*Biochimica et Biophysica Acta*, 657 (1981) 517—529


BBA 69197

# NEUTRAL PROTEINASES FROM ARTICULAR CHONDROCYTES IN CULTURE

## I. A LATENT COLLAGENASE THAT DEGRADES HUMAN CARTILAGE TYPE II COLLAGEN

CHARLES J. MALEMUD [a], DAVID P. NORBY [a], ASHER I. SAPOLSKY [b], KUNIO MATSUTA [b], DAVID S. HOWELL [b] and ROLAND W. MOSKOWITZ [a]

[a] *The Cartilage Research Laboratory, Departments of Medicine and Orthopaedics, Case Western Reserve University, Cleveland, OH 44106 and* [b] *The Departments of Medicine and Biochemistry, University of Miami School of Medicine, Miami, FL (U.S.A.)*

(Received March 20th, 1980)
(Revised manuscript received October 9th, 1980)

*Key words. Proteinase; Collagenase; Collagen degradation; (Articular chondrocyte)*

## Summary

Culture media collected from secondary monolayer and spinner cultures of rabbit articular chondrocytes showed evidence of collagenolytic activity by the following criteria: (1) Amicon PM-10 concentrates of culture medium released [$^{14}$C]glycine from reconstituted rabbit skin collagen fibrils at 37°C; (2) medium concentrated by lyophilization decreased the relative viscosity of human cartilage collagen in solution. The loss in viscosity was partially inhibited if medium was preincubated with *o*-phenanthroline, and (3) degradation of human cartilage collagen after 60 h incubation at 24°C was characterized primarily by the appearance of 75 000 dalton ($TC_A$) and 25 000 dalton ($TC_B$) products.

The majority of the collagenase (EC 3.4.24.3) from cultured chondrocytes was secreted in latent form, since preincubation with either trypsin or *p*-aminophenylmercuric acetate significantly increased activity against human cartilage collagen.

Chondrocyte collagenase may be important in mediating the normal slow turnover of cartilage collagen and may be particularly active in collagen destruction associated with early stages of synovial joint arthritides, before attack by non-cartilage cells or extra-articular soft tissues.

## Introduction

Collagen turnover in mammalian diarthrodial joints is believed to be extremely slow, with a half-life estimated between 50 and 300 days or longer [1]. Following articular cartilage injury, collagen surrounding chondrocyte lacunae appears to undergo transition from the normally occurring Type II species ($\alpha_1[II]_3$) to Type I ($\alpha_1[I]_2\alpha_2$) as indicated by immunofluorescent studies with monospecific collagen antibodies [2]. This switch in collagen phenotype may well be preceded by an enzyme-mediated resorption of the Type II collagen species. Indeed, Ehrlich et al. [3] have measured collagenolytic activity in osteoarthritic, but not in normal human cartilage. Activity was adjudged highest in osteoarthritic hips with moderately severe disease [4]. The above evidence [2,3] suggests that the chondrocyte is a source of collagenolytic activity, although collagenase (EC 3.4.24.3) in osteoarthritic specimens could have arisen from synovium after initial proteoglycan degradation. We report here that secondary monolayer and spinner cultures of normal rabbit articular chondrocytes secrete into the culture medium a latent metal-dependent collagenase with activity against both rabbit skin Type I and human articular cartilage Type II collagen.

## Materials and Methods

*Materials.* All culture media, balanced salt solutions, antibiotics, antimycotics and fungizone were obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. Fetal bovine serum (not heat-inactivated) was procurred from Associated Biomedic Systems, Buffalo, NY, U.S.A. Trypsin and trypsin/soybean trypsin inhibitor were purchased from Worthington Biochemicals, Freehold, NJ, U.S.A., and lactalbumin hydrolysate was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. [$^{14}$C(U)]glycine (99.6 mCi/mmol) was from New England Nuclear Corp., Boston, MA, U.S.A. All chemicals were of reagent grade or the highest purity available.

*Techniques of culture.* Chondrocytes were obtained from pooled articular cartilages (hip, knee and shoulder) of immature New Zealand White rabbits (less than 3 kg) as previously described [5,6]. Primary cultures were grown to confluency in Nutrient F-12 medium (Ham's) supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U and 100 $\mu$g/ml, respectively). After 7—10 days, the chondrocytes were passaged by brief treatment with trypsin, centrifugation and replating of the cells in 100 mm$^2$ cell culture dishes (Falcon Plastics), at a density of 5—7 · 10$^5$ cells in Dulbecco's Modified Eagle's medium and supplemented with 10% acid-treated fetal bovine serum/penicillin-streptomycin (0.1%)/fungizone (1%)/mycostatin (0.1%). Acid treatment of the serum was carried out by the method of Werb and Gordon [7] to destroy endogenous $\alpha$-2-macroglobulin, a potent inhibitor of most vertebrate collagenases. Secondary cultures (monolayer I, monolayer II) were maintained in the above medium for 7—10 days at which time the last medium change was decanted and the cell layer washed several times with Dulbecco's phosphate buffered saline, pH 6.9. Dulbecco's Modified Eagle's medium containing 0.2% lactabumin hydrolysate ('serum-free' medium) was layered over the cells. 24 h

later, this medium was recovered and frozen. The chondrocytes were refed with serum-free medium and a second medium collection made 24 h later.

