Acta Dermatovener (Stockholm) 54: 265–269, 1974

COLLAGENOLYTIC ACTIVITY IN RHEUMATOID NODULES

I. *Effect on Acid-soluble Tropocollagen*

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Abstract. Homogenates of subcutaneous rheumatoid nodules (SRN) are capable of cleaving acid-soluble tropocollagen at neutral pH. Viscometry, polarimetry and polyacrylamide gel electrophoresis revealed that SRN homogenates (*a*) reduced the viscosity of acid-soluble tropocollagen without affecting its triple helical structure at 27°C, a temperature well below that required for collagen denaturation; (*b*) decreased the stability against heat of the tropocollagen fragments produced thus lowering their melting temperature (T_m) by about 8°; (*c*) cleaved tropocollagen at specific loci along the macromolecule. The data suggest that a multienzyme system might be involved in the necrobiosis of the nodules.

Several studies using tissue culture technique demonstrated the presence of collagenase activity in synovial tissues (2, 9) as well as synovial fluid (5) from patients with rheumatoid arthritis. The enzyme from both materials was isolated and reasonably purified. However, studies on subcutaneous rheumatoid nodules are few.

In histological sections the center of subcutaneous rheumatoid nodules show collagen degeneration, commonly called fibrinoid degeneration or necrobiosis (10).

The possibility that proteolytic activity or specific collagenolytic activity may play a role in such tissue necrosis was investigated in this study. In the present communication we report the presence of collagenolytic activity in homogenates of subcutaneous rheumotid nodules.

MATERIALS AND METHODS

Subcutaneous rheumatoid nodules. Three subcutaneous rheumatoid nodules (SRN) were removed surgically from the arms and elbows of rheumatoid arthritis patients hospitalized at the Memphis V. A. Hospital—the clinical history of these patients is described elsewhere (7). After subcutaneous fat and normal collagen were removed, the rest of the nodule was sectioned and either used separately or pooled with other nodules which had been similarly treated.

Preparation of homogenate supernatant. Individual or pooled nodules were homogenized in a ground-glass homogenizer at 0–4°C with about five times their volume of 0.05 M Tris-HCl/buffer, pH 7.6, containing 0.04 M $CaCl_2$. The homogenates were then centrifuged at 30 000 *g* for 30 minutes at 4°C. Quantitative determination of protein utilized ultraviolet absorption of the supernatant at 280 nm or Lowry's method (11) with bovine serum albumin as a standard.

Preparation of substrate. Uniformly labeled ^{14}C-proline in two doses of 25 μCi each was injected intraperitoneally into randomly bred white guinea pigs. The animals were killed by decapitation 48 hours after the injection. The skin was removed and subcutaneous tissue was scraped off mechanically. Neutral salt-soluble collagen was extracted by the method of Gross (4). The final products were lyophilized and stored at −20°C. ^{14}C-proline was obtained from Schwarz/Mann, Orangeburg, N.Y.

Preparation of incubation mixture. Reconstituted collagen was prepared from the ^{14}C-proline incorporated, lyophilized salt-soluble collagen, according to the method of Nagai et al. (12). Aliquots of 0.5 ml of the collagen solution (0.2 %) were pipetted into plastic centrifuge tubes and allowed to gel in a 37°C water bath for at least 12 hours. Prior to admixture with the tumor homogenate, the collagen gel was disrupted with a steel needle in order to insure a good contact with the homogenate (12), after which 0.2 ml of the supernatant of the pooled homogenate (crude enzyme solution) was added to each tube. All tubes were incubated at 37°C for 18 hours with constant agitation. After the incubation, tubes were centrifuged at 59 000 *g* at room temperature for 30 minutes to sediment undissolved collagen. An 0.5 ml aliquot of the supernatant was added to 10 ml of Insta-Gel (Packard), and the radioactivity was counted in a liquid scintillation spectrometer.

Tropocollagen preparation. Purified acid-soluble calf skin tropocollagen was prepared according to the method of Rubin et al. (14) as modified by Dabbous et al. (1). Fractions 2A and 2B were used.

