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ABSTRACT A collagenase and a neutral protease have been insolated and characterized from primary cultures obtained from rheumatoid subcutaneous nodules. Release of both active enzymes was maximal between the 3rd and 7th days of culture and was stimulated by the presence of small amounts of colchicine (0.1 μg/ml) added to the culture medium.

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Since rheumatoid nodules grow centrifugally at the expense of the palisading fibroblast layer it seems possible that the central necrotic areas are caused by release of collagenase and protease from the highly cellular palisading zone resulting in the destruction of the extracellular collagen matrix.

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INTRODUCTION

The pathology of subcutaneous rheumatoid nodules is well documented (1, 2). In the well-formed nodule there is a central area of necrosis rimmed by a corona of palisading fibroblasts which in turn is surrounded by a dense proliferation of collagen along with perivascular collections of chronic inflammatory cells (1, 2).

Although there are data from careful histologic sections of nodules at very early stages of development which suggest that the development of the peripheral nodule, as well as the synovitis, is mediated through affected arteries to the terminal vascular bed of the tissues (3), there are few other data which provide any unifying hypothesis for the pathogenesis of both rheumatoid nodules and rheumatoid synovitis, or for the development of central necrosis in the former lesions. In addition, there are virtually no biochemical studies of rheumatoid nodules, contrasted with the many metabolic, enzymatic, and immunologic studies of rheumatoid synovium and synovial fluid.

Since the mesenchymal cells of rheumatoid synovium seem to be capable of synthesizing and releasing in vitro (4–7) and in vivo (8, 9) specific collagenolytic enzymes which may have a primary role in the mechanism of articular destruction in rheumatoid arthritis (10, 11), we searched for collagenolytic enzyme production in primary cultures of rheumatoid nodules, using the same techniques that have been applied to synovium.

METHODS

Patients and handling of specimens. Nodule tissue was removed from the olecranon bursae of two female patients in whom they were causing discomfort. Both patients had classic, seropositive rheumatoid arthritis. In neither was there skin ulceration over the nodules. All tissue cultures were initiated within 1 hr after nodule excision using sterile precautions. The dense fibrous capsule of each nodule was incised. Fluid from the necrotic centers of each nodule was discarded. Firm tissue bordering on the central necrotic areas but not including the tough, dense outer capsule was

cut into approximately 2 × 2-mm pieces and cultured in vitro using techniques similar to those used for short-term cultures of synovial tissue (4, 7). Samples of the cultured tissue were fixed and sectioned for microscopic examination and were then stained. Colchicine (U.S.P., Eli Lilly and Company, Indianapolis, Minn.) was added to the medium in one-half of the flasks at a concentration of 0.1 μg/ml. Medium was changed at least every other day. After 9 days of culture the tissue was harvested and the DNA content in each group of flasks was determined (12).

Assay procedures. Collagenolytic activity was assayed using substrates of guinea pig collagen in solution at 20–24°C and ^{14}C-labeled collagen which had been reconstituted at 37°C to form native fibrils as described earlier (8). Neutral protease activity was determined using the same ^{14}C-labeled collagen after it had been denatured to gelatin (7). Caseinolytic activity (13) and protein concentration (14) were measured in some samples.

Enzyme purification. Pooled tissue culture medium was concentrated using Aquacide and applied to a column (1.5 × 55 cm) of Bio-Gel A-1.5 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 0.1 M Tris-HCl, pH 7.6, 0.005 M $CaCl_2$. Fractions containing peaks of protease and collagenase activity were pooled separately and then subjected to chromatography on a column (1.0 × 165 cm) of Sephadex G 150. Fractions containing collagenase activity were pooled and concentrated.

Affinity chromatography. Sepharose 4B (Pharmacia) was activated by cyanogen bromide as described by Cuatrecasas, Wilchek, and Anfinsen (15) and was bound to collagen in solution (purified as described previously [7]) using the technique of Bauer, Eisen, and Jeffrey (9). The Sepharose-collagen was washed and then equilibrated with 0.05 M Tris-HCl, pH 7.6, 0.005 M $CaCl_2$ at 4°C, as was the enzyme. The enzyme was applied to a 1 × 4 cm column of the Sepharose-collagen. After washing the column with 10 bed-volumes of the starting buffer, collagenase was eluted with 1.0 M NaCl in 0.05 M Tris HCl, pH 7.6, 0.005 M $CaCl_2$.

Miscellaneous. Enzyme inhibitors were purchased from Sigma Chemical Co. (St. Louis, Mo.) and from Calbiochem (Los Angeles, Calif.). Acrylamide disc gel electrophoresis was carried out by the methods of Davis (pH 8.3) (16) and Nagai, Gross, and Piez (17). Segment-long-spacing (SLS) aggregates of intact collagen and of the enzymatic digestion products were prepared as described previously (8), except that the SLS formation was initiated by dialyzing samples against adenosine triphosphate (ATP) rather than by addition of ATP to the samples. Specimens prepared on carbon-coated grids were examined and photographed by Dr. Charles Faulkner using a Siemens Elmiskop IA electron microscope (Siemens Corp., Iselin, N. J.). Use of a Gilford gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) was kindly provided by Leonard Malkin and Rhona Mirsky.

