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Research Article

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Collagenase and Collagenase Inhibitors in Osteoarthritic and Normal Human Cartilage

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ABSTRACT In advanced osteoarthritis, all of the cartilaginous components are lost from the joint surface. Although mechanisms exist for proteoglycan degradation, there is not known to be any system for removal of the collagen. This study suggests that the loss of the collagen components may be a function of articular cartilage collagenase. The enzyme in normal human cartilage is bound to an inhibitor and appears to be present in very small amounts.

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Electrophoresis of the degradation products of the enzyme-collagen complex incubated at 37°C revealed breakdown was complete to small dialyzable fragments, while at 25°C larger fragments were split off.

INTRODUCTION

In advanced or "end-stage" osteoarthritis, all or most of the cartilage is lost and the articulating surface consists of eburnated, sclerotic bone. Although one can theorize that this destruction of cartilaginous tissue occurs as a result of mechanical debridement, the more likely explanation is that at least some of the degradation is a result of enzymatic action. Prior studies from this and other laboratories have demonstrated locally synthesized proteases and other enzymes, principally lysosomal in origin, which have as their substrate the proteoglycan component of the articular cartilage and these appear to be significantly increased in quantity in osteoarthritis (1).

The other major component of the cartilage, the collagen, has been thought to undergo only minimal turnover under normal circumstances but is known to be actively destroyed in rheumatoid and septic arthritis. Prior studies by Evanson et al. (2), have shown that a synovial collagenase is responsible for the destruction seen in rheumatoid arthritis and similar enzymes arising from the leukocyte appear to be responsible for destruction seen in septic disease (3). To date, however, there has been no demonstration of a locally synthesized autolytic collagenase in normal or osteoarthritic articular cartilage.

The purpose of this report is to describe some experimental data which indicate the presence of a collagenase in osteoarthritic articular cartilage, which either does not exist in normal tissue or is present in such minute quantities as to be undetectable by the methods available. The collagenase can only be demonstrated in culture media and evidence is presented to show that it is bound to an inhibitor.

METHODS AND RESULTS

Human articular cartilage was obtained from freshly resected normal and osteoarthritic tissues obtained at the time of arthroplasty (osteoarthritic) or removal of the femoral head for fractures. Histologic confirmation was made of

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the diagnosis. In addition, each surgeon stated that the diagnosis was not secondary to a septic process or rheumatoid arthritis.

The tissue obtained was immediately frozen in liquid nitrogen, lyophilized in a cryostat at −30°C, and stored until utilized.

The technique utilized to demonstrate the presence of a collagenase is dependent on the release of [^{3}H]-glycine from prelabeled salt and acid-extractable rat skin collagen (2). This substrate was prepared by four injections of 21-day-old Sprague-Dawley rats with 50 μCi of [^{3}H]glycine i.p. at 12-h intervals, harvesting the collagen from skin by salt and acid extraction, and reconstituting the material as a gel (4). All materials thought to contain the enzyme were incubated for 15–20 h with 25 μl (4 mg/ml) of the labeled collagen at 37°C in 1 ml 50 mM Tris-HCl buffer, pH 7.6, containing 6 mM of $CaCl_2$. After incubation, the preparation was centrifuged at 40,000 for 40 min in a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at 4°C and a 0.5-ml aliquot of the supernatant fluid was assayed for radioactivity using a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Preliminary metabolic studies

Attempt to assess the presence of collagenase in freshly ground osteoarthritic tissue and in the lysosomal fraction. Osteoarthritic and normal cartilage slices were quick frozen in liquid nitrogen, cut in a Harris cryostat to 16-μm slices, at −30°C, and then lyophilized in the cryostat. The slices were then incubated with 25 μl of reconstituted collagen fibers as described previously for 15–20 h and assayed for radioactivity. Controls consisted of "heat-killed" cartilage (incubated at 80°C for 2 h) or buffer alone. As shown in Table I, no collagenolytic activity was detected. The total weight of cartilage used ranged from 25 μg to 1 mg.