Spinner culture (spinner I) was initiated from one-half of the primary culture cell pellet. Chondrocytes ($6.9 \cdot 10^4$ cells/ml medium) were placed in Dulbecco's Modified Eagle's medium without $MgSO_4$ or $CaCl_2$, but containing $MgCl_2$ (165 mg/l) and 10% acid-treated fetal bovine serum. After 2 days, the cells were pelleted by centrifugation and the supernatant discarded. The spinner chondrocytes were washed with saline (vide supra) and cultured in serum-free medium. Media was collected for two consecutive 24-h periods.

*Processing of media.* In preliminary experiments, serum-free monolayer medium without the lactalbumin hydrolysate was concentrated by passing medium through an Amicon PM-10 filter membrane (Amicon Corp., Boston, MA) under $N_2$ pressure at 4°C. The PM-10 concentrate (approx. 20-fold concentration) was used to assay for collagenolytic activity in the reconstituted rabbit skin collagen fibril assay (see below).

For most other experiments, the media were lyophilized and then dissolved (50—100 mg/ml) in 0.005 M Tris-HCl, pH 7.25. The solution was dialyzed against 100 vol. 0.005 M Tris-HCl, pH 7.25/0.2 M NaCl/0.01 M $CaCl_2$/0.2% sodium azide in 2000 molecular weight cut-off membranes (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) at 4°C.

*Collagen substrates.* Radioactive rabbit skin collagen was prepared as follows: A single immature New Zealand White rabbit was injected intraperitoneally with β-aminoproprionitrile (500 mg) for 24 h. [$^{14}$C]Glycine (167 μCi/kg) together with a second injection of the lathyrogen was administered intraperitoneally and the animal killed 24 h later. The skin was removed and purification of skin collagen subsequent to extraction with 0.5 M acetic acid was carried out as previously described [8]. The acid-soluble skin collagen fraction used in these experiments contained 1034 cpm/mg protein.

Human cartilage collagen was prepared by the following procedure. Cartilage removed at autopsy from 25 patellae free of joint disease was used. 50 g were suspended in 10 vol. 0.005 M phosphate buffer, pH 8.0 and homogenized in a Virtis homogenizer at high speed for 5 min. The homogenate was clarified by centrifugation (4500 × *g* for 1 h) and the pellet obtained was stirred overnight at 4°C in 10 vol. 0.05 M Tris-HCl buffer, pH 7.4/2 M $CaCl_2$. After centrifugation (6000 rev./min for 1 h), the pellet was washed with 10 vol. distilled water and extracted with 10 vol. 0.5 M acetic acid overnight at 4°C. The pH of the extract was adjusted to 2.5 and the extract was incubated with pepsin (2 mg/ml) for 48 h at 4°C. The reaction mixture was centrifuged, and the pellet was re-extracted with pepsin for 48 h in the cold. After centrifugation, bulk NaCl was added to the supernatant to give a final concentration of 0.9 M and the suspension again centrifuged. The resulting pellet was dissolved in 0.05 M Tris-HCl, pH 7.5/1 M $CaCl_2$ and stirred in the cold overnight. Following centrifugation, the supernatant was dialyzed against 0.02 M disodium phosphate, recentrifuged and the pellet re-extracted with 0.5 M acetic acid. After centrifugation the supernatant was dialyzed against 0.5 M acetic acid and lyophilized.

*Preparation of cyanogen bromide peptides of human cartilage collagen.* Cyanogen bromide peptides of purified α1(II) chains from 300 mg cartilage collagen were prepared by the method of Miller et al. [9].

*Collagenase assays.* Collagenolytic activity of chondrocyte culture media was measured by three different assay procedures:

1. Collagenase activity in serum-free medium was measured in a reconstituted collagen fibril assay using [$^{14}$C]glycine rabbit skin collagen by the method described by Golub et al. [10]. The reaction mixture consisted of 400 μg [$^{14}$C]glycine reconstituted collagen fibrils and varying amounts of PM-10 concentrate incubated at 37°C for 3 h.

2. The change of human cartilage collagen viscosity in solution was measured by incubation of reconstituted serum-free medium and substrate directly in a microviscometer as previously described [11]. Cartilage collagen was first dissolved in 0.1 M acetic acid to give a final concentration of 0.4%. When a cloudiness developed, the collagen solution was centrifuged and the clear supernatant was used for viscometric assay, since it retained sufficient collagen. Thus, the collagen concentration eventually mixed with culture medium varied from one preparation to another, but comparative analysis within one experiment were performed with the same preparation. The substrate was then dialyzed at 4°C against 0.05 M Tris-HCl, pH 7.4/0.2 M NaCl/0.05 M lysine/0.01 M $CaCl_2$/0.02% sodium azide. Dialysis continued until the pH of the collagen solution was 7.25. The collagen (0.15—0.25 ml) and medium (0.1 ml) were mixed and incubated at 24°C. In some cases, the incubation mixture became cloudy on standing at 24°C, but cleared at 4°C. When possible, therefore, viscosities were measured at room temperature and at 4°C after temperature equilibration for 10 min. Results were obtained by comparing the flow time of collagen alone to that obtained in the presence of collagen and serum-free chondrocyte medium.

3. Analysis of human cartilage collagen degradation products were performed as follows: Substrate was incubated with serum-free medium for 20—60 h at 24°C. Following incubation, samples (0.2 ml) were diluted to 1.2 ml with saturated $(NH_4)_2SO_4$. After centrifugation, the pellet was mixed with 2% sodium dodecyl sulfate (SDS) (0.2 ml) and an equal volume of 2% mercaptoethanol and incubated at 37°C overnight. Electrophoresis of the reaction mixture was performed on 10 × 0.4 cm 7.5% polyacrylamide gels containing 0.1% SDS in Tris-glycine buffer, pH 8.9 generally for 4—6 h.