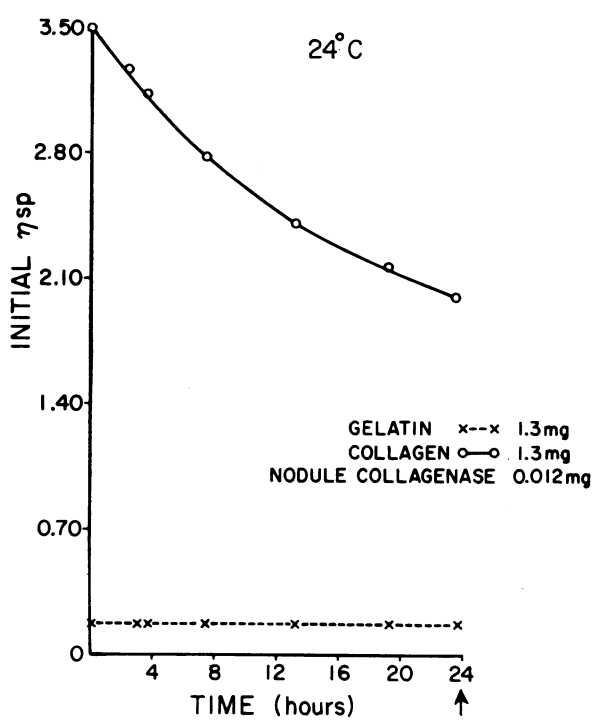

FIGURE 1 Viscosity changes during incubation of gelatin and collagen with purified rheumatoid nodule collagenase.

Specific viscosity

$$(\eta sp) = \eta \text{ rel} - 1 ; \eta \text{ rel} = \frac{\text{sample time}}{\text{water time}}.$$

RESULTS

Histologic examination of the nodules cultured in these studies showed classic changes of central necrosis and a dense cellular layer arranged radially. This layer gradually became less cellular at the periphery where the cells and collagen assumed a circumferential orientation and where there were small foci of perivascular lymphocytes.

During the first 3 days of culture no significant amounts of collagenolytic activity were found in the medium from either control or colchicine-treated tissue.[1] However, from the 3rd through the 7th days of culture considerable collagenolytic activity was found in the medium in the tissues from both patients. The colchicine-containing medium from one set of cultures had collagenolytic activity calculated at 15,000 dpm/mg DNA per hr, while enzyme from the medium not containing colchicine degraded substrate at a rate of 8,000 dpm/mg DNA per hr. The medium from eight rheumatoid *synovial* cultures were assayed using the same substrate and only one quarter to one-third as much collagenolytic activity was found. The increase in activity related to the presence of colchicine in the medium was similar to that seen in the synovial cultures.

When the pooled, concentrated crude collagenolytic activity was passed through a column of Bio-Gel A 1.5, collagenase activity was eluted in the area corresponding to 20,000–40,000 daltons. 95% of the enzyme activity was present in 8% of the initial protein applied to the column. Protease activity was eluted in a broad peak closer to the void volume of the column. After the pooled

[1] The colchicine had been added because of our previous observations that small concentrations (0.1 μg/ml) of this compound stimulate synthesis of collagenase (6) and protease (7) by rheumatoid synovial tissue.

protease and collagenase peaks were passed through the Sephadex G 150 column, there was no collagenolytic activity found in the protease fractions, and negligible protease in the collagenase. The partially purified collagenase was then subjected to affinity chromatography and a total of 50 μg of protein containing collagenolytic activity was isolated. 25 μg of this ran as a single, faint band when subjected to acrylamide disc gel electrophoresis (7.5% acrylamide, pH 8.3). The remainder was divided into two, 12-μg portions, each in 0.1 ml of 0.1 M Tris-HCl, pH 7.6, 0.005 M $CaCl_2$ in 10% glycerol. One portion was added to 1.3 mg collagen in 0.9 ml 0.1 M Tris-HCl, pH 7.6, 0.005 M $CaCl_2$ and 0.1 M NaCl in a Manning semimicro viscometer, and the other portion of enzyme was placed in a similar viscometer with the same amount of substrate in denatured form.

The course of the viscosity assay is shown in Fig. 1. The specific viscosity (ηsp) of the collagen dropped to 57% of its original value in 24 hr. The ηsp of the gelatin solution did not rise, indicating that there was no renaturation of the gelatin. The reactions in both viscometers were halted by the addition of EDTA (0.1 M, 50 μl). Aliquot portions of the reaction products were applied to acrylamide gels for disc electrophoresis. Densitometer scans of the stained gels are shown in Fig. 2. The collagen/enzyme reaction products showed intact β- and α-chains as well as the TC^A fragments and the TC^B fragments.[2] The site of cleavage was also demonstrated by direct visualization of TC^A SLS and TC^B SLS by electron microscopy. In the gelatin/enzyme digest, the $TC^A\beta$- and $TC^A\alpha$-bands were less well-defined than in the collagen/enzyme digest but the distribution indicated that about 90% of the substrate was converted into $TC^A\beta$, $TC^A\alpha$, and TC^B; 10% of the gelatin/collagenase reaction products ran with the buffer front, where peptide fragments of less than 10,000 daltons are found.

