Studies on Cathepsin B in Human Articular Cartilage

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The thiol proteinase cathepsin B (EC 3.4.22.1), previously called cathepsin B_1, was assayed in human articular cartilage by its hydrolysis of the synthetic substrate α-N-benzoyl-DL-arginine 2-naphthylamide. The enzyme was activated by cysteine and EDTA and completely inhibited by iodoacetamide and $HgCl_2$. It was also partially inhibited by whole human serum. Human osteoarthrotic cartilage had increased activity when compared with normal cartilage. Cathepsin B activity of normal cartilage was age-related, being high in juveniles and declining to low values in adult and elderly individuals. Cathepsin D and cathepsin B both exhibited a zonal variation through the cartilage depth; the surface cells appeared to contain more activity than those close to the subchondral bone.

Since the work of Lucy *et al.* (1961), it has been widely held that cathepsin D is the lysosomal enzyme responsible for the breakdown of rabbit, chick and human cartilage proteoglycans during autolysis (Dingle *et al.*, 1972). However, Woessner (1973*a,b*) has shown that highly purified cathepsin D from cartilage and uterus will not degrade cartilage proteoglycans above pH 6.0. Thus the role of cathepsin D under physiological conditions was questioned. Ali (1964) and Ali *et al.* (1967) demonstrated that rabbit cartilages were rich in the thiol-dependent enzyme cathepsin B and suggested that this proteinase might be important in cartilage autolysis. However, unlike rabbit cartilages, the enzymes in human articular cartilage would not hydrolyse the synthetic substrates used (Ali & Evans, 1973). Dingle *et al.* (1972), studying the effect of inhibitors on the autolysis of human articular cartilage, found a proportion that was inhibited by thiol-enzyme inhibitors. This suggested a cathepsin B-like enzyme with maximum activity at pH 6.0. Sapolsky *et al.* (1974, 1976) have demonstrated metal-dependent neutral and acid proteinases in human articular cartilage, but no thiol-dependent enzymes.

By using a more sensitive assay method it is now possible to demonstrate conclusively the presence of cathepsin B activity in human articular cartilage. The variation of activity with age, arthritic disease and the association of the enzyme with certain chondrocytes found in different sagittal levels of articular cartilage is outlined.

Experimental

Materials

Bovine haemoglobin was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. α-N-Benzoyl-DL-arginine 2-naphthylamide and Fast Blue B (tetra-azotized di-*o*-anisidine) were supplied by Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Activators and inhibitors of cathepsin B activity, namely cysteine, EDTA, $HgCl_2$ and iodoacetamide, were obtained from BDH Chemicals, Poole, Dorset, U.K. Chloroquine phosphate was obtained from ICI, Wilmslow, Cheshire, U.K.

Cartilage sections

Normal human articular cartilage was obtained within 1 h of operation, from femoral-head fracture specimens and amputation specimens. Osteoarthrotic cartilage (types IV and VI in the nomenclature of Ali & Bayliss, 1974) was taken from femoral heads removed for total hip replacement. The specimens were washed with cold sterile 0.9% NaCl, and full-thickness cartilage was removed with a scalpel and cut into pieces of $2mm^2$ cross-sectional area on moist glass Petri dishes at 4°C. Portions of cartilage (0.3 g wet wt.) were then frozen at −20°C in cylindrical blocks with 0.5 ml of sterile water and sectioned at −20°C on a cryostat at 18 μm as described by Ali (1967) and Evans *et al.* (1967). The frozen cartilage sections were collected quantitatively in sterile Universal bottles and incubated directly with the various enzyme substrates as described by Ali (1967), Ali *et al.* (1967) and Ali & Evans (1969).

For studies on the distribution of enzyme activities through the depth of cartilage, individual full-thickness pieces of tissue ($3mm^2$ cross-sectional area) were mounted on a cryostat chuck with 0.02 ml of sterile water and sectioned (18 μm) from the articular surface, and every ten sections were collected in sterile conical glass centrifuge tubes. This procedure was repeated for five pieces of cartilage. Each tube, which contained sections from each level

pooled from the five specimens, was then assayed directly for the cathepsin D or cathepsin B activity by scaled-down methods.

