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THE DEGRADATION OF ARTICULAR COLLAGEN BY NEUTROPHIL PROTEINASES

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Summary

The action of the serine proteinase (EC 3.4.21.—) of human neutrophil leucocytes, elastase and cathepsin G, on cartilage and tendon was investigated. With cartilage, both enzymes first degraded the proteoglycan, then solubilized collagen by an attack on the terminal peptides, destroying the inter- and intramolecular cross-links. There was little degradation of the helical region of the type II collagen. Elastase also solubilized type I collagen from tendon, though this was less susceptible than cartilage collagen, and attacked the terminal peptides and perhaps the helical region of type I skin collagen in solution. Cathepsin G had little or no effect on type I collagen of skin or tendon. Since massive infiltration of joint tissues by neutrophil leucocytes is a prominent feature of inflammatory joint disease, it may well be that elastase and cathepsin G make a significant contribution to the tissue damage that occurs.

Introduction

The damage to articular cartilage caused by proteolytic attack is an important feature of inflammatory joint diseases. The two structural components of articular cartilage known to be essential for its unique physical properties are the proteoglycan aggregates and the collagen fibres [1,2]. The proteoglycan is susceptible to attack by many proteinases (EC 3.4.21.—). Once removed it may be replaceable by newly synthesized material, although the rate at which this occurs has been questioned [3]. The collagen fibres, in contrast, are relatively resistant to proteolytic attack, but there is evidence that once degraded they cannot be replaced [4]. The importance of collagen degradation in joint disease is further illustrated by the extensive damage to tendons often seen in rheumatoid arthritis [5].

In septic arthritis, gout and the acute inflammatory episodes of rheumatoid arthritis, there is a massive infiltration of the joint by neutrophil leucocytes. On

encountering bacteria, urate crystals, immune complexes or activated complement factors in the inflamed joint, the leucocytes are capable of releasing lysosomal enzymes extracellularly [6—9]. The major neutral-acting proteinases of human neutrophil leucocytes are the specific collagenase [10] and the lysosomal serine proteinases, elastase and cathepsin G [11,12]. Neutrophil collagenase is unable to release collagen from intact cartilage [13] and is much less effective in degrading collagen fibrils than collagen in solution [10]. Even the collagenase from rheumatoid synovium has very little action on mature articular cartilage collagen, though it has some activity against insoluble tendon collagen [14,15]. It therefore appeared of interest to consider whether the neutrophil serine proteinases, elastase and cathepsin G, have any action on the collagen of articular cartilage and tendon, as well as on the cartilage proteoglycan.

Materials and Methods

Neutrophil elastase and cathepsin G were isolated from human spleen [16] or blood leucocytes [17]; the enzymes were pure by the criteria described in ref. 16. Pancreatic elastase (porcine pancreas, twice crystallized, Type I) and porcine trypsin (porcine pancreas, crystallized, Type IX) were from Sigma (London) Chemical Co., Ltd., Kingston-upon-Thames, Surrey, KT2 7BH, U.K.; bovine trypsin was from Miles Laboratories, Stoke Poges, Slough SL2 4LY, U.K.; chymotrypsin A_4 was from Boehringer (London) Corp. Ltd., London W5 2TZ, U.K. and pepsin (porcine stomach, twice crystallized) was from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Pig articular cartilage from the metacarpal-phalangeal joints, and achilles tendon were obtained from 6- to 12-month-old animals within 6 h of killing in a local abattoir, and stored at −20° C. Porcine laryngeal cartilage [18], largely depleted of proteoglycan by extraction at 4° C with 1 M NaCl, 0.05 M Tris, pH 7.4, for 24 h, was given by Dr. D.R.Eyre, Kennedy Institute of Rheumatology, Hammersmith, U.K. Human articular cartilage was visually normal tissue obtained from joints removed in the course of operations at Addenbrooke's Hospital, Cambridge, and was stored at −20° C until used. Acid-soluble rabbit skin collagen was prepared by standard methods [19]. Heat-denatured collagen was prepared by incubating a solution of the rabbit skin collagen in 0.05 M Tris · HCl buffer, pH 7.5, at 45° C for 30 min.