Viscometry. Viscosity of the reaction mixture was measured at timed intervals during incubation at 27°C±0.1° in a temperature-regulated water bath. Measurements were

Table I. *SRN collagenolytic and caseinolytic activities on salt-extracted, proline-^{14}C labeled guinea pig skin collagen*

	Tissue protein (mg/ 0.5 ml	CPM[a] solubilized (above blank)	Collagen gel lysed (%)	% Lysis mg protein	Caseinolytic activity[b]
Subcutaneous rheumatoid nodules (SRN)	800 μg	500	11.3	13.1	6.7
Normal skin	4.7	60	1.4	0.3	3.0

[a] Total radioactivity of incubation mixture for SRN and the normal skin was 4 305 cpm.
[b] Expressed as μg trypsin equivalent/mg crude enzyme protein.

carried out using Ostwald viscometers with flow times for water of 75–90 seconds. The reaction mixture consisted of 4–5 mg of lyophilized acid-soluble calf skin tropocollagen (as prepared above) in 3 ml of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.04 M $CaCl_2$ and 1.0 ml of supernatant solution of SRN homogenates. The reaction mixtures were incubated at 27°C±0.1° and the viscosity was measured after various periods of incubation. The enzyme action was terminated by lowering the pH of the reaction mixture to 3.5–3.8 with acetic acid and adding EDTA to a final concentration of 0.01 M.

Polyacrylamide gel electrophoresis. Collagenolytic activity of the homogenates on acid-soluble calf skin tropocollagen was analysed by electrophoresis on polyacrylamide gels. The method of Nagai et al. (13) as modified by Dabbous et al. (1) was used.

Optical rotation and melting curves. Optical rotation was measured in a Carl Zeiss Polarimeter equipped with a hydrogen light source (365 nm) using water-jacketed 1-dm polarimeter tubes. The concentration of acid-soluble calf skin tropocollagen solutions was calculated from the optical rotation measurements obtained for denatured samples with −460° as the specific optical rotation of denatured gelatin. Melting curves were obtained by monitoring the optical rotation of samples to which EDTA was added to a final concentration of 0.01 M (to terminate the enzyme action), at pH 3.8, as the temperature was increased from 27° to 45°C; before each measurement the samples were allowed to stand at that temperature for 20 minutes.

RESULTS

Release of radioactivity. Collagenolytic activity of the pooled homogenate supernatant of three SRNs was calculated in terms of counts per minute of solubilized collagen gel, percentage lysis of collagen gel, and percentage lysis of collagen gel per milligram tissue protein (Table I). Collagenolytic activity of the normal skin control was also tabulated similarly (Table I). It was noted that the collagenolytic activity of SRNs is significantly higher than that of the normal skin control. For example, percentage lysis of collagen gel per milligram tissue protein of SRNs was 13.1, whereas that of the normal skin control was 0.4 (Table I). Caseinolytic activity of the SRN supernatant as expressed in mg trypsin equivalent per mg tissue protein was 6.7, while that of the normal skin control was 3.0 (Table I).

Viscometry. Incubation of acid-soluble calf skin collagen with the 30 000 *g* supernatant of SRN homogenates resulted in a decrease in its viscosity. At 27°C, the specific viscosity of dilute tropocollagen solutions at pH 7.6 decreased by 15% (SRN-1) and by 54% (SRN-2) after 16 hours of incubation. Fig. 1 shows the progress of loss of specific viscosity of tropocollagen as a function of time during incubation. In both samples (SRN-1 and SRN-2) a lag was observed which was followed by a continuous loss of viscosity for a certain period but a limiting value seemed to be reached within 16 to