*Preincubation procedures.* Serum-free chondrocyte medium was preincubated with porcine pancreatic trypsin (20 μg/ml) at 24°C for 30 min followed by 10-fold excess of soybean trypsin inhibitor prior to incubation with [$^{14}$C]glycine reconstituted rabbit skin collagen fibrils. When testing activity against soluble human cartilage collagen, activation of the serum-free medium was carried out by preincubating medium with *p*-aminophenylmercuric acetate, a thiol binding reagent (0.5 mM), for 15—30 min at 24°C or with trypsin and soybean trypsin inhibitor. Medium was treated with trypsin (100 μg/ml) for 30 min at 24°C, followed by soybean trypsin inhibitor (500 μg/ml) prior to addition of activated medium to collagen. In all activation experiments, *p*-aminophenylmercuric acetate or trypsin/soybean trypsin inhibitor without culture medium was included as a control in the collagenase assay.

*Column chromatography of monolayer medium on Bio-Gel P-30 or Sephadex G-100.* Molecular sieve chromatography of *p*-aminophenylmercuric acetate-activated medium or medium receiving no pretreatment was carried out

on Bio-Gel P-30 or Sephadex G-100 (100 × 0.6 cm) which was equilibrated and eluted with 0.005 M Tris-HCl buffer, pH 7.25/0.1 M $CaCl_2$/0.2% sodium azide. Fractions showing enzyme activity by viscometric analysis were rechromatographed and SDS-polyacrylamide gel electrophoresis carried out on pooled fractions as indicated in Results.

## Results

*Effects of monolayer chondrocyte medium on reconstituted rabbit skin collagen fibrils*

Serum-free monolayer culture medium (without lactalbumin hydrolysate) was harvested from two successive 24 h periods and concentrated by Amicon PM-10 ultrafiltration. The retained fraction was assayed for collagenase activity against [$^{14}$C]glycine reconstituted rabbit skin collagen fibrils (Table I). Chondrocyte medium caused a concentration-dependent release of radioactivity. Release of radioactivity by buffer or trypsin was negligible, indicating a lack of interfering non-collagenous radioactive substrate and suggesting specificity of degradation for the helical region of the collagen molecule. Preincubation of concentrated medium with trypsin did not increase collagenolytic activity.

*Effect of serum-free monolayer and spinner culture medium on the viscosity of human cartilage collagen*

Serum-free chondrocyte medium (with lactabumin hydrolysate) was collected for two successive 24 h periods and lyophilized. The medium was reconstituted in 0.005 M Tris-HCl, pH 7.25/0.01 M $CaCl_2$/0.2% sodium azide to give a final concentration of 50—100 mg/ml. The medium was dialyzed against the above buffer and 0.1 ml medium was mixed with human cartilage collagen for

TABLE I

EFFECT OF CHONDROCYTE MONOLAYER MEDIUM CONCENTRATED BY AMICON PM-10 ULTRAFILTRATION ON RECONSTITUTED RABBIT SKIN COLLAGEN FIBRILS

The reaction mixture [$^{14}$C]glycine reconstituted rabbit skin collagen fibrils (100 μl) was incubated with either buffer (0.05 M Tris-HCl/0.2 M NaCl/0.01 M $CaCl_2$, pH 7.6)/trypsin (20 μg/ml) or PM-10 concentrate of chondrocyte monolayer medium for 3 h at 37° C. The amount of radioactivity released from duplicate samples into the clear supernatant was measured by liquid scintillation spectrometry. The collagen substrate contained 1034 cpm/mg protein. 1 unit collagenase activity = 1 μg collagen degraded/h.

| Reaction Mixture | PM-10 concentrate (μl) | Percent [$^{14}$C]glycine released/3 h | Units of activity |
|---|---|---|---|
| Buffer + collagen | 0 | 2.9 | 0.24 |
| Trypsin + collagen | 0 | 1.3 | 0.11 |
| Monolayer + collagen | 50 | 25.8 | 2.15 |
| | 100 | 52.9 | 4.40 |
| | 150 | 64.9 | 5.40 |
| Monolayer + trypsin * + collagen | 100 | 51.1 | 4.25 |

* Chondrocyte monolayer PM-10 concentrate was preincubated with trypsin (20 μg/ml) for 30 min at 24° C, followed by addition of 10-fold excess of soybean trypsin inhibitor. The mixture was then incubated with radioactive reconstituted rabbit skin collagen fibrils for 3 h at 37° C.

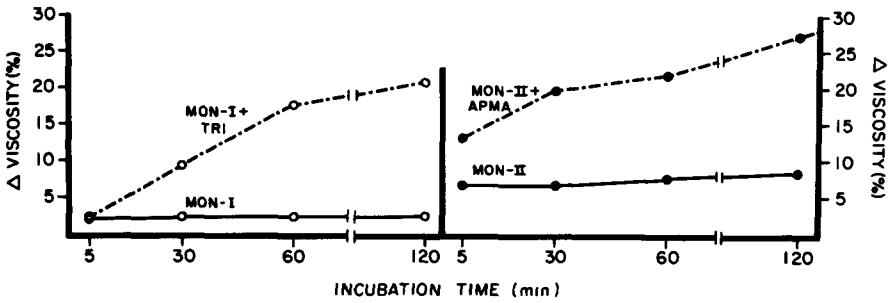


Fig. 1. Analysis of human cartilage collagen viscosity after incubation with serum-free medium from chondrocyte monolayer cultures. Serum-free medium was collected from the last two consecutive 24 h periods of culture and lyophilized. After reconstitution, the medium was dialyzed against Tris-HCl buffer, pH 7.25/0.2 M NaCl/0.01 M $CaCl_2$/0.02% sodium azide. Human cartilage collagen was incubated with medium (50—100 mg/ml) for 5—120 min at 24° C. In some experiments, medium was preincubated with trypsin/soybean trypsin inhibitor (TRI) or *p*-aminophenylmercuric acetate (APMA). The loss in collagen viscosity was measured over 0.01 min in a microviscometer [11]. Results are expressed as the percent difference in collagen viscosity in the presence of medium or trypsin/soybean trypsin inhibitor or *p*-aminophenylmercuric acetate-activated medium as compared to collagen alone, calculated as follows:

Δ% = 100 X (change in viscosity with monolayer or *p*-aminophenylmercuric acetate + monolayer — change in viscosity without monolayer or *p*-aminophenylmercuric acetate + monolayer) ÷ (change in viscosity of collagen without monolayer or *p*-aminophenylmercuric acetate + monolayer).

periods ranging from 5—120 min and 20—60 h. Some serum-free monolayer medium (monolayer I and monolayer II) caused measurable, albeit small decreases in viscosity of Type II collagen (Fig. 1). Preincubation of medium with either trypsin/soybean trypsin inhibitor (left panel) or *p*-aminophenylmercuric acetate (right panel), markedly augmented the decrease in substrate viscosity. Trypsin/soybean trypsin inhibitor or *p*-aminophenylmercuric acetate alone had little or no effect. In general, collagenolytic activity in the *p*-aminophenylmercuric acetate-activated medium was greater than in trypsin/soybean trypsin inhibitor-activated samples after 30—60 min of incubation.

In a separate experiment, monolayer medium (monolayer II) was activated by preincubation with *p*-aminophenylmercuric acetate and then further treated with the metal-chelator, *o*-phenanthroline (5 mM) prior to incubation with cartilage collagen. A 38.7% inhibition of the loss in collagen viscosity occurred. This result indicated that the collagenolytic activity was at least partially metal-dependent.

Medium from different cultures expressed variably low or undetectable intrinsic activity (Table II). Insufficient numbers of samples were analyzed to attach significance to differences between spinner and monolayer cultures. Substantial activation of collagenolytic activity by *p*-aminophenylmercuric acetate was seen with culture media from both types of cultures.

*Analysis of human cartilage collagen degradation products by SDS-polyacrylamide gel electrophoresis*

The products of a 60 h digestion were subjected to electrophoresis (Fig. 2) for 5 h (tracks 1—3) or 3.5 h (track 4). In the control reaction (track 1),

TABLE II

VISCOMETRIC ANALYSES OF HUMAN COLLAGEN AFTER INCUBATION WITH CONDROCYTE MONOLAYER OR SPINNER MEDIUM

The reaction mixture consisted of 100 μl culture medium (100 mg lyophilized medium/ml) alone in buffer (0.05 M Tris, pH 7.25/0.2 M NaCl/0.01 M $CaCl_2$/0.2% sodium azide) or with collagen (100 μl). Reaction was for 60 min at 24° C. Viscometric analyses were run either at room temperature or 4° C.

| Reaction mixture | Loss in viscosity (Δ%) | |
|---|---|---|
| | 24° C | 4° C |
| Collagen | 0 | 0 |
| Monolayer — I + Collagen | 0 | 0 |
| Monolayer — I + *p*-aminophenylmercuric acetate + Collagen | 29.7 | 28.5 |
| Monolayer — II + Collagen | 11.2 | 7.4 |
| Monolayer — II + *p*-aminophenylmercuric acetate + Collagen * | 19.5 | 17.4 |
| Spinner — I + Collagen | 9.3 | 0 |
| Spinner — I + APMA + Collagen * | 16.7 | 10.7 |

* Monolayer or spinner medium was preincubated with *p*-aminophenylmercuric acetate (APMA) (0.5 mM) for 30 min at room temperature prior to incubation with collagen.

Δ(%) = 100 × (decrease in viscosity with monolayer or spinner — decrease in viscosity without monolayer or spinner) ÷ (decrease in viscosity without monolayer or spinner)

collagen was incubated with *p*-aminophenylmercuric acetate. No degradation of α chains was detected. Incubation of collagen with *p*-aminophenylmercuric acetate-activated monolayer medium (250 and 150 μg/ml, tracks 2 and 3, respectively) resulted in the appearance of bands corresponding to the sizes of $TC_A$ and $TC_B$ fragments typical of mammalian collagenases. The $TC_B$ band is faint in these gels, and may be due to further degradation by contaminating nonspecific protease(s) in the culture medium. In this regard, a diffuse band (dp) appeared migrating just behind the bromophenol blue, which may represent the degradation products. The $TC_B$ band shows more clearly in track 4.

A comparison of the activity of monolayer and spinner culture collagenase activity was made by incubating *p*-aminophenylmercuric acetate-activated medium from both culture regimens and assessing their effects on cartilage collagen degradation (Fig. 3). These results indicated that monolayer medium contained some intrinsic collagenase activity (gel 2); that both monolayer and spinner culture collagenase activity was increased by preincubation with *p*-aminophenylmercuric acetate (gels 3 and 4); but that spinner medium had no effect on collagen degradation unless first activated with *p*-aminophenylmercuric acetate (gel 5).

