiment between 10 and 20 sections of each limb-bone rudiment were examined. Cells and cartilage matrix did not autofluoresce when examined under the conditions employed in these experiments.

In explants cultured with control sera and without additional retinol, extracellular immunofluorescent staining for IgG was diffuse and confined to the perichondrium and the periosteum. Particulate extracellular staining was also observed in those explants cultured with the antiserum to chicken cathepsin D. It was seen mainly in the peri-
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Epiphyseal chondrocytes

Articular chondrocytes

Inner chondrocytes

Bone

Periosteal fibroblasts

Hypertrophic cartilage

Perichondrial fibroblasts

Fig. 2. Diagram of part of a longitudinal section of an 8-day-old embryonic chick limb-bone rudiment illustrating the different zones described in this paper (after Glauert et al. 1969).

chondrium (Figs. 4A, 5) and in the periosteum (Fig. 4C). A very low density of particulate staining was detected in the epiphyseal cartilage which was too weak to photograph. A narrow band (about 2 cells deep) of weak extracellular particulate staining was also seen in the epiphyses after 8 days of culture; it was situated either adjacent to the perichondrium or parallel to it, but separated by an unstained band up to 3 cells deep. This banding was not observed in explants examined after a shorter period in culture (6 days).

The tissues cultured without added retinol exhibited intense metachromatic matrix staining with toluidine blue (Table 2), whereas tissues cultured with 3 μg of retinol/ml showed an extensive loss of metachromasia (Table 2) similar to that previously described by Fell & Dingle (1963).

In retinol-treated tissues cultured with the antiserum to chicken cathepsin D a much higher density of both diffuse and particulate extracellular immunofluorescent staining for IgG was seen throughout the explants. This was particularly evident in the hypertrophic cartilage (Fig. 4D) and, in some experiments (5 out of 9), in the epiphyseal cartilage (Figs. 4B, 6, 10A). Intense particulate immunofluorescent staining for IgG was always found in cartilage which exhibited little or no metachromatic staining with toluidine blue. When explants were cultured with the control sera (As-RD or Ns) only
Table 2. Summary of extracellular staining for IgG and metachromatic staining with toluidine blue in cultured chick limb-bone rudiments

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>No. of expts</th>
<th>Control Sera (Ns or As-RD)</th>
<th>Antiserum (As-CD)</th>
<th>Metachromatic Staining (As-CD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perichondrium</td>
<td>Nil</td>
<td>9</td>
<td>D+</td>
<td>D++</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>9</td>
<td>D+</td>
<td>D++</td>
<td>P+</td>
</tr>
<tr>
<td>Epiphyseal cartilage</td>
<td>Nil</td>
<td>9</td>
<td>0</td>
<td>D+</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>9</td>
<td>D+</td>
<td>D++</td>
<td>P+</td>
</tr>
<tr>
<td>Periosteum</td>
<td>Nil</td>
<td>9</td>
<td>D+</td>
<td>D+</td>
<td>P+</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>9</td>
<td>D+</td>
<td>D++</td>
<td>P+</td>
</tr>
<tr>
<td>Hypertrophic cartilage</td>
<td>Nil</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>9</td>
<td>D+</td>
<td>D++</td>
<td>P+</td>
</tr>
</tbody>
</table>

Extracellular staining for IgG and with toluidine blue of 6-μm-thick frozen fixed sections of chick limb-bone rudiments cultured for 8 days with and without additional retinol (3 μg/ml) for the first 6 days. This culture period was divided into 3 stages. In the first 4 days the standard BGJ6 medium was supplemented with 5% Cs. For the next 2 days, the control rudiments were grown with 10% (v/v) Ns or As-RD whilst other explants were exposed to the 10% (v/v) antiserum to chicken cathepsin D (As-CD). In order to remove unreacted immunoglobulin to chicken cathepsin D, all explants were cultured for a further 2 days with only 5% Cs. Frozen sections were prepared, fixed and stained for IgG with Ras-F and counterstained with EB. Extracellular staining for IgG was recorded as diffuse (D) or particulate (P). Only explants cultured with As-CD were stained with toluidine blue. The relative intensities of both types of staining were recorded as indicated (+); 'o' indicates no staining. For further observations see text.

weak diffuse staining for IgG was seen in these sites and there was no evidence of particulate staining.