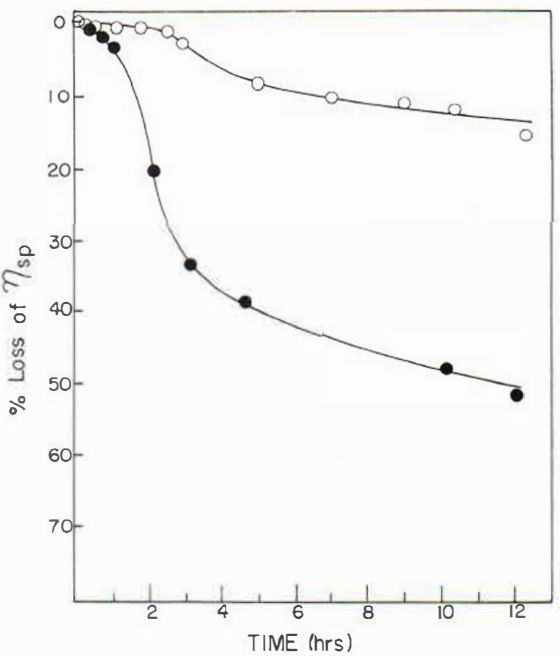

Fig. 1. Viscosity change during incubation of the supernatant of SRN homogenates with acid-soluble calf skin collagen (1 ml/4 ml reaction mixture) at 27±0.1°. The collagen concentration was 0.1% in 0.05 M Tris-HCl containing 0.04 M $CaCl_2$, pH 7.6. The percentage loss in specific viscosity is plotted as a function of time. Curves (○—○) and (●—●) represent two different SRN homogenates, described in the text.

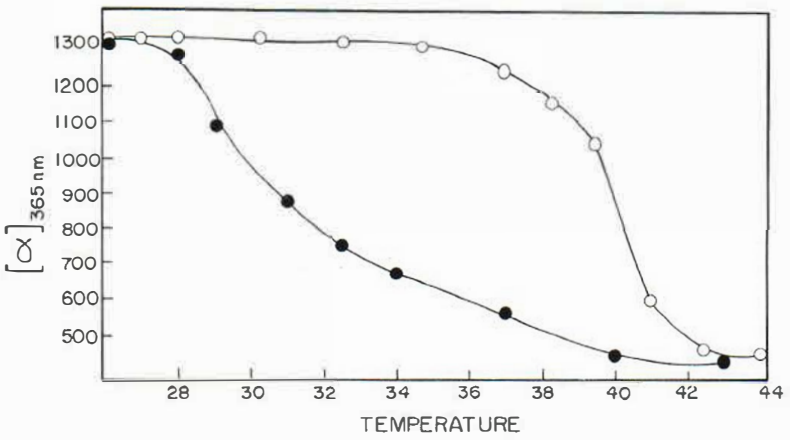

Fig. 2. Melting curves of acid-soluble calf skin tropocollagen (○—○) and SRN-treated tropocollagen (●—●). Samples were dissolved in 0.05% acetic acid and the temperature was raised gradually as described under Materials and Methods.

24 hours (varied from one sample to another). However, during the following 48 hours of incubation a continuous but relatively slow loss of viscosity was also observed, indicating a continuous breakdown of the products of incubation at a temperature well below that of tropocollagen denaturation. During one experiment, a loss of 70% of the original specific viscosity of tropocollagen was obtained after 55 hours of incubation with SRN-1.

Melting curve. Incubation of acid-soluble collagen with SRN-1 or SRN-2 at 27°C for 24 hours did not result in any detectable change in optical rotation. This indicated that the tropocollagen macromolecule was not denatured to any significant extent during this treatment. However, the negative optical rotation of the treated tropocollagen solutions was gradually decreased during the stepwise increase in temperature of the reaction mixture above 28°C, as shown in Fig. 2. The denaturation temperature midpoint (melting temperature, T_m) of native tropocollagen (40°C) dropped by about 8–9° as a result of incubation with the supernatant of rheumatoid nodule homogenates.