Quantities of tissue (cartilage and tendon) are given as wet weights. Proteoglycan was determined as glucuronic acid [20] after papain digestion and precipitation with cetylpyridinium chloride [21]. Collagen was assayed as hydroxyproline [22].

Results

Action on porcine articular cartilage

Slices (about 0.5 mm thick) of porcine articular cartilage (about 20 mg containing about 600 μg of hydroxyproline and 240 μg of uronic acid) were incubated with 0.3 ml of 0.3 M NaCl in 0.1 M Tris · HCl buffer, pH 7.5, containing 0.2% NaN_3, and 0.3 ml of a solution of neutrophil elastase or cathepsin

G in 0.1 M sodium acetate; controls contained no enzyme. Tubes were rotated (1 rev./min) at 37°C for 18 h. The enzymes were then inactivated by introduction of phenylmethanesulphonylfluoride to 1 mM concentration and further incubation for 1 h. The tubes were centrifuged (1400 × *g*, 5 min) at 22°C, and the pellets and supernatant fluids assayed for proteoglycan and collagen.

Dose vs. response curves (Figs. 1a and 1b) showed that both proteinases caused essentially complete release of proteoglycan from the pig cartilage at 0.1 μg of enzyme/mg of cartilage, but larger amounts of enzyme were necessary to solubilize the collagen.

The results of further experiments in which the neutrophil enzymes and porcine trypsin were used at about 1.3 μg/mg of cartilage are shown in Fig. 2. Although all three proteinases caused almost complete release of proteoglycan, the solubilization of collagen was 0.5% (control), 4.5% (trypsin), 21.5% (elastase) and 17.9% (cathepsin G). The result with trypsin shows that very little if any of the cartilage collagen had been denatured and that solubilization of collagen does not automatically follow the removal of proteoglycan from cartilage, but requires an attack on the collagen molecules themselves. Of the collagen fragments solubilized by the neutrophil proteinases, 40—80% were precipitable with 15% (w/v) trichloroacetic acid suggesting that they were of molecular weight >5000 [23], whereas none of the fragments released by trypsin were precipitable with trichloroacetic acid.

To investigate the nature of the collagen remaining insoluble after incubation with the neutrophil proteinases, the cartilage residues were treated either with 1 M NaCl at 4°C for 18 h, or with 1% sodium dodecyl sulphate (SDS) and 0.5% 2-mercaptoethanol at 100°C for 10 min.

A further 10% of the total collagen was solubilized by the NaCl whereas about 50% became soluble in SDS. After incubation with trypsin or buffer alone less than 5% of the collagen was solubilized by either treatment. These results indicated that elastase and cathepsin G, as well as solubilizing about 20%

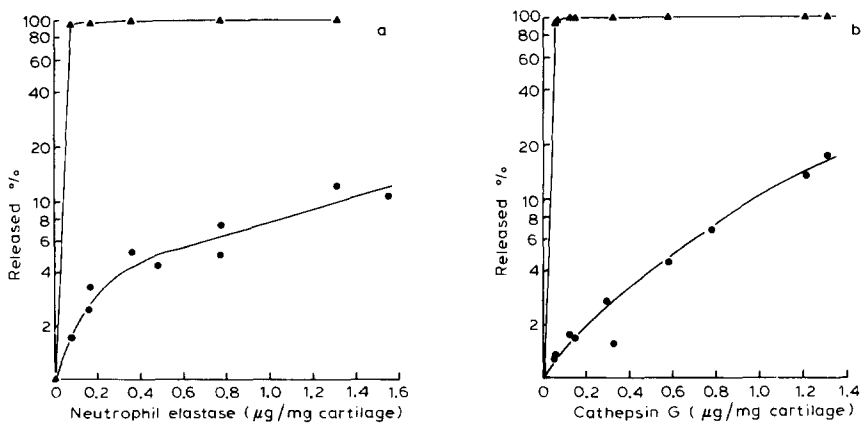

Fig. 1. Dose-response curve for the solubilization of porcine articular cartilage by (a) neutrophil elastase and (b) cathepsin G. Slices of porcine articular cartilage (approx. 20 mg) were incubated at pH 7.5, 37°C for 18 h in the presence of varying amounts of elastase or cathepsin G. The solubilization of proteoglycan (▲) and of collagen (●) was measured.