In all experiments, less diffuse extracellular staining was seen in explants cultured for a final 2 days in the absence of sheep serum, after previous culture with the antiserum to chicken cathepsin D; against this reduced background the particulate staining was more clearly demonstrable.

Rate of appearance of extracellular particulate staining. A study was made of the development of extracellular staining in chick tissues which had been previously cultured with added retinol. After 4 days, explants were transferred to culture medium containing either Ns or As-CD. After 1 h of culture with As-CD, intense, mainly diffuse extracellular staining for IgG was observed in and under the perichondrium and periosteum. At 6 h some particulate staining was observed and the peripheral band of staining had increased in thickness. Much more particulate staining was seen after 24 h. By 48 h, it had further increased and had the distribution previously described. Cultures exposed to Ns exhibited weak diffuse staining in the same sites.

Concentration of antiserum required to produce detectable particulate extracellular staining for IgG. Immunoprecipitates were most commonly seen at 10% As-CD.
Extracellular cathepsin D in cartilage

**Table 3. Summary of extracellular staining for IgG and metachromatic staining with toluidine blue in cultured rabbit ear cartilage**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>No. of Expts</th>
<th>Control sera (Ns or As-CD)</th>
<th>Antiserum (As-RD)</th>
<th>Metachromatic staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perichondrium</td>
<td>Nil</td>
<td>7</td>
<td>D+</td>
<td>D+ P+</td>
<td>o</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>7</td>
<td>D+</td>
<td>D+ P++</td>
<td>o</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Nil</td>
<td>7</td>
<td>o</td>
<td>P±</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>7</td>
<td>D+</td>
<td>D+ P++</td>
<td>+</td>
</tr>
<tr>
<td>Fibroblastic tissue</td>
<td>Nil</td>
<td>7</td>
<td>D+</td>
<td>D+ P+</td>
<td>o</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>7</td>
<td>D+</td>
<td>D+ P++</td>
<td>o</td>
</tr>
</tbody>
</table>

Extracellular staining with toluidine blue and for IgG in 6-µm-thick frozen fixed sections of rabbit ear cartilage cultured for 10 days with and without additional retinol (3 µg/ml). Control explants were cultured with 5% (v/v) Ns or As-CD, whilst other explants were cultured with 5% (v/v) antiserum to rabbit cathepsin D (As-RD). Frozen sections were fixed, and stained for IgG with RaS-F. Only explants cultured with As-RD were stained with toluidine blue. Extracellular staining was recorded as described in Table 1. For further observations see text.

Concentration without subsequent culture in the absence of antibody. They were much less common at 1% and only just visible at 0.25%. At 0.1% only diffuse staining was detectable, which was comparable to that seen in cultures with 10% Ns, but much more intense than that seen at 0.1% Ns.

**The extracellular distribution of cathepsin D in rabbit ear cartilage**

When discs of rabbit ear cartilage were cultured for 10 days they often exhibited a dense peripheral outgrowth of fibroblastic tissue from the perichondrium, although the cut edges of the cartilage usually remained free of fibroblastic tissue. The matrix of cultured cartilage stained intensely with toluidine blue (Fig. 7A).

The distribution of extracellular staining for IgG in these explants is summarized in Table 3. In each experiment between 5 and 10 sections of each explant were examined. Only in tissues exposed to the antiserum to rabbit cathepsin D was particulate extracellular staining observed.

In explants cultured without added retinol particulate extracellular staining was seen in the fibroblastic tissue in the perichondrium, around chondroblasts (counterstained with EB) and some peripheral chondrocytes (Fig. 8) and others at the cut edges of the cartilage.