Since it seemed possible that the enzyme could be in the lysosomal envelope, the experiment was repeated after grinding the cartilage with a Duall tissue grinder (Kontes Co., Vineland, N. J.), in an ice bath for 15-s periods to release lysosomal enzymes, and then 1 ml of suspension was added to the collagen-buffer substrate. In another study, the lysosomal fraction was partially separated from the ground cartilage by centrifugation at 20,000g. The supernate so obtained was added to the medium. In both of these experiments, lysosomal activity of the supernate was confirmed by measuring acid phosphatase activity. As shown in Table I, only a slight increase in collagenolytic activity was observed with these preparations, but as noted in Fig. 1, no increase in activity was noted with increasing quantities of cartilage.

TABLE I
Lysosomal Effect on Collagen Degradatin

Condition	Increase over control values	n‡
	%	
Osteoarthritic		
Intact lyophilized cartilage	+10.5±2.8*	6
Ground cartilage suspension	+8.0±4.7*	5
Lysosomal fraction	+6.6±4.9*	5
Nonarthritic		
Intact lyophilized cartilage	+3.7±1.8*	(n = 7)‡

Effect of lyophilized and ground cartilage in solutions on collagen substrate. There is only a minimal increase of activity from the osteoarthritic cartilage compared with the controls.

* SEM.

‡ n = number of patients.

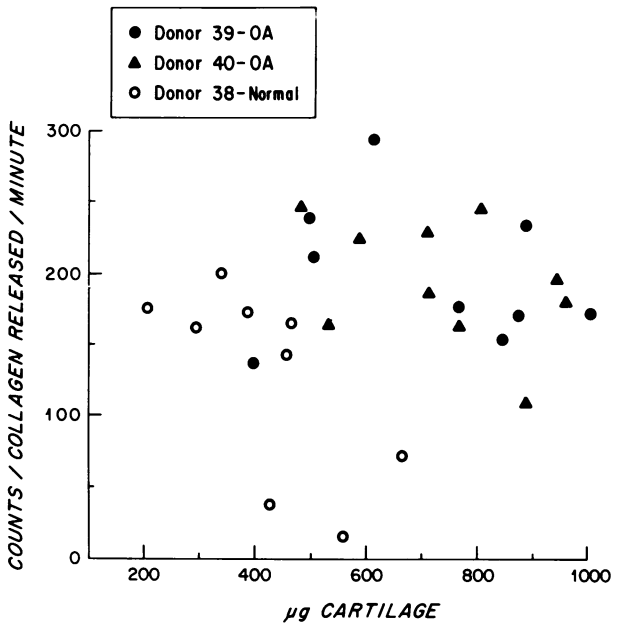


FIGURE 1 Correlation of collagen degradation with increasing amount of cartilage as a source of enzyme. There is no correlation.

Attempts to discern the enzyme by culture of cartilaginous tissues. As has been previously noted (5), collagenase may be demonstrated in some tissues only after several days of culture. Under these circumstances, the enzyme is released into the medium. In this study, sterile normal and osteoarthritic cartilage obtained in the operating room were incubated with 5× the vol of Eagle's media containing 1,500 U of aqueous sodium penicillin, 3 mg of streptomycin, and 0.39 mg L-glutamine/ml. All of the articular cartilage present on the femoral head was used for the culture except for that tissue clearly identifiable as representing osteophytic cartilage.

The media containing the cartilage was gassed with 95% O_2 and 5% CO_2 in a rotating drum incubator at 37°C with daily collections and media changes for 7 days. The pooled media so obtained were filtered on Millipore filters and stored at −20°C until used. Controls consisted of heat-killed cartilage and Eagle's media incubated without cartilage, to which 10 μg/ml of trypsin had been added (serving as a nonspecific protease).

As can be noted by examination of Table II, there was no demonstrable collagenase activity in the media after 7 days of cartilage incubation and all values were less than those for the trypsin-containing control media.