Determination of α-N-benzoyl-DL-arginine 2-naphthylamide hydrolysis

Cartilage sections (0.3 g in 0.5 ml of sterile water) were incubated in 11.5 ml of 2.5 mM-α-N-benzoyl-DL-arginine 2-naphthylamide and 8.0 ml of 0.2 M-sodium acetate buffer, pH 5.0, containing 25 mM-EDTA and 5 mM-cysteine at 37°C. Samples (3.5 ml) were removed during incubation and the reaction was stopped by the addition of 0.15 ml of 75 mM-p-chloromercuribenzoate. The pH of the sample was then adjusted to 7.0 by adding 0.85 ml of 0.4 M-sodium phosphate buffer, pH 7.4, and the naphthylamine released was measured by coupling it with 1 ml of 0.4% (w/v) Fast Blue B in 2% (v/v) Tween 40 solution as described by Nachlas et al. (1964). The chromogenic reaction was allowed to continue for 10 min and then 0.5 ml of 4 M-HCl was added. The acid conditions brought the p-chloromercuribenzoate out of solution and the red azo dye was extracted by adding 4 ml of butan-1-ol as described by Barrett (1972). Absorption was measured at 520 nm. When the pH maximum for hydrolysis was determined, 0.1 M Universal buffer (Ellis, 1961) was used. For the scaled-down system the cartilage sections were incubated with 1 ml of 0.2 M-acetate buffer, pH 5.0, containing 20 mM-EDTA and 4 mM-cysteine and 1 ml of 2.5 mM-α-N-benzoyl-DL-arginine 2-naphthylamide at 37°C for 24 h. Duplicate 0.5 ml samples were removed and the reaction was stopped with 0.1 ml of 20 mM-p-chloromercuribenzoate. After addition of 0.4 ml of 0.4 M-phosphate buffer, pH 7.4, and 0.2 ml of the above Fast Blue/Tween solution the colour was left to develop for 10 min. The reaction was stopped with 0.1 ml of 4 M-HCl and the solution was extracted with 0.6 ml of butan-1-ol. The A_{520} of the butanol layer was read in micro-cuvettes of 1 cm light-path.

Determination of haemoglobin hydrolysis by cartilage cathepsin

The hydrolysis of urea-denatured bovine haemoglobin at pH 4.0 by large samples (0.3 g) of human articular cartilage was carried out exactly as described by Ali et al. (1967). When the activity at different levels of cartilage was estimated, a slightly modified assay system was used. Cartilage sections were incubated in 1.3 ml of 0.2 M-acetate buffer, pH 4.0, and 0.7 ml of 4% (w/v) bovine haemoglobin in aq. 6 M-urea at 37°C for 5 h. Samples (0.25 ml) were removed from the incubation medium and precipitated with 0.25 ml of 10% (w/v) trichloroacetic acid, heated at 60°C for 15 min, and centrifuged at 1000g for 15 min to sediment denatured protein. The tyrosine released was measured by the Folin–phenol method described by Anson (1938). To 0.25 ml of the

supernatant was added 0.5 ml of 1 M-NaOH and 0.25 ml of diluted (1:2, v/v) Folin–Ciocalteau reagent. The A_{670} was read after 10 min.

Determination of acid phosphatase and alkaline phosphatase activity

Cartilage sections (0.3 g) were incubated in 10 ml of 0.1 M-acetate buffer, pH 5.0 (acid phosphatase), or 10 ml of 0.1 M-Tris/HCl buffer, pH 9.0 (alkaline phosphatase), containing 0.1 M-sodium β-glycerophosphate at 37°C. Duplicate 0.6 ml samples were removed at intervals and precipitated with 2.5 ml of 8% (w/v) trichloroacetic acid (Ali & Evans, 1969). After centrifugation at 1000g for 15 min the P_i content in the supernatant was measured by the method of Fiske & SubbaRow (1925).

Results

Hydrolysis of α-N-benzoyl-DL-arginine 2-naphthylamide by cartilage sections

When sections of human articular cartilage (0.3 g) were incubated with α-N-benzoyl-DL-arginine 2-naphthylamide in buffers of various pH values (containing 2 mM-cysteine and 10 mM-EDTA) for 5 h at 37°C, maximum hydrolysis of the substrate occurred at pH 5.0 (Fig. 1). This activity was completely abolished by preheating the cartilage sections in water at 80°C for 15 min before incubation at 37°C (Fig. 2).