*Partial separation of chondrocyte collagenase from other chondrocyte neutral proteases*

Reconstituted serum-free monolayer medium was chromatographed on Bio-Gel P-30 with or without preincubation with *p*-aminophenylmercuric acetate. Enzyme activity was eluted with 0.005 M Tris-HCl buffer, pH 7.25/0.1 M NaCl/0.01 M $CaCl_2$/0.2% sodium azide. Fractions containing enzyme activity were rechromatographed on Bio-Gel P-30 and eluted as above. Viscometric

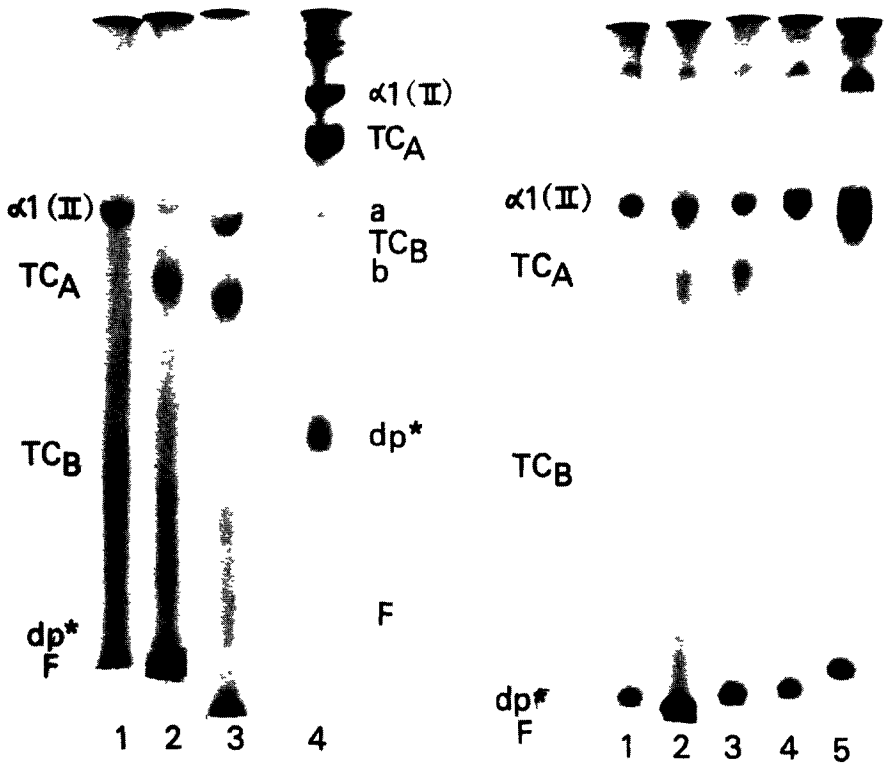


Fig. 2. SDS-polyacrylamide gel electrophoresis of reaction products resulting from the incubation of human cartilage collagen with serum-free monolayer medium. Collagen was incubated with reconstituted *p*-aminophenylmercuric acetate-activated serum-free monolayer medium or *p*-aminophenylmercuric alone for 60 h at 24° C. The reaction products were analyzed on 7.5% polyacrylamide gels containing 0.1% SDS. Gels were electrophoresed in Tris-glycine buffer, pH 8.9 for 5 h. The gels were stained with 0.15% Coomassie blue and destained in 7% acetic acid. 1. Collagen + *p*-aminophenylmercuric acetate (blank). 2. Collagen + medium (250 μg/ml) + *p*-aminophenylmercuric acetate. 3. Collagen + medium (150 μg/ml) + *p*-aminophenylmercuric acetate. 4 Same as 3, except running time was 3.5 h instead of 5 h. Small molecular weight proteins migrate (dp — degradation products) to a point directly behind bromphenol blue dye marker (F) in tracks 1—3 and are clearly separated from F when gels are electrophoresed for a shorter time period (track 4).

Fig. 3. A comparison of the collagenolytic activity of monolayer and spinner medium by SDS-polyacrylamide gel electrophoresis. Monolayer and spinner media with or without *p*-aminophenylmercuric acetate (0.5 mM) pretreatment were incubated with cartilage collagen for 60 h at 24° C. Incubation conditions were similar to that given in Fig. 2. 1. Collagen (no *p*-aminophenylmercuric acetate). 2. Monolayer (no *p*-aminophenylmercuric acetate) + collagen. 3. Monolayer + *p*-aminophenylmercuric acetate + collagen. 4. Spinner + *p*-aminophenylmercuric acetate + collagen. 5. Spinner (no *p*-aminophenylmercuric acetate) + collagen.

analyses and SDS-polyacrylamide gel electrophoresis were carried out on pooled fractions. The decrease in viscosity after incubation of the indicated pooled fraction (Table III) with collagen shows that most of the enzyme activity resided at 25—35 ml of the Bio-Gel P-30 column eluate.

The products of incubation of human cartilage collagen with various chromatographic fractions from the Bio-Gel P-30 column elution of *p*-aminophenylmercuric acetate and untreated monolayer medium were analyzed on 7.5% polyacrylamide gels containing 0.1% SDS. *p*-Aminophenylmercuric

TABLE III

CHROMATOGRAPHY OF CHONDROCYTE CULTURE MEDIA ON BIO-GEL P-30

Incubations were for 60 h at 24°C. *p*-Aminophenylmercuric acetate pretreatment was as described in Table II. Gel filtration on Bio-Gel P-30 (100 × 0.6 cm). The elution buffer was 0.005 M Tris-HCl, pH 7.25/0.1 M NaCl/0.01 M $CaCl_2$/0.02% sodium azide. Fractions were lyophilized, reconstituted with distilled water and incubated with collagen.

| Incubation conditions | Bio-Gel P-30 fractions (ml) | Loss in viscosity (Δ%) (4°C) * |
|---|---|---|
| Control assays | | |
| Collagen | — | 0 |
| Monolayer-I + collagen | — | 2.2 |
| Collagen + *p*-aminophenylmercuric acetate | — | 2.2 |
| Chromatography of monolayer-I after *p*-aminophenylmercuric acetate activation | | |
| Monolayer-I + *p*-aminophenylmercuric acetate + collagen | 18—25 | 8.0 |
| | 25—35 | 16.0 |
| | 39—46 | 8.0 |
| | 49—63 | 8.0 |
| Chromatography of monolayer-I without *p*-aminophenylmercuric acetate activation | | |
| Monolayer-I (no *p*-aminophenylmercuric acetate) | 28—32 | 15.5 |

* Viscosity was read at 4°C after temperature equilibration, (see Table II for calculation of Δ%).