After culture with added retinol, there was a reduction in matrix staining with toluidine blue. In some experiments it was almost absent (Fig. 7B), but in other cases some staining persisted in the more central regions; in explants cultured with the antiserum to rabbit cathepsin D this was associated with the appearance, in unstained or weakly toluidine blue-stained matrix, of extracellular particulate immunofluorescent staining for IgG around many, but not all, chondrocytes (Figs. 9, 10B). Particulate
staining for IgG was seen all over the sectioned extracellular surfaces of some chondrocytes whereas others stained only at discrete sites at their surfaces. An increase in the density of fluorescent particulate staining for IgG was also observed in the fibroblastic tissue and the perichondrium.

The identity of extracellular particulate staining for IgG observed in cartilage cultured with antiserum to cathepsin D

Since particulate extracellular staining for IgG was only observed after culture with species-specific anti-serum to cathepsin D, it was considered to represent discrete immunoprecipitates of cathepsin D and antibody of the kind seen in immunoprecipitin lines. In further experiments the identity of the immunoprecipitates was tested by exposing them to an excess of antigen.

Unfixed frozen sections of chick and rabbit tissues cultured with species-specific antisera to cathepsin D and added retinol were exposed at 2–4 °C for 18 h at neutral pH to either chicken or rabbit cathepsin D in PBS, conditions which permit cathepsin D to express its species-specific antigenicity but not its enzymic activity. Sections were then washed with PBS, fixed and stained as usual with RaS-F. Similar experiments were made with rabbit tissues.

Extracellular particulate staining was observed in sections of chick tissues exposed to PBS or rabbit cathepsin D (Fig. 11 A, B). However, no particulate staining was seen in sections previously treated with the same amount of chicken cathepsin D (Fig. 11 C) (on an enzyme activity or protein basis), although some diffuse extracellular staining was observed in these sections. Particulate extracellular staining was clearly observed in sections of rabbit ear cartilage which had been similarly pretreated with PBS or chicken cathepsin D (Fig. 11 D, E). Only pretreatment with the same amount of rabbit cathepsin D led to a considerable reduction or the complete absence of the particulate staining (Fig. 11 F).

DISCUSSION

Several biochemical studies have clearly demonstrated the extracellular release of cathepsin D from tissues in organ culture (Fell & Dingle, 1963; Dingle et al. 1967; Dingle, Fell & Glauert, 1969). In order to determine the role of this enzyme in the turnover of matrix macromolecules, it is necessary to establish which cells release cathepsin D and the spatial relationship of this release to matrix degradation. However, until now there has been no way of directly detecting cathepsin D histochemically in extracellular sites. The particulate extracellular staining for IgG was only observed in the presence of species-specific antiserum to cathepsin D, and it was largely absent from sections exposed to an excess of species-specific antigen. Hence it is concluded that it represents immunoprecipitation of extracellular cathepsin D with antibody to cathepsin D. It is believed that the particulate staining observed in this study is the first microscopic demonstration of the release of a lysosomal proteinase to an extracellular environment.

The immunoprecipitates resembled in both size and appearance those seen in
Extracellular cathepsin D in cartilage

...immunoprecipitin lines. The formation of discrete immunoprecipitates large enough to be detected with darkground fluorescence microscopy was probably preceded by micro-immunoprecipitation to form, by antibody accumulation, the intense diffuse staining which was observed initially. The progressive accumulation of antibody with time, as cathepsin D was released to extracellular sites, clearly resulted in the formation of demonstrable immunoprecipitates.

The sizes of the immunoprecipitates are probably influenced by the relative amounts of antigen and antibody present in a given micro-environment. Differences in size were observed not only in different parts of precipitin lines but also between rabbit and chick tissues cultured with species-specific antibodies. Whereas in the case of rabbit ear cartilage cultured with added retinol, immunoprecipitates may have been formed at or near equivalence (as in the middle of a precipitin line) the smaller sizes of those observed in chick limb-bone rudiments cultured with added retinol suggest that their formation may have been influenced by a local excess of cathepsin D or antibody to cathepsin D. Micro-immunoprecipitates may also aggregate to form larger structures of the kind observed in the perichondria of chick limb-bone rudiments.