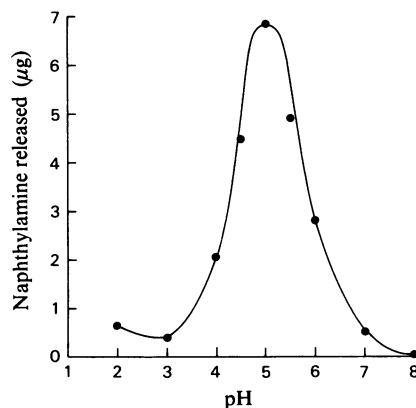It was not possible to detect the hydrolysis of α-N-benzoyl-DL-arginine 2-naphthylamide in the absence

Fig. 1. *Effect of pH on cathepsin B activity in human articular cartilage*
Cartilage sections (0.3 g) were incubated at 37°C for 5 h in 0.1 M Universal buffer, at various pH values, containing 10 mM-EDTA, 2 mM-cysteine and 2.6 mM-α-N-benzoyl-DL-arginine 2-naphthylamide. The naphthylamine released was determined as described in the text.

of cysteine or EDTA. Even when the activators were present individually the enzyme activity was still very low. However, when both activators were present in the incubation medium the substrate hydrolysis was considerably enhanced (Fig. 2).

Further experiments with inhibitors were also indicative of a thiol-dependent enzyme. Thus iodoacetamide (2 mM) and HgCl₂ (1 mM) completely inhibited the enzyme, and chloroquine also caused extensive inhibition at relatively high concentrations. Normal human serum also inhibited the hydrolysis of α-N-benzoyl-DL-arginine 2-naphthylamide by 60–70% at a concentration of 2% (v/v); however, higher concentrations did not result in further inhibition (Table 1). All of these results were consistent with cathepsin B activity.

Aging and pathological changes in cathepsin B activity

The hydrolysis of α-N-benzoyl-DL-arginine 2-naphthylamide by normal human articular cartilage was proportional to the time of incubation and the rate was linear with time for up to 7 h. A comparison of the enzyme activity in normal and osteoarthrotic cartilage was therefore carried out, based on the amount of naphthylamine released during 5 h incubation at 37°C. Fig. 3 shows that there was a progressive decrease in cathepsin B activity with increasing age, adults having only one-third of the enzyme activity of that found in juvenile tissue. Some osteoarthrotic cartilage (type IV)

Table 1. *Effect of inhibitors on cathepsin B activity in human articular cartilage*

Cartilage sections (0.3 g) were incubated with the inhibitors at 37°C for 24 h in 0.1 M-acetate buffer, pH 5.0, containing 2.6 mM-α-N-benzoyl-DL-arginine 2-naphthylamide, 2 mM-cysteine and 10 mM-EDTA. The amount of substrate hydrolysed was then determined as described in the text.

Compound	Concn. in assay (mM)	Inhibition (%)
Iodoacetamide	0.2	100
	2.0	100
HgCl₂	0.1	100
	1.0	100
Chloroquine	10	22
	20	53
	40	75
	Concn. in assay (%, v/v)	
Normal human serum	1	21
	2	65
	3	57
	4	59
	5	64

samples also had slightly elevated activity, but osteophytic cartilage (type VI) from the same femoral heads was up to 12 times as active as normal age-matched cartilage (Table 2).

Changes such as these have been reported in detail for other enzymes in similar cartilage samples (Ali & Bayliss, 1974, 1975; Bayliss, 1976) and a summary of the results is shown in Table 2. All four enzyme activities studied are significantly elevated in osteoarthrotic and osteophytic cartilages.

Enzyme activities in sagittal zones of articular cartilage

An attempt was made to determine whether the chondrocytes in different sagittal zones of articular cartilage varied in their catheptic activity. Full-thickness pieces (3 mm²) of normal human articular cartilage were sectioned on a cryostat from the articular surface towards the subchondral bone as described in the Experimental section. The activities of both cathepsin D and cathepsin B were highest in the surface layers and decreased progressively through the cartilage depth (Fig. 4). This indicated that there was heterogeneity in chondrocyte proteolytic activity.