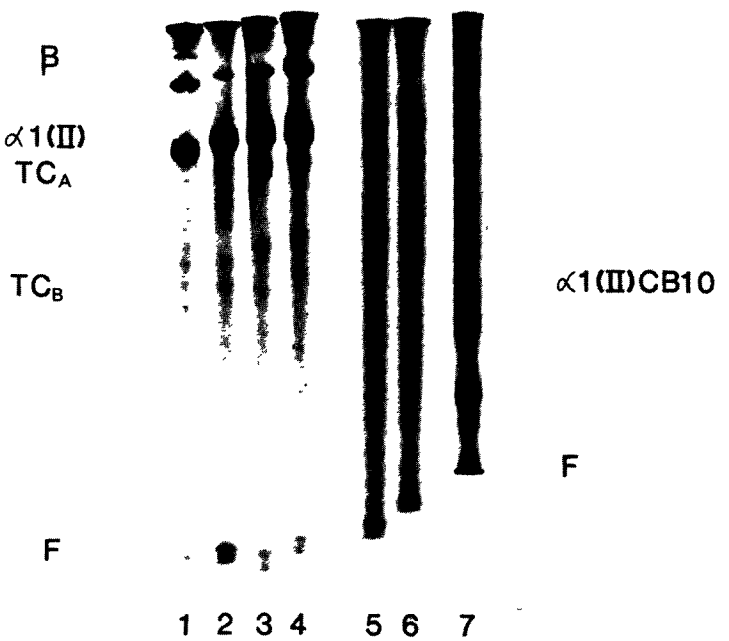


Fig. 4. Analysis of collagenolytic activity after molecular sieve chromatography on Bio-Gel P-30. Reconstituted monolayer medium activated by pretreatment with *p*-aminophenylmercuric acetate (0.5 mM) or not activated, was eluted from Bio-Gel P-30 with 0.005 M Tris-HCl pH 7.25/0.1 M NaCl/0.01 M $CaCl_2$/0.02% sodium azide. Fractions (3.5 ml) were collected, pooled as indicated, lyophilized and incubated with cartilage collagen for 60 h at 24°C. The products of the reaction were analyzed by SDS-polyacrylamide gel electrophoresis as described in Fig. 2. 1. Collagen alone. 2. Bio-Gel P-30 (25—35 ml). 3. Bio-Gel P-30 (36—45 ml). 4. Bio-Gel P-30 (46—63 ml). 5. Bio-Gel P-30 (25—32 ml *). 6. Bio-Gel P-30 (33—45 ml *). 7. CNBr peptides of α1(II) chains showing α1(II) CB-10 with a molecular weight of 25 000 migrating similar to protein band marked $TC_B$.

* Non-activated monolayer medium.

acetate-activated pooled medium fractions eluting at 25—35 ml and 36—45 ml (gels 2 and 3), contained considerable collagenase activity (Fig. 4). Little or no activity was obtained in earlier fractions (data not shown) or later fractions (46—63 ml, gel 3) of the column. When non-*p*-aminophenylmercuric acetate-treated monolayer medium was chromatographed on Bio-Gel P-30, fractions at 25—32 ml and 35—45 ml (gels 5 and 6) showed collagenase activity (Fig. 4), but the activity of fractions eluting at 25—32 ml were more active as shown by the deeper intensity of staining in the $TC_A$ fragment and relative loss of staining from the $\alpha$1(II) chains. The location of the 25 000 (0.25) dalton fragment ($TC_B$) was verified by comparison of its migration with authentic cyanogen bromide peptide $\alpha$1(II) CB-10 of human cartilage collagen with an approx. molecular weight of 25 000 [12].

The apparent molecular weight for chondrocyte collagenase was obtained by gel filtration of *p*-aminophenylmercuric acetate-activated monolayer medium on a calibrated column of Sephadex G-100 eluted with 0.005 M Tris-HCl/0.1 M $CaCl_2$/0.2% sodium azide. The active collagenase eluted before the proteoglycanase activity in a position somewhat later than the ovalbumin standard (42 000 mol. wt.). A tentative molecular weight of 40 000 was assigned to the collagenase. After Sephadex G-100 chromatography, a 32-fold enrichment in collagenase activity was obtained.

## Discussion

The experimental data reported provide initial evidence for the presence of a metal-dependent vertebrate-type collagenase secreted by normal monolayer and spinner cultures of rabbit articular chondrocytes. This enzyme activity is apparently distinct from other chondrocyte neutral proteinases, i.e., proteoglycanase [13]. The chondrocyte collagenase released [$^{14}$C]glycine in the reconstituted rabbit skin collagen fibril assay and degraded $\alpha$1(II) chains of human cartilage collagen. A metal requirement for full enzyme activity was shown by partial inhibition of activity after incubation of *p*-aminophenylmercuric acetate-activated medium with 5 mM *o*-phenanthroline.