Attempts to define the presence of an inhibitor. Control and experimental media obtained as described above were incubated with 25 μl of labeled collagen and bacterial collagenase (Worthington Biochemical Corp.,

TABLE II
Degradation Effect of 7-Day Culture

Media	Degradation	n‡
	counts/10 min	
Osteoarthritic cartilage		
Experimental media	365.0±34.0*	23
Heat-killed control	369.2±35.4*	20
Pooled media control	343.1±43.7*	23
Trypsin control	954.0±99.6*	20
Nonarthritic cartilage		
Experimental media	356.8±63.2*	6
Heat-killed control	361.3±55.7*	6
Pooled media control	332.3±54.2*	6
Trypsin control	1,149±189.8*	5

Effect of media from cultured cartilage tissue on collagen substrate. There is no evidence of increased degradation compared to the controls.
* SEM.
‡ n = number of patients.

Freehold, N. J.) in a concentration of 10 μg/ml. Released counts were determined as previously described and demonstrated that the culture media from the arthritic tissues significantly inhibited the collagenolytic action of the bacterial enzyme, while no such inhibition was noted with media from incubation of normal cartilage (Table III). This strongly suggested the presence of an inhibitor in the osteoarthritic cartilage which could act not only on the locally synthesized collagenase but on the exogenous bacterial material as well.

The media were also treated by dialysis in an attempt to remove the inhibitor. In three experiments, the dialyzed experimental media, still inhibited bacterial collagenase by an average of 37%, (1,656 counts/10 min vs 2,709). Because of the possibility that the inhibitor might represent collagen that had leached out from the cartilage, the media was centrifuged at 20,000 g for 40 min at 4°C. When combined in a series of four experiments with bacterial collagenase, the average number of counts released by the collagenase alone was 2,750/10 min, but with the addition of the supernate of the culture media, there was still only 1,735 counts/10 min or 37% inhibition. Neither technique was successful, suggesting that the inhibitor was of high molecular weight and probably protein in nature.

TABLE III
Inhibition Studies

	Osteoarthritic tissue	Normal tissue
	counts/10 min	
Experimental media and collagenase	2,181.3±309.8*	1,866.0±561.8*
Collagenase control	2,672.1±348.6*	1,947.0±580.9*
	$P < 0.01$‡	$P < 0.50$‡
Inhibition, %	18.4	4.2
n	21	5

Inhibition of bacterial collagenase by media from cultured cartilage. There is significant inhibition of the collagenase by the arthritic cartilage but not by the normal cartilage.
* SEM.
‡ Paired t test.

In another approach, however, in a concentration of 10 μg/ml, trypsin was added to the 7-day culture medium of osteoarthritic and normal articular cartilage to measure the effect on the release of counts from labeled collagen gel compared with trypsin in media alone (without cartilage). As seen in Table IV, there was a highly significant increase in the counts released from the osteoarthritic cartilage media by trypsin treatment (not present in normal tissue), indicating that trypsin could possibly act to destroy the collagenase inhibitor, thus freeing the enzyme to act on the gel. It is possible that the trypsin could serve to activate a procollagenase. To further suggest that this effect was not a nonspecific effect of the two proteolytic enzymes, trypsin was preincubated in a concentration of 20 μg/ml with 200 μl of the cartilage culture media for 1 h and then 80 μg/ml of trypsin soybean inhibitor was added. The results shown on Table V indicate a sevenfold increase in activity compared to the culture media not pretreated with trypsin or the heat-killed media. There is a fourfold increase over the effect of trypsin alone.