Discussion

Early results of Ali (1964, 1967) and Ali & Evans (1969) implicated cathepsin B in cartilage degradation. The enzyme was sufficiently active in all rabbit cartilages to hydrolyse α-N-benzoyl-α-amide and
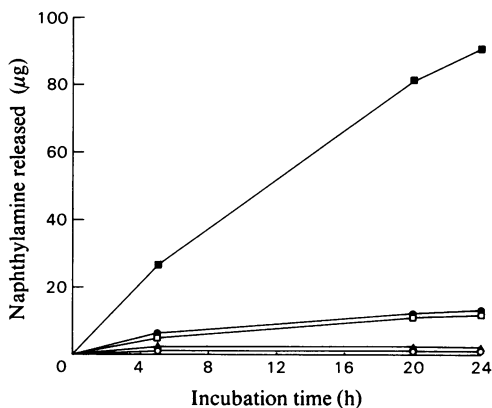

Fig. 2. *Effect of cysteine and EDTA on the hydrolysis of α-N-benzoyl-DL-arginine 2-naphthylamide*

Cartilage sections (0.3 g) were incubated with 2.6 mM-α-N-benzoyl-DL-arginine 2-naphthylamide in 0.1 M-acetate buffer, pH 5.0 at 37°C. The release of naphthylamine was determined as described in the text after incubation in the presence of buffer alone (▲), 2 mM-cysteine (●), 10 mM-EDTA (□), 2 mM-cysteine+10 mM-EDTA (■) or after heat-inactivation (80°C for 15 min) of cartilage in the presence of 2 mM-cysteine and 10 mM-EDTA (○).
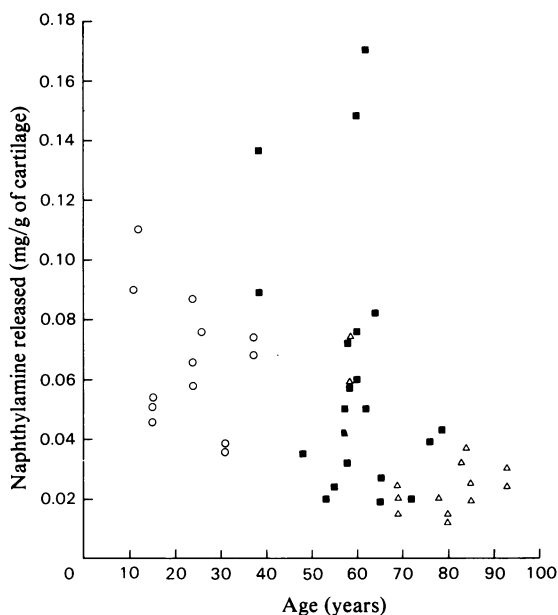
Fig. 3. *Cathepsin B activity in normal and osteoarthrotic human articular cartilage*
Cartilage sections (0.3 g) were incubated at 37°C for 5 h in 0.1 M-acetate buffer, pH 5.0, containing 2.6 mM-α-N-benzoyl-DL-arginine 2-naphthylamide + 2 mM-cysteine + 10 mM-EDTA. $\bigcirc$, Normal cartilage (type I) from amputation specimens; $\triangle$, normal cartilage (type II) from femoral-head fracture specimens; $\blacksquare$, osteoarthrotic cartilage (type IV) from total-hip-replacement specimens.
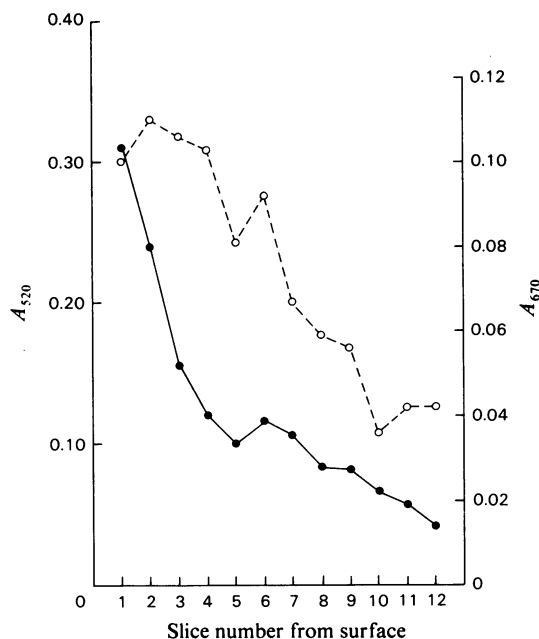