The demonstration of cathepsin D in cartilage matrix is dependent upon the penetration of antibody IgG. However, studies in this and other laboratories have indicated that normal cartilage matrix is relatively impermeable to IgG (Maroudas, 1970; Lotke & Granda, 1972; Poole, Barratt & Fell, 1973a), although it is permeable to cathepsin D (R. M. Hembry, J. T. Dingle, A. R. Poole & A. J. Barrett, unpublished results). The latter authors have also shown that after a partial loss of matrix components it becomes readily permeable to IgG. Hence this imposes a limitation on the sensitivity of this method for the demonstration of extracellular cathepsin D in normal cartilage, although in the present experiments sufficient antibody penetrated to enable the demonstration of some cathepsin D in parts of the matrix of normal chick and rabbit cartilage.

The present study of the extracellular localization of cathepsin D in cartilage draws further attention to the involvement of this enzyme in extracellular catabolism, in both normal and pathological situations. Although biochemical measurements made of cathepsin D activity in culture media have demonstrated that added retinol stimulates an increased release of this enzyme from chondrocytes in chick and rabbit cartilage, the present studies indicate that this occurs in sites where proteoglycan has been degraded and lost from the tissue. It was also notable that this enzyme was not released from all cells to the same extent, judging by the presence and density of immunoprecipitates: many cells in degraded cartilage matrix released little or no detectable enzyme.

Pericellular staining, seen in both chicken and rabbit cartilage treated with retinol, may have resulted from the capture of cathepsin D by antibody as it was released to an extracellular site. The discrete nature of the immunoprecipitates seen at the surfaces of some cells in explants cultured with added retinol may in part be accounted for by the cathepsin D contents of individual lysosomes forming nucleation centres for the formation of immunoprecipitates. This would be consistent with the proposal that primary lysosomes may fuse with the plasma membrane and then discharge their...
contents to an extracellular site (see Dingle, 1968; Dingle et al. 1969) and that this fusion may sometimes be restricted to certain areas of the plasma membrane. Further immunocytochemical work at the ultrastructural level may help to clarify this point.

The extracellular release of cathepsin D as demonstrated here is likely to be correlated with the release of other lysosomal enzymes: previous workers have reported a parallel release of these enzymes from cultured tissues (Vaes, 1968; Dingle et al. 1969). However, it is not known whether these enzymes are all released at the same site or even at the same time. Some of these other enzymes are probably also involved in matrix degradation. The availability in this laboratory of specific antisera to cathepsin B1, hyaluronidase, β-glucuronidase and collagenase should enable us to examine this problem further.

Cathepsin D may play a role in the normal turnover of proteoglycans in cartilage (Mankin & Lipiello, 1969; Dingle, 1973) and this is supported by the demonstration of its presence in undegraded cartilage. A controlled extracellular release of this enzyme probably normally occurs producing limited extracellular activity sufficient to contribute to the mobilization of proteoglycan macromolecules associated with remodelling and growth.

It is generally believed that the embryonic growth of the epiphysis takes place from the perichondrium. Appositional growth of this kind would account for the banded distribution of immunoprecipitates seen in chick bones at 8 days under the perichondrium: immunoprecipitates formed in the perichondrium would be left in sub-perichondrial sites with continued outgrowth from the perichondrium.

With this new method for demonstrating extracellular enzyme it has been possible to extend our studies of the extracellular involvement of lysosomal proteinases in tissue catabolism. Besides being involved in the intracellular digestion of certain proteins within lysosomes (Dingle, Poole, Lazarus & Barrett, 1973), cathepsin D can be seen to be secreted by cells to extracellular sites. Capture of extracellular cathepsin D with inhibitory antisera also partially inhibits the pathological degradation of matrix proteoglycan (J. T. Dingle, R. M. Hembry, A. J. Barrett & A. R. Poole, unpublished results). This points to an involvement of cathepsin D in the degradation of proteoglycan of the matrix of cartilage whether it be of an experimental nature as described here, or naturally occurring, as in rheumatoid arthritis in man.