Metabolic studies

Sakamoto et al. (6, 7) recently demonstrated that bone collagenase could be freed of its inhibitors by use of heparin-coupled affinity chromatographic technique using a cyanogen bromide-activated Sepharose 4B column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The technique, slightly modified, was as follows:

5 g of washed dry Sepharose 4B was exposed to 60 ml of 100 mg/ml CnBr in H_2O. After activation and washing, the CnBr-Sepharose was dried in a sintered glass filter. A heparin buffer solution with 50,000 U heparin in 50 mM Tris-HCl, pH 7.6, with 5 mM $CaCl_2$, was used to couple the heparin to the activated Sepharose. A 1.4 × 5-cm column was poured (8). The total quantity of solution from the 7-day incubation medium, collected as previously discussed, and containing the collagenase and inhibitor, was added to the column and eluted with a series of discontinuous gradient buffers. After initial runs, only two buffers were added: buffer 1, 0.0125 M Ca-acetate and 0.0125 M NaCl; and buffer 3, 0.25 M Ca-acetate and 0.25 M NaCl. These were prepared in 50 mM Tris-HCl, pH 7.6, with 5 mM $CaCl_2$ added. 5-ml fractions were collected.

The optical density of the fractions were read for protein at OD 280 on a Hitachi-Perkin-Elmer spectrophotometer. 0.1-ml samples were then added to 100 μl of labeled gel, incubated for 4 h at 37°C and assayed for released radioactivity as described. Control solutions consisted of buffer 3, trypsin 10 μg/ml, and bacterial collagenase, 10 μg/ml. When collagenase activity was demonstrated, the material was assayed for casein degradation to rule out nonspecific protease activity at pH 7.6, for 20 min at 37°C (9). The results of the affinity column chromatographic study of five osteoarthritic cartilage specimens and four normals are shown in Table VI. As can be noted, the 7-day osteoarthritic media, treated by the heparin coupled cyanogen-bromide Sepharose 4B affinity column, released over 42% of the total counts in the gel while normal cartilage 7-day media and trypsin-control media averaged only 10.1 and 6.42%, respectively. These values for osteo-

TABLE IV
Trypsin Effect

	Osteoarthritic tissue	Normal tissue
	counts/10 min	
Experimental media and trypsin	1,232.8±146.6*	1,261.4±276.5*
Trypsin control	954±99.6*	1,114.4±189.8*
	$P < 0.02$‡	$P > 0.50$‡
Increase, %	29.2	13.2
n	20	5

Effect of the addition of trypsin to the media from the osteoarthritic and normal tissues. Trypsin activity is significantly enhanced by the addition of media from the osteoarthritic tissues.
* SEM.
‡ Paired *t* test.

TABLE V
Effect of Preincubation of Cartilage with Trypsin

	Heat-killed media with trypsin and soybean inhibitor	Heat-killed media with trypsin	Experimental media with trypsin and soybean inhibitor
		counts/10 min	
Mean	167.8±8.6*	293.3±8.6*	1,256.8±50.0*
Number	6	6	6
		(——— $P < 0.01$‡ ———)	
	(——————— $P < 0.01$‡ ———————)		

Collagenase activity from media preincubated with trypsin to which trypsin soybean inhibitor was then added. There is a sevenfold increase in activity compared to the heat-killed control.
* SEM.
‡ Student's *t* test.

arthritic cartilage preparations are shown in Fig. 2A–E. As can be noted, the eluant with buffer 3 contained the collagenase and was present in high quantities in the osteoarthritic specimens, lower in the cartilage from one patient with osteonecrosis, and below trypsin controls in two normals.

In those cases where collagenase activity could be demonstrated, assays for casein degradation were performed. There was negligible enzymatic breakdown of casein when compared to trypsin standards. (OD values of 0.0010, 0.0075, and 0.0045; trypsin, 0.0635.)

Because of the noted calcium dependence by other mammalian collagenases (2), another experiment was performed in which 100 μl (0.15 M) EDTA was added to 1 ml of trypsin-activated culture media. The mean of the trypsin-stimulated media was 637.6 cpm ±16.07 (SEM), while the EDTA media had 134±5.6 cpm ($P < 0.01$).