Fig. 4. *Zonal variations in cathepsin D and cathepsin B activity*
Cartilage sections in 180 μm sagittal zones of human articular cartilage were prepared as described in the text. Cathepsin D ($\bigcirc$) was assayed by measuring the tyrosine released, at 670 nm, after incubation at 37°C for 5 h. Cathepsin B ($\bullet$) was assayed by measuring the naphthylamine released, at 520 nm, after incubation at 37°C for 24 h.

Table 2. *Enzyme activities in different types of human articular cartilage*
The results are expressed in μmol of substrate hydrolysed/h per g wet wt. of cartilage.

Enzyme	Cartilage ...	Adult normal (type II)	Osteo-arthrotic (type IV)	Osteo-phytic (type VI)
Cathepsin D		0.027	0.046	0.054
Cathepsin B		0.032	0.086	0.295
Acid phosphatase		0.90	2.35	1.90
Alkaline phosphatase		0.25	2.75	13.50

α-N-benzoyl-DL-arginine 2-naphthylamide extensively even in the absence of cysteine and EDTA. However, in human articular cartilage, cathepsin B activity could not be detected with α-N-benzoyl-DL-arginine 2-naphthylamide as a substrate, and when cysteine was added in an attempt to activate the enzyme there was interference in the measurement of the hydrolysis product 2-naphthylamine. Ali &

Evans (1969) first tried to overcome this interference by adding the thiol-blocking agent p-chloromercuribenzoate at the end of the incubation period; however, this also inhibited the colour reaction. By modifying an assay system described by Barrett (1972), which included p-chloromercuribenzoate, it is now possible to show that the enzyme in human cartilage sections does hydrolyse α-N-benzoyl-DL-arginine 2-naphthylamide. Inhibition of the hydrolysis by $HgCl_2$ and iodoacetamide, together with the activation brought about by EDTA and cysteine, confirmed the presence of cathepsin B in human articular cartilage. These observations are consistent with the characteristics of cathepsin B isolated from other sources (Otto, 1971; Barrett, 1973; Swanson et al., 1974), as was the inhibition of cathepsin B by whole human serum (Starkey & Barrett, 1973; Snellman & Sylven, 1974).

Inhibition by chloroquine also suggested cathepsin B activity. The action of chloroquine on the autolysis of cartilage at pH 5.0 was first observed by Whitehouse

& Cowey (1966). Ali *et al.* (1967) also confirmed this effect on rabbit ear-cartilage autolysis, but in addition demonstrated inhibition of cathepsin B. Wibo & Poole (1974) observed that chloroquine inhibited cellular protein degradation by rat fibroblasts, and, although cathepsin D was not affected by the drug, cathepsin B was severely inhibited. Chloroquine also has well-known effects on membrane stability (Weissman, 1966); however, the incubation system described here would have resulted in disruption of the lysosomal membrane. The inhibition of proteoglycan-subunit degradation by chloroquine has also been reported for particulate-free cartilage extracts (Woessner, 1973a; Sapolsky *et al.*, 1973, 1974). A possible mechanism for chloroquine inhibition of cathepsin B was suggested by Ali *et al.* (1967), based on the work of Schubert (1947), and involves the interaction of the quinone with protein thiol groups.