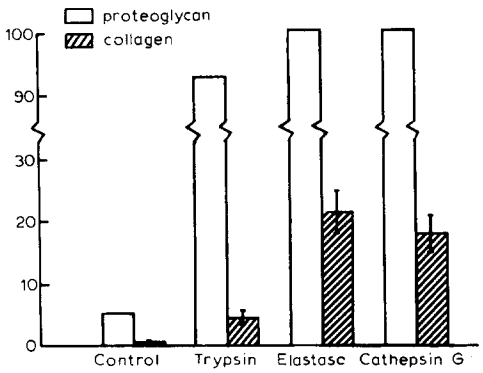

Fig. 2. Solubilization of porcine articular cartilage by neutrophil elastase and cathepsin G, and porcine trypsin. Slices of porcine articular cartilage (approx. 20 mg) were incubated at pH 7.5, 37°C for 18 h with 25 μg enzyme, or buffer alone. The solubilization of proteoglycan and of collagen (± S.E.; n = 4) is shown.

of the cartilage collagen, had partially degraded a large proportion of that collagen which remained insoluble.

Action on porcine laryngeal cartilage

A similar experiment was made with porcine laryngeal cartilage from which the proteoglycan had been largely removed with 1 M NaCl. The collagen solubilized directly by incubation with elastase or cathepsin G, or subsequently extracted with 1 M NaCl, formed highly viscous solutions at concentrations of a few mg/ml, and formed a gel at low ionic strength. These properties resemble those of soluble collagen that has not been appreciably degraded in the helical region.

Samples of the material extracted in buffer or 1 M NaCl were analysed by SDS electrophoresis in polyacrylamide gradient (4—10% T; 3% C) slab gels, in the Tris/borate/sulphate buffer system of Neville [24] (Fig. 3). Type II collagen solubilized from the porcine cartilage with pepsin (1 mg pepsin/62 mg cartilage in 0.5 M acetic acid at 4°C for 18 h) was run for reference. No collagen was detectable in the control extracts without enzyme. Almost all of the material solubilized directly by elastase and cathepsin G ran, after denaturation, as α chains of mobility similar to that of the material solubilized by pepsin (known to give modified α1 (II) chains). No fragments of lower molecular weight than the α chains were seen on the gels, but the elastase-solubilized collagen did show higher molecular weight β components. The amount of collagen extracted in 1 M NaCl was about 60% of that solubilized directly, but it appeared qualitatively similar in electrophoresis.

Action on human articular cartilage

Experiments were made with human articular cartilage under the conditions described for porcine cartilage except that the incubation was for 36 h. Both young and adult human cartilage gave results qualitatively similar to those obtained with porcine cartilage, though the human cartilage collagen appeared to be relatively more susceptible to degradation by trypsin (see Table I). The