It is likely that other tissue proteinases in addition to cathepsin D are also involved synergistically in the extracellular catabolism of the main macromolecular components of matrix, namely proteoglycan and collagen. The use of enzyme-capture methods of the kind described here should prove useful in elucidating the relative importance of these enzymes in the degradation of cartilage matrix.

This work was supported by grants from the Nuffield Foundation, the Medical Research Council, Arthritis and Rheumatism Council and the Wellcome Foundation. We thank Miss Christine Camus for her skilled technical assistance. We would like to thank Dr A. J. Barrett for supplying the purified chicken and rabbit cathepsin D.
REFERENCES


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Fig. 3. An immunoprecipitin line is shown in A which was formed within 24 h at room temperature in a gel containing 1-0 % agarose in PBS. Well A contained 200 μg of fluorescein-labelled pig immunoglobulins against rabbit serum IgG (Mercia, Watford, Middlesex) in 20 μl of 1 M sodium chloride. Well B contained 20 μl non-immune rabbit serum diluted 1 in 32 with PBS. The gel was frozen in liquid nitrogen for 80 s and frozen sectioned at 6 μm. Sections of the precipitin line were mounted unfixed and examined with fluorescence microscopy as described in the text. The precipitin line was composed of fluorescent micro-immunoprecipitates as shown in Fig. 3B. Fig. 3A, × 10-3; Fig. 3B, × 1184.

Figs. 4-6, 8-11. Darkground fluorescence micrographs of frozen, fixed sections stained with RaS-F, and EB where indicated.

Fig. 4. Distribution of IgG in 10-μm-thick longitudinal sections of chick limb-bone rudiments (femora) cultured with (a and b) and without (c and d) added retinol. After 4 days of culture with 5 % Ca ± 3 μg/ml retinol, rudiments were cultured for 2 days with, in addition, 10 % As-CD. After culture without added retinol, diffuse and particulate extracellular staining for IgG was mainly confined to (4a) the perichondrium (p) and (4c) the periosteum; diffuse and particulate extracellular staining was also seen in parts of (4b) the epihyseal cartilage (e) and in (4d) the hypertrophic cartilage (h). o, osteoblastic layer of periosteum; pf, fibroblastic layer of periosteum. × 200.
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Fig. 5. Extracellular localization of IgG (6-μm-thick longitudinal sections counterstained with EB) in the perichondrium (p) of chick tibiae cultured for 4 days with 5% (v/v) Cs and for 2 days with, in addition 10% (v/v) Na or As-CD, plus 2 days with 5% (v/v) Cs. A total of 56 micro-immunoprecipitates were measured in the perichondria of these sections as described in Table 1: the mean diameter and S.E.M. were 2.26 ± 0.13 μm. e, epiphyseal cartilage.

a, Cultured with Na. Perichondrial (p) extracellular diffuse staining for IgG was too weak to record photographically. Cell cytoplasms were stained with EB as in Fig. 6. × 1300.

b, Cultured with As-CD. Extracellular particulate staining for IgG (micro-immunoprecipitates) was also seen in the perichondrium. × 1300.

Fig. 6. Extracellular localization of IgG (in 6-μm-thick longitudinal sections counterstained with EB) in the epiphyses of chick femora cultured with 10% (v/v) As-CD or Na, as described in Fig. 5, with, in addition, 3 μg/ml of retinol for the first 6 days. A total of 105 micro-immunoprecipitates were measured in these sections as described in Table 1: the mean diameter and S.E.M. were 0.70 ± 0.03 μm.

a, Cultured with Na. Weak extracellular staining for IgG was seen in the epiphyseal cartilage (e) beneath the perichondrium (p) but at this magnification it was too weak to record photographically. Cells stained with EB fluoresced red as in Fig. 6b. × 850.

b, Cultured with As-CD. Intense extracellular particulate and diffuse staining for IgG was detected throughout the epiphyseal cartilage (e). Particulate staining (micro-immunoprecipitates) was observed both remote from cells and in pericellular sites (arrowhead). × 850.
Fig. 7. Light micrographs of the metachromatic staining with toluidine blue of rabbit ear cartilage cultured for 10 days with 5 % (v/v) Ns and either (A) without, or (B) with 3 μg/ml of retinol. These transverse wax sections are 6-μm-thick. In explants cultured with additional retinol, there was very little matrix staining. c, chondroblasts; f, fibroblasts; p, perichondrium. A, ×400; B, ×288.
Extracellular cathepsin D in cartilage
Fig. 8. Extracellular localization of IgG in 10-μm-thick transverse sections of rabbit ear cartilage cultured for 10 days with either 5% (v/v) Ns or As-RD.