No differences were found in pH maxima (4), estimated molecular size (8), or inhibitor effects comparing the synovial enzymes (7) and the nodule enzymes (both collagenase and protease). Both collagenases, for example, were inhibited 90% by 0.01 M dithiothreitol, D-penicillamine, α, α'-dipyridyl, and 1,10-phenanthroline, and by pooled normal human serum at 1:40 dilution. 1,10-phenanthroline inhibited both collagenases better at low concentrations (78% at 10^{-4}M, 36% at 10^{-5}M) than did the other inhibitors. Cysteine and L-histidine inhibited both enzymes to 60% of control values at 10^{-2} concentrations.

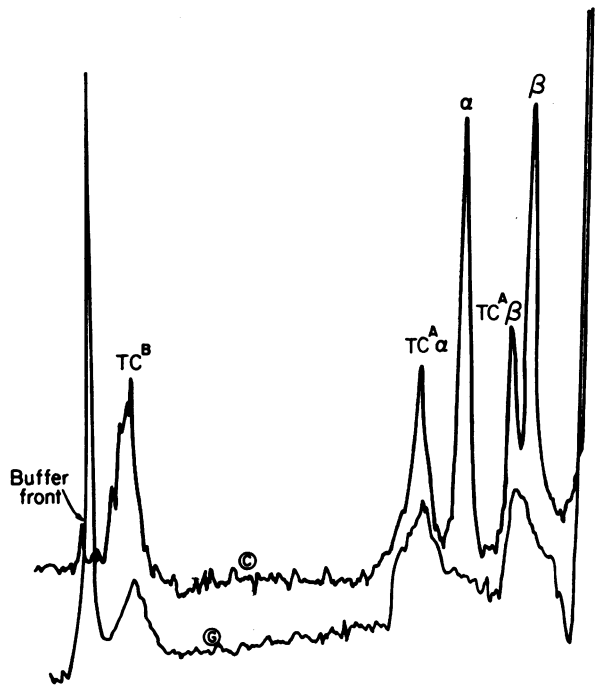

FIGURE 2 Densitometer scans after acrylamide gel electrophoresis of the reaction products from viscometric assays shown in Fig. 1. Line C is collagen/collagenase reaction products. Line G is the gelatin/collagenase mixture. Note that the G gel had broad, diffuse bands which nevertheless had peak density in areas corresponding to $TC^A\beta$, $TC^A\alpha$, and TC^B. Material staining at the buffer front represents gelatin fragments of < 10,000 daltons.

DISCUSSION

Previous studies of rheumatoid synovial tissue (7) showed that the collagenase partially purified from primary cultures of these tissues had little capacity to degrade gelatin to fragments of < 10,000 daltons while the neutral protease found in those same cultures readily digested gelatin to fragments of about 5,000 daltons. In the present studies, highly purified rheumatoid nodule collagenase did not degrade gelatin significantly more than it degraded native collagen. Our hypothesis is that, in vivo, after the initial cleavage of collagen into two fragments by collagenase and the postulated spontaneous denaturation of these fragments at 37°C to gelatin fragments (10), subsequent breakdown of these primary products of collagenolysis is accomplished by proteases.

It is likely that there are no significant differences between the collagenolytic system produced by rheumatoid synovial and nodule tissues. In all ways tested, the two nodule enzymes were similar to the collagenase and protease found in synovial cultures (4, 7). It is unlikely that

[2] α-Chains are the basic polypeptide component of collagen; 3 α-chains comprise one collagen molecule. β-Chains are 2 α-chains joined by a covalent cross-link near the amino-terminus. Thus, cleavage of intact collagen by mammalian collagenases at the locus 75% from the amino-terminus produces $TC^A\alpha$ and $TC^A\beta$ from α- and β-chains, respectively, and the carboxyterminal TC^B fragment from both α- and β-chains.

we have isolated enzymes which are present in normal subcutaneous tissue. Using primary cultures similar to ours, Eisen found negligible collagenolytic activity in the lower dermis and subcutaneous tissue of human skin (18).

A role in the pathogenesis of articular destruction in rheumatoid arthritis has been proposed for the synovial enzymes (11); similarly, it is possible that the collagenolytic enzyme system contributes to the central necrosis which develops in rheumatoid nodules. The rheumatoid nodule has been thought to grow by extension of the central necrotic area at the expense of the palisade layer of cells which recedes centrifugally. Collagen fragments have been identified by electron microscopy as comprising a large part of the necrotic center of these nodules (19). Perhaps the enzymes described here released by the palisading layer of cells are sufficient to result in destruction of the extracellular matrix collagen around the cells, leading to their death and subsequent necrosis.

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