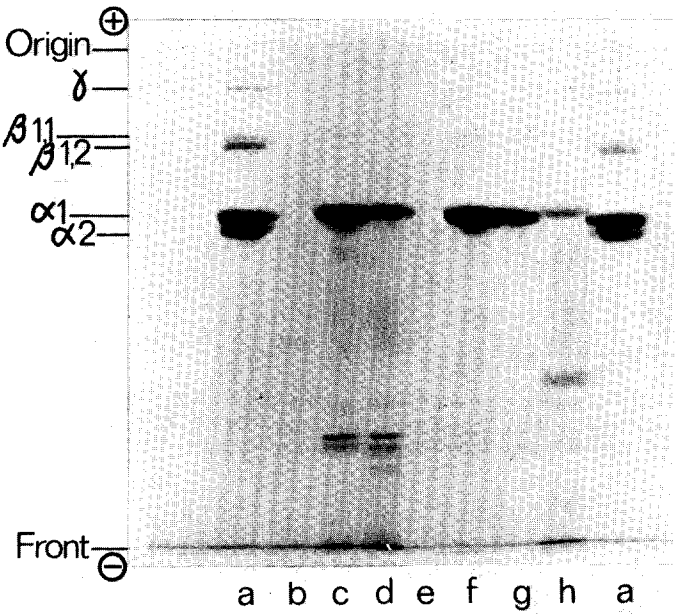

Fig. 3. Solubilization of collagen from porcine laryngeal cartilage seen in SDS gel electrophoresis. Samples were type I collagen from rabbit skin, for reference (a), collagen solubilized from porcine laryngeal cartilage in buffer alone (b), by neutrophil elastase (c), and by cathepsin G (d); and material subsequently extracted in 1 M NaCl from the residues of (b—d), (e—g), and type II collagen pepsin solubilized from the cartilage for reference (h).

rate of solubilization of collagen was slower than for the young porcine cartilage. Electrophoresis (Fig. 4) showed that the collagen solubilized from the human tissue contained a larger amount of β and γ components than we had seen with the young pig tissue, probably because the human material was more highly cross-linked.

Action on porcine tendon

To compare the susceptibilities to the neutrophil enzymes of tendon collagen (type I) and cartilage collagen (type II), samples (about 20 mg) of porcine achilles tendon or articular cartilage were incubated with elastase or cathepsin G at pH 7.5, 37°C for 18 h as described above, and the release of collagen was determined.

TABLE I

SOLUBILIZATION OF COLLAGEN FROM HUMAN CARTILAGE

For experimental details see the text.

Source of cartillage (age)	Solubilization of collagen (percent release hydroxyproline)			
	Control	Bovine trypsin	Elastase	Cathepsin G
Knee (55 years)	0.05	7.3	7.2	3.3
Costal (12 years)	1.0	2.0	12.0	8.0
Knee (67 years)	0.4	8.7	13.6	5.5