The collagen breakdown products were dialyzed by an electrophoretic technique. Using the method of Neville (10), a 5% polyacrylamide gel was poured and 100 μl, containing about 40-μg sample protein of collagen solution (that had been incubated for 4 h at 37°C), was added. This was repeated at 25°C with a 12-h incubation. Upper reservoir buffer was 0.04 M boric acid, 0.041 M Tris, pH 8.64, and 0.1% sodium dodecyl sulfate. The lower gel buffer was 0.424 M Tris, pH 9.5, and 0.31 M HCl. Collagen samples were exposed to 8 mg sodium dodecyl sulfate/mg protein for 1 min and heated to 100°C, then dialyzed against the upper gel buffer with 0.1% sodium dodecyl sulfate, 2% sucrose, and a trace of bromophenol blue in upper gel buffer with 0.541 M Tris and 0.267 M H_2SO_4 at pH 6.10. The current was run at 0.5 mamp/tube. The gels were stained with Coomassie Blue for 30 min and destained with methanol, 7%, acetic acid, and H_2O. The incubated mixture was also dialyzed against distilled water and the dialysis solution assayed for free hydroxyproline.

Electrophoresis of the incubation product showed marked reduction of the amounts of collagen present in the alpha, beta, and gamma cross-linked groups when incubation was at 37°C (Fig. 3). The breakdown products were sufficiently small, in that they did not appear on the gel. Bacterial collagenase, in the amount of 10 μg/ml showed the same breakdown pattern. Dialysis of the degradation products from the 37°C incubation showed a release of 40% of the incorporated radioactivity into the dialyzing medium, and free hydroxyproline could be measured in the dialyzing media. However, with incubation at 25°C, two nondialyzable breakdown products appeared in the gels, and no free hydroxyproline could be measured in the dialyzing media (Fig. 4).