Disc electrophoresis. Denatured tropocollagen solutions gave the normal electrophoretic pattern; the fast-moving $\alpha 2$ and $\alpha 1$ chains, respectively, followed by the β-bands (β_{12} and β_{11}, respectively) with intermediate mobility and then the slow moving γ- and higher molecular weight aggregates (H) (close to the top of the upper separating gel layer) as shown in Fig. 3 (gels A). The electrophoretic pattern of SRN-treated tropocollagen similarly denatured after

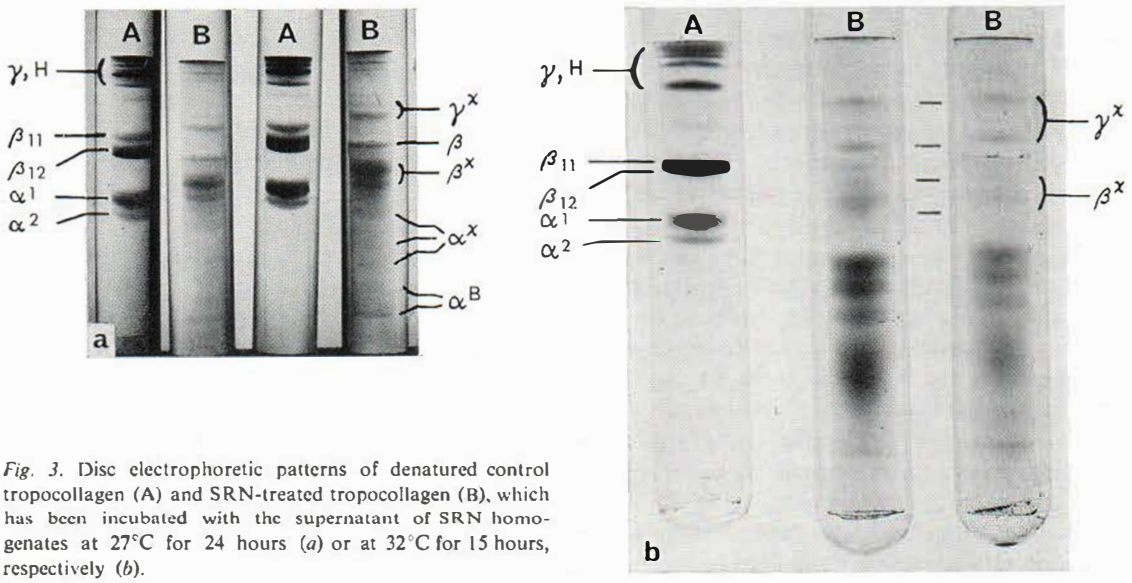

Fig. 3. Disc electrophoretic patterns of denatured control tropocollagen (A) and SRN-treated tropocollagen (B), which has been incubated with the supernatant of SRN homogenates at 27°C for 24 hours (*a*) or at 32°C for 15 hours, respectively (*b*).

incubation (Fig. 3*a*) (gels B) showed that only trace amounts of α-, β- and γ-chains were present. The major dense bands marked γ^{α}, β^{α} and α^{α} represent breakdown products of the original γ-, β- and α-chains, respectively. The relative intensities of the bands corresponding to the β^{α} and γ^{α} components indicated that these are the major products. The intensities and number of bands with mobility faster than that of the α-components suggested that the latter had undergone multiple cleavage during incubation with the supernatant of SRN homogenates, thus leading to several peptide fractions with molecular weights less than that of the α-chains.

When the temperature was raised to 32°C during incubation for 15 hours and the products were subjected to disc electrophoretic analysis the pattern shown in Fig. 3*b* was obtained. The major bands represent degradation products with molecular weights less than that of α-chains. Bands corresponding to the normal α-, β-and γ-chains were not detectable. Trace amounts of products with molecular weights less than γ- and less than β- are represented by the bands marked γ^{α} and β^{α}, respectively. In general, the relative intensity of the bands indicated an extensive degradation, certainly more than that produced at 27°C.

DISCUSSION

The first demonstration that collagenase was produced in vivo without tissue culture techniques was described by Harris et al. (5) in synovial fluid. Recently Yamanishi et al. (15) reported the presence of collagenase activity in homogenates of basal cell epithelioma of the skin. The present study provides evidence for the presence of collagenolytic activity in subcutaneous rheumatoid nodule homogenates (SRN-homogenates). The amount of radioactivity solubilized from ^{14}C-labeled reconstituted collagen gels incubated with SRN-homogenates was significantly higher than that released from similar gels incubated with normal skin tissue homogenates. This indicated that a relatively higher level of collagenolytic activity was present in vivo in the SRN homogenates. Viscometric studies showed that the supernatant solution of SRN-homogenates is capable of reducing the specific viscosity of acid-soluble collagen to a value which varied from one specimen to another.