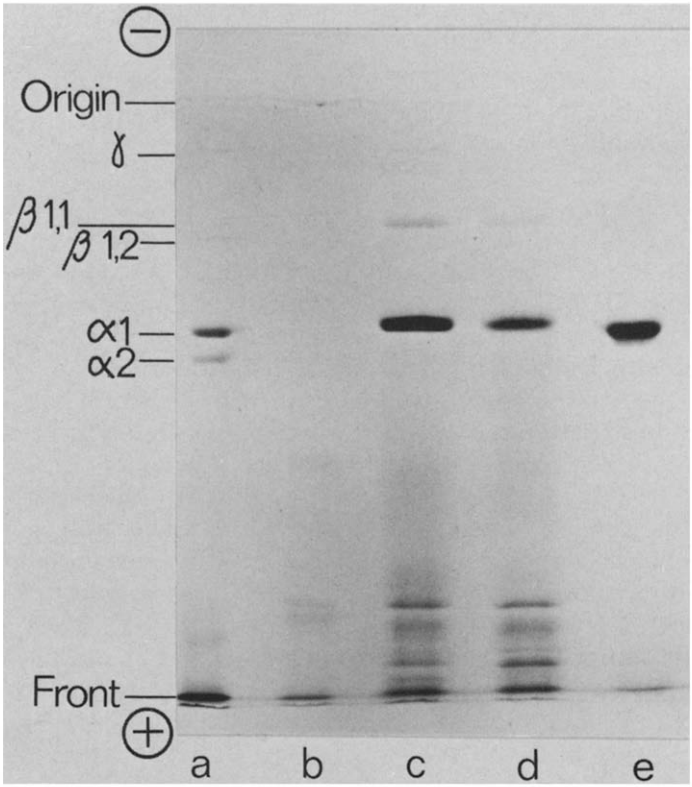

Fig. 4. Solubilization of collagen from human articular cartilage as seen in SDS gel electrophoresis. Samples were type I collagen from rabbit skin, for reference (a), collagen solubilized from human articular cartilage in buffer alone (b), by neutrophil elastase (c) and by cathepsin G (d), and type II collagen pepsin solubilized from human articular cartilage for reference, given by Dr. E.J. Miller (e).

The results (see Table II) show that for a given wet weight of tissue the collagen in tendon was about 5-fold more resistant to degradation by the neutrophil enzymes than was the collagen of cartilage; cathepsin G was almost completely ineffective in solubilizing type I tendon collagen. The difference in susceptibility of the two types of collagen was somewhat less marked if they were compared on the basis of μg enzyme/mg collagen in each tissue: treatment with

TABLE II

SOLUBILIZATION OF COLLAGEN FROM PORCINE ARTICULAR CARTILAGE AND ACHILLES TENDON BY NEUTROPHIL ELASTASE AND CATHEPSIN G

Cartilage or tendon (20 mg) was incubated with the stated amounts of each enzyme, or with buffer alone (total volume 0.6 ml), at pH 7.5, 37°C for 18 h.

Sample	Solubilization of collagen (percent release hydroxyproline)				
	Control	Elastase		Cathepsin G	
		7 μg	28 μg	6 μg	24 μg
Articular cartilage	0.4	4.3	12.9	2.2	15.5
Achilles tendon	0.2	0.5	2.4	0.3	0.6

about 5 μg of elastase/mg of collagen solubilized 2.5% of the tendon collagen and 6% of the collagen of cartilage.

The collagen fragments released from the tendon by elastase were not precipitable with 15% (w/v) trichloroacetic acid and were therefore taken to be of molecular weight less than 5000.

Action on rabbit skin collagen in solution

Acid-soluble, rabbit skin collagen (0.15 mg) was incubated in a total volume of 0.2 ml containing 0.1—50 μg of enzyme, 0.15 M NaCl, and 0.25 M glucose in 0.05 M Tris · HCl buffer, pH 7.5, at 28, 32 or 37°C for 18 h. Before samples for electrophoresis were treated with SDS and 2-mercaptoethanol at 100°C, the enzymes were inactivated with 1 mM phenylmethanesulphonylfluoride.

Electrophoretic analysis of the products of incubation with neutrophil elastase at all three temperatures (pattern at 28°C shown in Fig. 5) demonstrated almost complete elimination of γ and β components with a concomitant increase in the amount of free (modified) α chains. This indicated that elastase is capable of cleaving the amino-terminal peptides which contain the intramolecular cross-links. Cathepsin G (not shown) and the pancreatic serine proteinases, elastase, trypsin and chymotrypsin, even at several-fold higher concentrations, had little or no effect.