A, Cultured with Ns. Faint diffuse extracellular staining for IgG in fibroblastic tissue and at the periphery of the cartilage (c) was too weak to record. No particulate staining was seen. Cartilage matrix exhibited a fawn-coloured autofluorescence (a). × 390.

B, Cultured with As-RD. Intense staining for IgG was seen in association with chondroblasts (c) and bounding some chondrocytes (arrowheads) at the edge of the cartilage. × 390.

Fig. 9. Legend as for Fig. 8 except that 3 μg/ml of retinol were also present in the culture medium. Autofluorescence (a) was commonly seen. A total of 100 micro-immunoprecipitates were measured in these sections around chondrocytes as described in Table 1: the mean diameter and S.E.M. were 1.12 ± 0.05 μm.

A, Cultured with Ns. Faint diffuse staining for IgG was detected in the peripheral fibroblastic tissue and at the edge of the cartilage (c) but was too weak to record. Only autofluorescence (a) was seen in the matrix. × 400.

B, Cultured with As-RD. Much intense particulate staining for IgG (arrowheads) was present around chondroblasts and chondrocytes, in many parts of the cartilage. Autofluorescence (a) in the matrix was also recorded. × 400.
Extracellular cathepsin D in cartilage
Fig. 10. Extracellular localization of IgG in 6-μm-thick sections of (a) chicken tibia cultured with As-CD and retinol as described in Fig. 6 and (b) rabbit ear cartilage cultured with As-RD and added retinol as described in Fig. 9.

A, Green extracellular micro-immunoprecipitates can be seen in the matrix between chick chondrocytes counterstained with EB, and (b) around unstained rabbit chondrocytes. Matrix autofluorescence is also visible. A, × 330; B, × 200.
Extracellular cathepsin D in cartilage
Fig. 11 A, B, C. Effect of chicken and rabbit cathepsin D on the extracellular particulate staining for IgG detected in hypertrophic cartilage of chick limb-bone rudiments cultured with As-CD and retinol. Limb-bones were cultured for 4 days with 3 μg/ml of retinol, 5 % Cs and 10 % As-CD, then for 24 h in Cs only. Frozen unfixed 10-μm-thick longitudinal serial sections were exposed at 4 °C for 18 h to PBS, or PBS containing 1300 units/ml of either rabbit or chicken purified cathepsin D; they were then washed in PBS, fixed, washed and stained with RaS-F and EB.

A, PBS only. Extracellular particulate staining for IgG can be seen. × 750.
B, PBS with rabbit cathepsin D. Staining as in A. × 750.
C, PBS with chicken cathepsin D. Extracellular particulate staining was not visible although some fairly intense diffuse staining was observed. × 910.

Fig. 11 D, E, F. Effects of chicken and rabbit cathepsin D on pericellular particulate staining detected in rabbit ear cartilage cultured for 9 days with 5 % As-RD and 3 μg/ml of retinol followed by 24 h with 5 % Cs and no added retinol. Serial frozen transverse sections 10-μm-thick were exposed at 4 °C for 24 h to PBS or PBS containing either 1300 units/ml of purified rabbit or chicken cathepsin D. They were then washed in PBS, fixed, washed stained with RaS-F. a, matrix autofluorescence; f, fibroblasts.

D, PBS only. Pericellular particulate staining for IgG is shown. × 400.
E, PBS with chicken cathepsin D. Staining as in D. × 390.
F, PBS with rabbit cathepsin D. Much less pericellular particulate staining is present. × 430.
Extracellular cathepsin D in cartilage