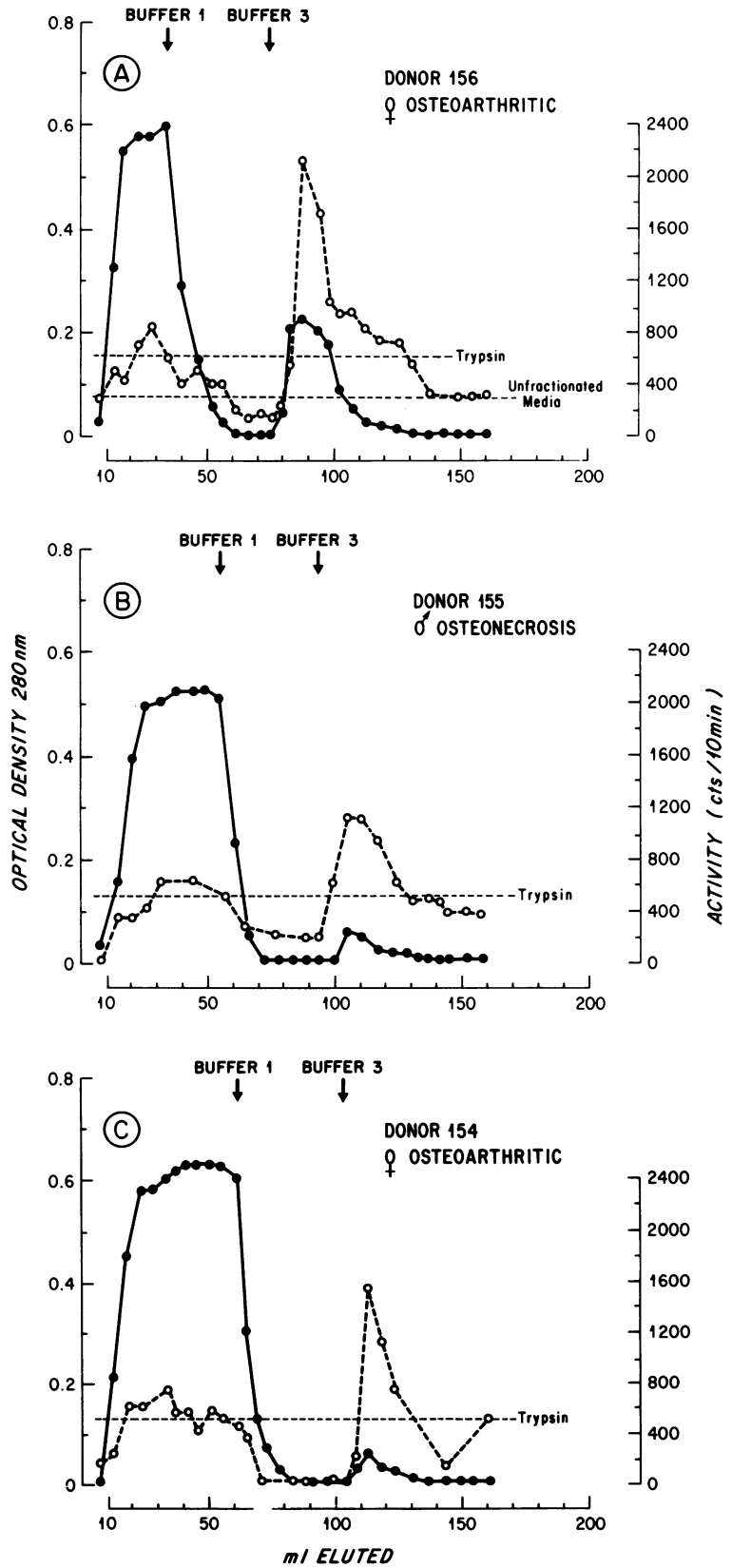
DISCUSSION

The experimental data reported provide the first evidence for the existence of an autogenous collagenase in osteoarthritic articular cartilage. The material is believed to be extra-lysosomal in origin and probably is bound to a trypsin-sensitive inhibitor, which makes demonstration difficult. It is probably present in minute quantities as evidenced by the necessity of 7-day cultures to obtain sufficient activity to be demonstrable on the labeled reconstituted rat skin collagen gel system.

TABLE VI
Activity of Enzyme Peak from Affinity Column

	Osteoarthritic tissue	Normal tissue	Trypsin control
n	5	4	5
Mean, %	42.4±9.4*	10.1±4.2*	6.42±2.62*
Osteoarthritic: Normal, $P < 0.02$‡			
Osteoarthritic: Trypsin Control, $P < 0.01$‡			

Collagenase activity from affinity-chromatography. The osteoarthritic tissue shows far more activity than either the trypsin controls or the normal tissue.
* Counts released/total counts gel±SEM.
‡ Student's *t* test.


BUFFER 1
BUFFER 3
A
DONOR 156
♀ OSTEOARTHRITIC
Trypsin
Unfractionated Media
B
DONOR 155
♂ OSTEONECROSIS
C
DONOR 154
♀ OSTEOARTHRITIC
OPTICAL DENSITY 280nm
ACTIVITY (cts/10min)
ml ELUTED