Although measurable levels of intrinsic collagenase activity were demonstrated, significantly higher activities in serum-free medium were measured if the reconstituted medium was preincubated with trypsin or *p*-aminophenylmercuric acetate. *p*-Aminophenylmercuric acetate activation suggested that the inactive form of the enzyme may be an enzyme inhibitor complex [14], while activation by trypsin could have resulted by preferential degradation of the inhibitor suggested by Reynolds et al. [15] or conversion of a proenzyme. Similar evidence of latent mammalian collagenases has been presented for cultures of rabbit [16] and chick [17] bone explants and for the collagenase secreted by embryonic chick skin [18]. In the latter two studies, activation was accomplished by preincubation with either NaSCN [17] or NaI [18], indicative of a non-covalent enzyme inhibitor complex. Although the chemical nature of the chondrocyte collagenase latency is unknown at present, the ability of latent collagenase to bind to collagen prior to activation has been shown for the rheumatoid synovial enzyme [19]. In the present study, we have detected measurable, albeit small amounts of collagenase activity when

non-activated medium was incubated with Type II collagen or when medium was concentrated by Amicon PM-10 ultrafiltration. These results suggest that two forms of collagenase activities may exist concomitantly in the secreted culture medium.

Serum-free monolayer and spinner medium activated by pretreatment with *p*-aminophenylmercuric acetate, degraded Type II collagen from pooled human patellar cartilage. The reaction products were identified by SDS-polyacrylamide gel electrophoresis and corresponded to molecular weights of 75 000 and 25 000. The molecular weight of the latter was confirmed by its comigration with authentic α1(II) CB-10 peptide of human cartilage collagen with a molecular weight of 25 000 [12]. The results showed that the activated chondrocyte collagenase resembled vertebrate collagenases obtained in other culture systems [20], in that the helical region of the tropocollagen molecule was degraded at 24°C and at only one locus. The appearance of smaller molecular weight fragments of degraded α chains after incubation of monolayer medium with cartilage collagen at 24°C, does, however, indicate the presence of other more non-specific proteases in addition to collagenase. Other investigators [21,22], have reported significant degrees of degradation by trypsin of Type I collagen $TC_A$ and $TC_B$ fragments at or below the temperature under which the present assays were conducted. We have previously identified trypsin-like activity in chondrocyte monolayer medium against the synthetic substrate, benzoyl-D, L-arginine-*p*-nitroanilide [13].

Partial separation of chondrocyte collagenase from other chondrocyte neutral proteases has been achieved. The molecular weight of the chondrocyte collagenase appeared to be between 30 000—40 000 and thus resembled the molecular weight of collagenase seen in several other culture systems [17,18, 20,23]. In the present experiments, collagenase activity was increased 32-fold from the original reconstituted medium after Sephadex G-100 chromatography.

The relative ability of monolayer and spinner culture collagenase(s) to be activated and the effect of activated enzyme on Type II collagen may be related to several factors. One possibility would be that the two systems differ with respect to inhibitor vis-à-vis enzyme synthesis, with spinner collagenase being more difficult to activate because of a relatively higher rate of inhibitor to enzyme synthesis. Another factor involved in the control of collagenase synthesis may be related to significant differences in the phenotypic programs exhibited by monolayer and spinner cultures of chondrocytes [24]. Spinner chondrocytes resemble adult rabbit cartilage in several major characteristics. they fail to undergo DNA synthesis [25] and synthesize predominantly Type II collagen when serum is included in the medium [8]. In the present experiments, medium was collected from serum-free cultures (but containing lactalbumin hydrolysate). The type of the collagen synthesized may not be entirely Type II. In fact, Norby et al. [8] showed that when serum was excluded from the culture medium, the percentage of Type II collagen synthesized dropped from 85 to 50%. This percentage of Type II collagen, however, was still higher than found in monolayer cultures. In addition, the synthetic rate of collagen synthesis although higher in serum-free medium of spinner as compared to monolayer cultures was markedly reduced in comparison to serum-containing

medium. Taken together, these results suggest that the microenvironment of cultured chondrocytes may be primarily responsible for the appearance of collagenase activity.

The presence of a cartilage collagenase has been previously suggested. Reynolds et al. [15] indicated the presence of a latent collagenase in rabbit scapular cartilage, activatable by trypsin and *p*-aminophenylmercuric acetate and inhibited by a 30 000 molecular weight polypeptide. However, no definitive data has been presented on the ability of this collagenase to degrade cartilage collagen. Fullmer and Lazarus [26] presented evidence for collagenolytic activity in culture fluids of goat tibial articular cartilage and human patellar cartilage by virture of the ability of these cultures to reduce the viscosity of skin collagen relative to trypsin. The enzyme may be similar to the chondrocyte collagenase reported here.

The cellular compartmentalization of cartilage collagenase has been investigated. Monfort and Pérez-Tamayo [27] provided evidence for immunoreactive collagenase located intracellularly, but absent in the matrix of chick xiphoid process. The findings have been confirmed by Sakamoto et al. [28] in mouse tibial cartilage. In the present study, monolayer chondrocyte collagenase appeared to be rapidly secreted after synthesis, since the medium contained higher levels of activity than the cell lysate in the reconstituted rabbit skin collagen fibril assay.