After incubation of the skin collagen with neutrophil elastase at 28 or 32°C,

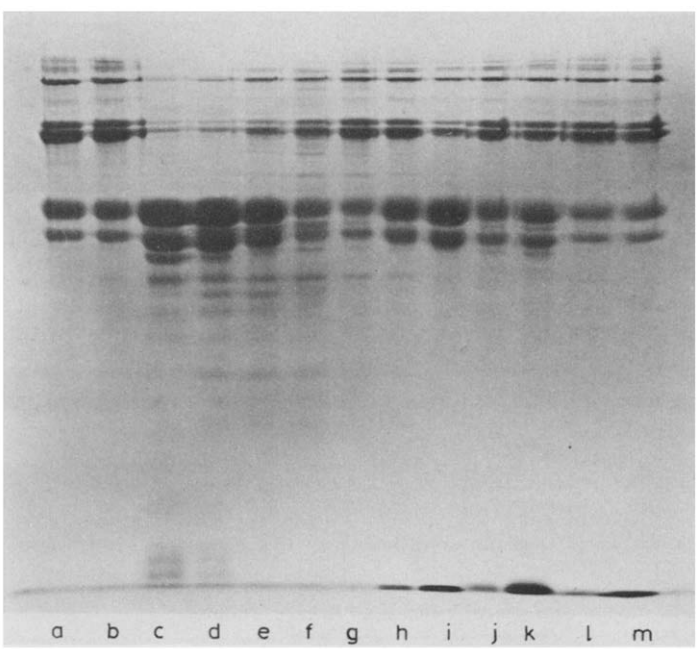

Fig. 5. SDS gel electrophoresis of the products of digestion of acid-soluble rabbit skin collagen by human neutrophil elastase, porcine pancreatic elastase and bovine trypsin and cymotrypsin. Collagen (150 μg) was incubated at pH 7.5, 28°C, for 16 h with buffer alone (b), with 8, 4, 1, 0.5 and 0.1 μg of human neutrophil elastase (c—g), with 10 and 40 μg of porcine pancreatic elastase (h,i), with 10 and 40 μg of bovine chymotrypsin (j,k) and with 10 and 50 μg of bovine trypsin (l,m). A sample of unincubated collagen is also shown (a).

in addition to the α chains, multiple sub-α components were sometimes visible on the gels, but their intensity was variable between experiments. These components did not correspond to the characteristic products of the action of a specific collagenase [25]. The observations would, however, be consistent with the possibility that neutrophil elastase attacks the helical region of skin collagen, the initial products being further degraded during the course of the incubation. Both elastase and cathepsin G were shown in a separate experiment to be able to degrade heat-denatured rabbit skin collagen, so we were not surprised to find that after incubation of collagen in solution with elastase at 37° C, the sub-α components, which would readily denature at this temperature [26], were not visible on the gels, only the band due to undegraded α chains being present.

Discussion

It is probable that degradation of cartilage matrix components in vivo occurs both intra- and extracellularly [22,27]. Under conditions of rapid turnover, fragments of connective tissue matrices produced as a result of mechanical wear or enzymic activity can be phagocytosed by neutrophil leucocytes or tissue cells [28,29], and then exposed to the acid-acting lysosomal proteinases including cathepsins B and D. Both enzymes can degrade proteoglycan at acid pH values (refs. 2, 30 and 31, and Roughley, P. and Barrett, A.J., unpublished), but only cathepsin B attacks native collagen [22].

Extracellular digestion of cartilage would be expected to involve proteinases maximally active at neutral pH. We suspect that very limited degradation is sufficient to impair the mechanical properties of the structural macromolecules, although the first products are too large to diffuse from the tissue. Further degradation produces smaller, diffusible fragments, and quantitation of these provides a minimal estimate of the degree of tissue damage.

The proteoglycan-solubilizing activity at neutral pH of the granules of human neutrophil leucocytes has been shown by use of selective inhibitors to be attributable at least in part to elastase and cathepsin G [32] although other workers have obtained less conclusive results in experiments with whole cells [33]. Subsequently, the digestion of proteoglycan by the purified neutrophil proteinases has been studied in detail (refs. 31 and 34 and Roughley, P. and Barrett, A.J., unpublished). The removal of proteoglycan from the cartilage is the initial stage of the degradation; it exposes the collagenous framework of the tissue to greater mechanical stress [2] and may increase its susceptibility to enzymic attack [35].