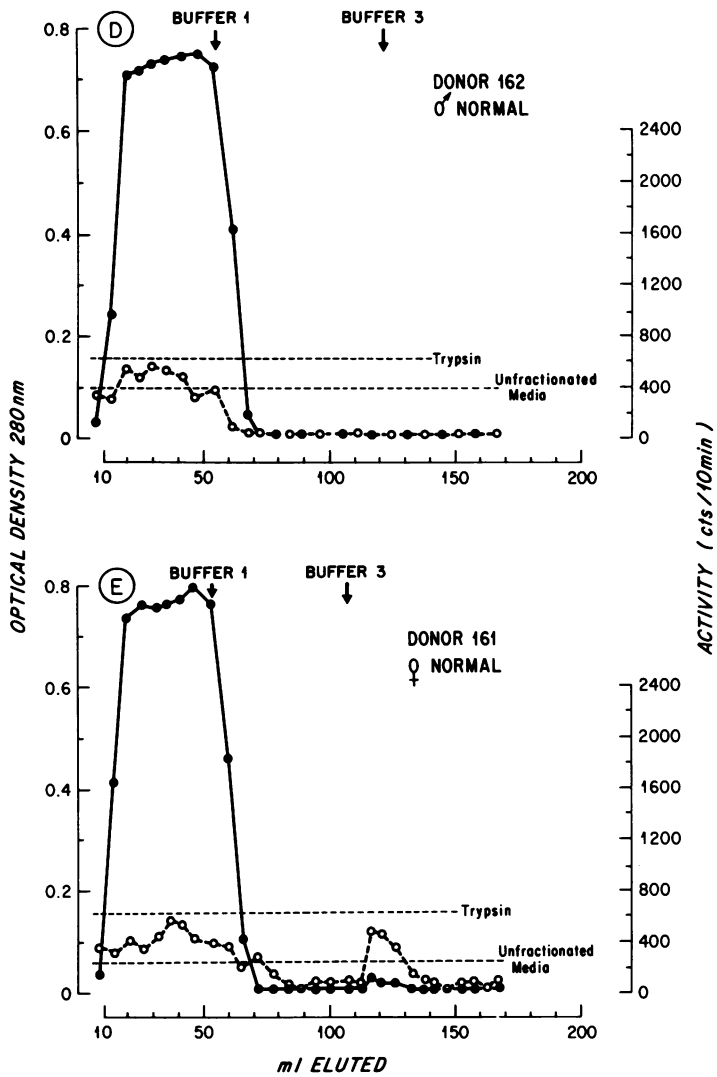


FIGURE 2 *D, E*: Normal donors. Although there is a small second peak at OD 280, degradative activity is less than that of the trypsin control.

Initial attempts to determine the presence of a collagenase were unsuccessful chiefly because of the relative stability of collagen in most tissues. In 1962, Gross and Lapiere (5) demonstrated the first animal collagenase which could act on native fibrils at a physiologic pH by culturing tadpole tails on collagen gels for a sufficient period of time to allow the enzyme to diffuse out of the tissue.

The presence of a collagenase in osteoarthritic articular cartilage but not in normal tissues is not surprising. Earlier studies from this laboratory demonstrated an 8-day half-life for a fraction of the proteoglycan of normal rabbit cartilage but suggested that the collagen was quite stable (11). Repo and Mitchell (12), using a [^{3}H]hydroxyproline assay after introduction of [^{3}H]proline, were able to postulate that the most rapidly turning over collagen of normal rabbit cartilage had a half-life of several months. In osteoarthritis, however, there has been ample demonstration of enhanced degradation of proteoglycan by lysosomal enzymes which appear to increase in quantity with advancing severity of

FIGURE 2 Optical density (●) and degradative enzyme activity (○) in collagen substrate with eluants from affinity column. *A*, *B*, and *C*: Osteoarthritic donors' activity from second affinity peak is considerably higher than trypsin or the buffer control. The first OD peak probably contains the inhibitor.

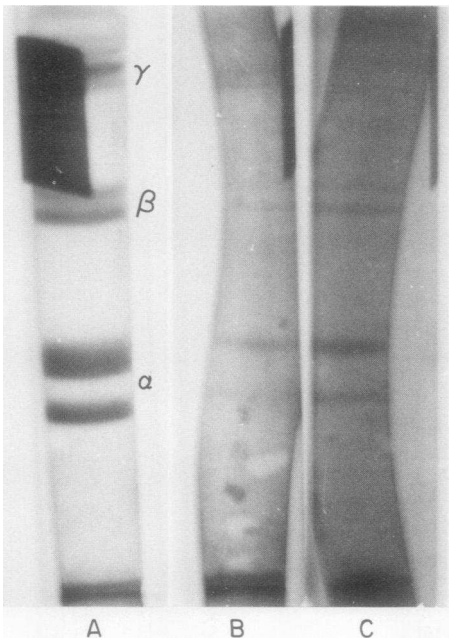


FIGURE 3 Sodium dodecyl sulfate-disk electrophoretic patterns of equal amounts of collagen.(A) Control incubated without enzyme and (B) after incubation with bacterial collagenase and (C) after incubation with osteoarthritic cartilage collagenase. There are considerable reductions in the α, B, and γ recombination peaks after both enzymes are used.

the disease (13). At end-stage osteoarthritis, the cartilage is totally lost from the surface, suggesting that while collagen is initially preserved (as compared with proteoglycan), it must be finally degraded to complete the process.