Under conditions which maintain the native conformation of tropocollagen, the decrease in specific viscosity was not accompanied by any significant loss of negative optical rotation. This indicated that the supernatant of SRN-homogenates contained collagenolytic enzyme(s) which cleaved tropocollagen without detectable denaturation of the triple helical structure of the macromolecule. However, there was a significant decrease in the stability against heat of the cleavage products in solution. The observed decrease in the melting temperature (*Tm*) of SRN-treated tropocollagen suggested that the amounts of stabilizing structures in the molecule, for example cross-linkages, were decreased as a result of this treatment. In this respect the SRN collagenolytic enzyme(s) behaved in a manner similar to those reported earlier for other tissue collagenases (2–5, 8–9, 15). However, the melting profile indicated a gradual loss of helicity with an average midpoint near 32°C. Disc electrophoresis of the reaction products showed that tropocollagen was cleaved to produce mainly several small peptides migrating faster than the normal α-chains in addition to a few discrete bands which appeared to migrate along the electropherogram with a mobility slightly faster than that of the usual β^{A} and γ^{A}, the 75% pieces of β-chains, and of tropocollagen molecules, respectively. This was further supported by electron microscopic data (7) which showed that the SLS crystallites obtained from the reaction mixture showed mainly fragments with 54% the length of tropocollagen from the A end and 20% pieces from the B end. Furthermore, the fragments cleaved along the β_2^a locus (at 3/4 the length of the molecule from the A end) showed chewing and progressive degradation at their end (7). In view of the rare detection of 75% pieces, the denaturation data and disc electrophoretic analysis, it is reasonable to speculate about the possibility of multiple enzyme activities in the supernatant of SRN-homogenate, for example, nonspecific proteases which further degrade the collagen molecules initially cleaved by specific collagenase activity at β_2^a locus. This is in agreement with a recent finding by Harris et al. (6) in tissue cultures of SRN of a collagenase and a protease. Although our data support their observation we have noted that incubation of tropocollagen with the supernatant of SRN-homogenates at 32°C for 15 hours resulted in extensive degradation rather than a more or less limited cleavage, as shown by the disc electrophoretic analysis of the reaction products. Harris et al. (6) reported that the products obtained by incubating denatured collagen (gelatin) with the

SRN enzyme preparation were mainly TC^A and TC^B (in approximately 60% yield). They concluded that the site of cleavage of tropocollagen by the SRN enzyme preparation is unrelated to the secondary structure of native collagen. The observed differences in the data presented here may be due to the fact that in the present investigation only a crude enzyme preparation was used while the above authors used a partially purified preparation. This possibility seems to be a more likely explanation since preliminary observations in our laboratory (unpublished data) have shown a more specific cleavage of substrate during the purification of the enzyme activity. Moreover, the question of identity or non-identity of enzymes from tissue cultures and from homogenates remains to be examined. Partial purification and characterization of the collagenolytic enzyme activity in SRN homogenates are being studied at present in our laboratory.

The significance of demonstrating collagenolytic activity in the homogenates of SRN is obvious in relation to the mechanism of connective tissue destruction in rheumatoid diseases. Ultrastructural evidence of this is described in the second paper of this series (7).

ACKNOWLEDGEMENTS

This work has been supported in part by A. C. S. University of Tennessee Institutional Grant IN85F and U. S. P. H. S. University of Tennessee Institutional Grant 243301–1534R10 (GRS Funds) (M. K. D. and H. H.) and by Medical Investigatorship Award (K. H.), Advanced Specialty Training Program in Dermatology (E. M.) and Designated Component Research Fund from the Veterans Administration (Y. Y.).

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Received November 5, 1973

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