The possible role(s) of chondrocyte collagenase in synovial joint metabolism remains to be defined. The enzyme may mediate the slow turnover of collagen in normal states, but may be particularly important in joint arthritides. Although Woolley et al. [29] failed to demonstrate immunoreactive collagenase in chondrocytes or cartilage matrix remote from the pannus of rheumatoid tissue, these investigations provide evidence for the presence rather than for the activity of the enzyme. Ehrlich et al. [3] have shown that moderately severe degenerative articular cartilage possessed detectable collagenase activity, which could not be demonstrated in normal cartilage. In the present study, chondrocyte collagenase degraded human Type II collagen. This result indicates that chondrocytes can synthesize an enzyme capable of degrading its own matrix collagen. In the present study, secretion of latent collagenase was observed without additional requirement for 'factor(s)' from other cell types (e.g., macrophages) as reported by others [30]. Naturally occurring activators of the latent enzyme have yet to be found.

## Acknowledgements

These studies were supported in part by National Institutes of Health (NIH) grants, AG-02205 (C.J.M.), RR-73604 (C.J.M.), AM-08662 (D.S.H.) and AG-01305 (R.W.M.). Support was also given by The W.L. McKnight Arthritis Fund. Preliminary portions of this work were supported by NIH grant, AM-17258 to Dr. Leon Sokoloff (State University of New York at Stony Brook, Stony Brook, NY, U.S.A.).

## References


1 Lane, J.M. (1978) Articular Cartilage Collagen in Health and Disease. in The Human Joint in Health and Disease. (Simon, W.H., ed.), pp. 31—36, University Pennsylvania Press, Philadelpia PA
2 Gay, S., Müller, P.K., Lemmen, C., Remberger, K., Matzen, K. and Kühn, K. (1976) Klin. Wsch. 54, 969—976
3 Ehrlich, M.G., Mankin, H.J., Jones, H., Wright, R., Crispen, C. and Vigliani, G. (1977) J. Clin. Invest. 59, 226—233
4 Ehrlich, M.G., Houle, P.A., Vigliani, G. and Mankin, H.J. (1978) Arthritis Rheum. 21, 761—766
5 Sokoloff, L., Malemud, C.J. and Green, W.T., Jr. (1970) Arthritis Rheum. 13, 118—124
6 Green, W.T., Jr. (1971) Clin. Orthop. 75, 248—260
7 Werb, Z. and Gordon, S. (1975) J. Exp. Med. 142, 346—360
8 Norby, D.P., Malemud, C.J. and Sokoloff, L. (1977) Arthritis Rheum. 20, 709—716
9 Miller, E.J., Epstein, E.H. and Piez, K.A. (1971) Biochem. Biophys. Res. Commun. 42, 1024—1029
10 Golub, L.M., Siegel, K., Ramamurthy, N.S. and Mandel, I.D. (1976) J. Dental Res. 55, 1049—1057
11 Sapolsky, A.I., Keiser, H., Howell, D.S. and Woessner, J.F., Jr. (1976) J. Clin. Invest. 58, 1030—1041
12 Eyre, D.R. and Muir, H. (1975) Connect. Tissue Res. 3· 165—170
13 Malemud, C.J., Weitzman, G.A., Norby, D.P., Sapolsky, A.I. and Howell, D.S. (1979) J. Lab. Clin. Med. 93, 1018—1030
14 Sellers, A., Cartwright, E., Murphy, G. and Reynolds, J.J. (1977) Biochem. J. 163, 303—307
15 Reynolds, J.J., Sellers, A. Murphy, G. and Cartwright, E. (1977) Lancet ii, 333—335
16 Sellers, A., Cartwright, E., Murphy, G. and Reynolds, J.J (1977) Biochem. Soc. Trans. 5, 227—229
17 Sakamoto, S., Sakamoto, M., Matsumoto, A., Goldhaber, P. and Glimcher, M. (1978) FEBS Lett. 88, 53—58
18 Shinkai, H., Kawamoto, T., Hori, H. and Nagai, Y. (1977) J. Biochem. 81, 261—263
19 Vater, C.A., Mainardi, C. and Harris, E.D., Jr. (1978) Biochim. Biophys. Acta 539, 238—247
20 Gross, J. (1976) Aspects of the Animal Collagenases. In Biochemistry of Collagen (Ramachandran, G.N. and Reddi, A.H., eds.), pp. 275—317, Plenum Press, New York
21 Sakai, T. and Gross, J. (1967) Biochemistry 6, 518—528
22 Harris, E.D., Jr. and Krane, S.M. (1974) New Engl. J. Med. 291, 557—563
23 Woolley, D.E., Glenville, R.W., Crossley, M.J. and Evanson, J.M. (1975) Eur. J. Biochem. 54, 611—622
24 Malemud, C.J. (1978) Cell culture in the study of mammalian chondrocytes. In The Human Joint in Health and Disease. (Simon, W.H., ed.), pp. 43—52, University Pennsylvania Press, Philadelphia, PA
25 Srivastava, V.M.L., Malemud, C.J. and Sokoloff, L. (1974) Connect. Tissue Res. 2, 127—136
26 Fullmer, H. and Lazarus, G. (1967) Israel J. Med. Sci. 3, 758—761
27 Montfort, I. and Pérez-Tamayo, R. (1975) J. Histochem. Cytochem. 23, 910—920
28 Sakamoto, M., Sakamoto, S., Goldhaber, P. and Glimcher, M.J. (1978) Trans. Orthop. Res. Soc. 2, 157
29 Woolley, D.E., Crossley, M.J. and Evanson, J.M. (1977) Arthritis Rheum. 20, 1231—1239
30 Deshmukh-Phadke, K., Lawrence, M. and Nanda, S. (1978) Biochem. Biophys. Res. Commun. 85, 490—496