Prior attempts to find a collagenase in normal or even osteoarthritic articular cartilage were probably unsuccessful because of the small quantity of enzyme present, its extralysosomal origin, and the presence of a high molecular weight inhibitor which made demonstration by the labeled collagen gel technique difficult. Subsequent studies have demonstrated collagenase activity in culture fluids of rat bone (6, 14) and uterus (15, 16), in human gingiva (17) and also bone (18), and skin (19) and synovia from patients with rheumatoid arthritis (20).

Within the last 4 yr, by use of special technique, collagenolytic activity has been demonstrated in homogenates at physiologic pHs by Woessner and Ryan (21, 22). It has also been shown to be present in homogenates of human granulocytes (3). Studies by Fullmer and Lazarus on a specimen of human patella suggested that human articular cartilage may contain a collagenase (18). In addition, evidence is now accumulating that an inhibitor (or inhibitors) of collagenase exists in many tissues and prevents demonstration of enzyme activity unless the inhibitors can be removed or be altered in some way. Two such techniques recently reported include inhibitor extraction by a thiocyanate preparation (23), and the heparin-bound cyanogen bromide activated, Sepharose 4B chromatography, reported by Sakamoto (6, 7) and used with slight modification in this study.

Characterization studies on enzymes obtained from the uterine homogenates indicate that it acts to release soluble digestion products from collagen fibrils at 37°C, although it still shows activity at 30°C. The optimum pH is 7.5, and the enzyme requires the presence of Ca^{++} ion in concentrations of 0.01–0.1 M although other workers have found good activity at lower concentrations (21, 22). The enzyme is inhibited irreversibly by EDTA in quantities sufficient to chelate the calcium ion (2). The mechanism of action of the tadpole tail enzyme appears to be splitting of the collagen chain at a point one quarter distant from the "B" end. The fragment, designated by Kang et al. as TC_B, has a molecular weight of about 24,000 and a triple helicoid structure (4).

The pattern of breakdown of collagen at 37°C to dialyzable fragments which we observed in electrophoresis and measured in the dialysate has recently been reported for rheumatoid synovial collagenase (24). It was noted that this reaction could proceed to complete digestion of the collagen where break-

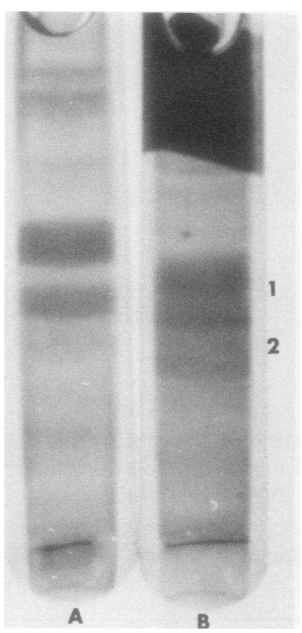


FIGURE 4 Electrophoresis of collagen (A) and collagen incubated with collagenase (B) from osteoarthritic cartilage at 25°C. There are two main digestion products (1, 2). (Each contains both α_1 and α_2 chains.)

down products were smaller than 8,000 daltons. However, at 25°C, nondialyzable digestion products appeared in the electrophoresis gels. The articular cartilage collagenase demonstrated the same pattern, but the nondialyzable fragments did not appear to be identical to the ones described by Kang et al. (4).

These data suggest that in pathologic states, human articular cartilage makes a collagenolytic enzyme. Future work remains necessary to characterize the enzyme and quantitate it in various degrees of severity of osteoarthritic cartilage.

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