The release of collagen from cartilage by the action of the neutrophil enzymes was not predictable from any previous work, apart from our preliminary study [36]. We conclude from our present results that both elastase and cathepsin G attack the terminal peptides of cartilage collagen which are essential for the integrity of the intra- and intermolecular cross-links [37]. A proposed mechanism for this degradation is illustrated schematically in Fig. 6. Part of the modified collagen becomes soluble in the buffer directly, whereas the remainder can be solubilized in vitro by salt or SDS. Almost all of the collagen solubilized in our experiments gave un-cross-linked α chains in SDS electro-

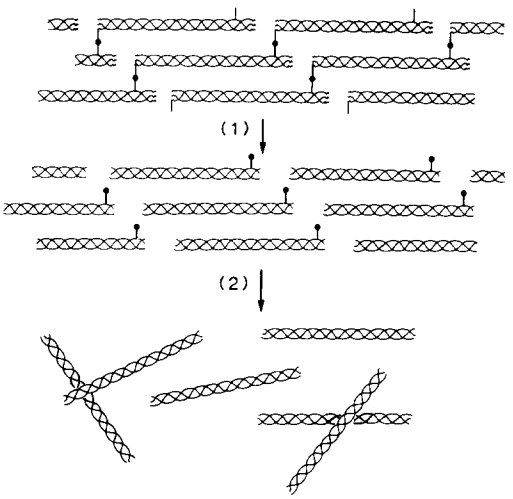

Fig. 6. The mode of action of neutrophil proteinases in degradation of articular cartilage collagen. In stage 1, the proteinases cleave the terminal peptides between the cross-links and the helical region, so that adjacent molecules are no longer linked through their terminal peptides. (The cross-link shown is the predominant, CB 4 X 9 link found in cartilage collagen [48—51]). In stage 2, the remaining non-covalent assocations between molecules are broken by mechanical stress or chaotropic agents, and the collagen becomes soluble.

phoresis, showing it to be monomeric collagen. Dr. D.R. Eyre (personal communication) examined the cyanogen bromide peptides [18] generated from this enzyme-solubilized collagen and confirmed that it was type II collagen.

Type I tropocollagen is expected to exist entirely in fibrillar form at 37°C in moderate conditions of pH and ionic strength, so it was surprising that a substantial proportion of the degraded cartilage collagen was soluble directly in the buffer, in our experiments. It is known, however, that removal of the terminal peptides greatly decreases the efficiency of fibril formation for type I collagen in 0.15 M phosphate (but not 0.3 M) [38] and the type II collagen was presumably affected in a similar way. Part of the degraded collagen did remain in the tissue to be extracted by 1 M NaCl, or SDS, which disrupt the non-covalent interactions. In vivo, many collagen molecules might remain in the cartilage despite removal of the terminal peptides, but the tissue would then resemble osteolathyritic cartilage, in which the enzyme formation of cross-link precursors has been blocked. The collagen fibres of such cartilage are excessively hydrated, and show a low resistance to solubilization and mechanical stress [39,40], and almost certainly increased susceptibility to enzymic attack. The fact that collagen from lathyritic cartilage is not reported to be unusually soluble at physiological pH and ionc strength (although it is highly soluble in 1 M NaCl), may be attributable to the more soluble component having diffused from the tissue in vivo.

Neither of the neutrophil enzymes appears to attack the helical region of type II collagen in the first instance, though at 37°C a proportion of the modified α1 (II) chains released from the cartilage would be expected to denature and become susceptible to further degradation, since both neutrophil enzymes hydrolyse gelatin to low-molecular weight peptides. The exact proportion of

the free α chains denatured could differ between experiments and account for the variable proportion of low- and high-molecular weight collagen fragments detected by precipitation with trichloroacetic acid.

The slight solubilization of cartilage collagen by trypsin is consistent with the report of Harris et al. [1] for dog cartilage, and is unlikely to be accounted for by the degradation of denatured collagen alone. In our work, the absence of electrophoretically detectable fragments in the trypsin-solubilized cartilage collagen, and the solubility of all the products in trichloroacetic acid, suggested that after the initial attack trypsin rapidly degraded the collagen fragments to small peptides.