The importance of cathepsin B in cartilage degradation was emphasized by the work of Morrison *et al.* (1973), who found that cathepsin B and cathepsin D, purified from human liver, were capable of degrading cartilage that had been washed free of endogenous lysosomal enzymes. Their results also demonstrated that the products of hydrolysis by cathepsin B were smaller than those of cathepsin D and that cathepsin B was still active at neutral pH, whereas cathepsin D showed no activity at pH 7.0. Similarly, Woessner (1973a,b) confirmed that highly purified preparations of cathepsin D have no effect on cartilage proteolgycans above pH 6. Thus, although cathepsin D is the enzyme mainly responsible for the autolytic breakdown of human articular cartilage at acid pH (Ali, 1970; Dingle *et al.*, 1971; Ali & Evans, 1973), cathepsin B may be the more important enzyme in the turnover of proteoglycans at physiological pH (Dingle *et al.*, 1972). An important feature of cathepsin B purified from bovine and human liver is its capacity to degrade soluble and insoluble collagen at acid pH (Burleigh *et al.*, 1974; Etherington, 1974, 1976). This further suggests that cathepsin B might play a very important role in cartilage metabolism.

Other evidence has also demonstrated neutral proteinase activity in human articular cartilage (Sapolsky *et al.*, 1974, 1976), but the relative importance of these enzymes has yet to be established. Even so, the activity can only account for a very small proportion of the autolysis of cartilage at pH 7.4, because Dingle *et al.* (1972) have shown that the pepstatin-resistant neutral enzyme activity is almost totally inhibited by thiol-enzyme inhibitors. Further, the neutral proteinases seem to be no more effective or specific in degrading proteoglycans than is cathepsin B (Morrison *et al.*, 1973; Sapolsky *et al.*, 1976). Sapolsky *et al.* (1973, 1974) were unable to detect cathepsin B activity in their cartilage extracts.

This finding was based on the lack of inhibition by iodoacetate or activation by cysteine. However, this does not exclude part of the neutral activity that they found being due to cathepsin B. Our results clearly demonstrate that both cysteine and EDTA must be present to activate the enzyme. It is also noteworthy that Sapolsky *et al.* (1974) found that chloroquine strongly inhibited their neutral proteolytic activity.

The elevated cathepsin B activity in some osteoarthrotic specimens is consistent with our findings for other cartilage lysosomal enzymes (Ali & Evans, 1973; Ali & Bayliss, 1974, 1975; Bayliss, 1976). By contrast, the age-related change in cathepsin B activity is not typical and is more akin to that of alkaline phosphatase (Ali & Bayliss, 1974, 1975). Osteophytic cartilage, which is a proliferating tissue with a juvenile-type chemical composition (Bayliss, 1976), is extremely rich in both cathepsin B and alkaline phosphatase activities. Evidence also suggests that proteoglycans from some osteoarthrotic-cartilage specimens have a more juvenile-type structure (Mankin & Lippiello, 1971; McDevitt *et al.*, 1974, 1977). Thus the elevated cathepsin B activity in some samples of type IV osteoarthrotic cartilage may reflect a juvenile-type metabolism in these specimens.

There are many examples of chrondrocyte heterogeneity. The variations in glycosaminoglycans through the depth of articular cartilage have been extensively studied histochemically and by chemical analysis (Collins & McElligott, 1960; Stockwell & Scott, 1965; Scott & Stockwell, 1967; Maroudas *et al.*, 1969; Kincaid *et al.*, 1972). Incorporation of radioactive sulphate is also less in the superficial cells of human articular cartilage (Collins & McElligott, 1960; Maroudas, 1975). Similarly, histochemical observations of NAD^+ reductase and $NADP^+$ reductase (Balogh *et al.*, 1961) and lactate dehydrogenase activities (Stockwell & Meachim, 1973) also suggest lower activities in the superficial zone than in the deeper layers. The observed variations in cathept ic activity in sagittal zones deeper than 200–300 μm from the articular surface are unlikely to be caused by changes in cell density, since Stockwell & Meachim (1973) have shown that, apart from the superficial zone of articular cartilage, little or no change in cell density occurs. The results therefore seem to reflect a true heterogeneity in chondrocyte activity and imply that the flat fibrocyte-type cells found near the surface of articular cartilage (Ali & Wisby, 1975) have a higher activity than deeper cells. The observed variations in enzyme activity may also explain the decrease in proteoglycan turnover through the cartilage depth observed by Maroudas (1975). Similarly, the raised lysosomal enzyme activities in some osteoarthrotic cartilage samples are consistent with the higher turnover rates measured in similar cartilage specimens (Maroudas, 1975).

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