The effectiveness of pepsin in solubilizing collagen of types I and II from tissues is already known to be due to its specific action on the terminal peptides [41,42], and the action on cartilage collagen has shown that few, if any cross-links occur directly between helical regions in this collagen [43]. The predominant reducible cross-link is between cyanogen bromide peptides 4 and 9 (the latter being from the helical region) [43,44], and the non-reducible cross-links are probably in the same position [45]. Our results indicate that the cross-link regions of type II collagen are very sensitive to both neutrophil elastase and cathepsin G, whereas those of type I collagen are less sensitive to elastase and insensitive to cathepsin G. The amino-terminal sequences of the α1(I), α2 and α1(II) chains are known to differ, though in general the sequence between the cross-link and the helical region is rich in hydrophobic residues [46]. The action of elastase [47] and cathepsin G [48] on the insulin B chain shows that the enzymes are specific for bonds involving hydrophobic residues. The specificities of the serine proteinases thus resemble that of pepsin, but are more restricted, which could explain the general similarity of action of the three enzymes on collagen, but also the greater selectivity of the two serine proteinases.

In the past it has often been assumed that the extracellular degradation of collagen fibres in living tissues is initiated exclusively by the specific collagenases, although Harris and co-workers [49—52] have pointed out that there are difficulties in this view. A particular problem is the marked resistance of fibrous cartilage collagen to the collagenases. Even non-cross-linked (lathyritic) cartilage collagen in solution has been found to be about 6-fold less susceptible to the action of collagenase from rheumatoid synovium than tendon collagen (in experiments at 25°C) [53]. This may be due to the high degree of glycosylation of the cartilage collagen, or to some other aspect of the structure of the helical region, but cartilage collagen also differs from that of skin and tendon in being more highly cross-linked [43,44]. It is clear that this must further decrease the sensitivity of mature fibres to the action of specific collagenase [54,55]. Very recently E.D. Harris, Jr. (Dartmouth Medical School, N.H., U.S.A.) (personal communication) has found that collagen cross-linked in vitro by lysyl oxidase becomes markedly more resistant to cleavage by specific collagenase above 0.25 cross-links/mol.

The possible involvement of a cross-link-degrading enzyme in collagen catabolism has been considered previously (for reviews see refs. 27 and 52). The neutrophil elastase and cathepsin G are the first enzymes recognized to be able to fill this role in extracellular digestion under physiological conditions, and

are probably identical with the neutrophil serine proteinases that act synergistically to allow neutrophil collagenase to degrade reconstituted collagen fibrils [10]. It has been pointed out by Dr. A.J. Bailey (personal communication) that while the resistance of the highly glycosylated cartilage collagen to collagenase is partially attributable to screening of the helical regions by carbohydrate residues, the cross-link regions sensitive to the non-specific proteinases are exposed in the "holes" between the ends of molecules in the "quarter-stagger" array (Fig. 6).

It is clear then that neutrophil elastase and cathepsin G have the capacity to degrade the collagen as well as the proteoglycan of articular cartilage, and that elastase can also attack type I collagen of tendon and skin. Much further work will be required to determine to what extent they do so in the disease processes. Complex factors to be taken into account include the effectiveness of the endogenous inhibitors of proteinases in the synovial fluid (notably α_1-proteinase inhibitor and α_2-macroglobulin), and the distribution of cell types in relation to the exact sites of tissue damage. The damage to articular cartilage in septic arthritis [56,57] has been attributed to activation of plasminogen by bacterial factors [58], or to the direct action of bacterial proteinases (cf. ref. 59), but it now seems that a possible role of serine proteinases liberated by the leucocytes should be considered. Since uptake of urate crystals causes release of granule enzymes from neutrophils, the serine proteinases should also be considered in connection with the tissue damage of gout [60]. In rheumatoid arthritis, in addition to the gross destruction which occurs in the immediate vicinity of the invading pannus, a generalized destruction of collagen and proteoglycan is detectable in the surface layer of the cartilage [61,62]. Few neutrophils are seen in the pannus, but the surface damage could well be due to synovial fluid leucocytes attracted by immune complexes and degranulating close to the cartilage [62].

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