THE PRESENCE OF PARTIALLY DEGRADED COLLAGEN FRAGMENTS IN THE RESORBING GRANULATION TISSUE IN RATS

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SUMMARY

Insoluble collagen of granulation tissue produced by carrageenin injection was solubilized by pepsin treatment and purified. The pepsin-solubilized insoluble collagen contained partially degraded collagen fragments and the amounts of these small fragments of collagen were much greater in the resorbing granulation tissues than in the growing tissues, suggesting that these small fragments were formed in the course of resorption of granulation tissue, including collagen breakdown.

INTRODUCTION

Although the general pathway of collagen breakdown in granulation tissue is not completely understood, collagenase may be the first enzyme to attack collagen fibrils in the matrix of granulation tissue in vivo (1). Donoff et al. (2) isolated granulation tissue collagenase from the medium during culture of mesenchymal tissue obtained from healing open cutaneous wounds in the rabbit. From ultrastructural studies of carrageenin granulomas, Perez-Tamayo (3) showed an extensive phagocytosis of collagen fibrils by the tissue fibroblasts. There is also a considerable rise in the activities of lysosomal enzymes such as cathepsin B1 during resorption of granulation tissue (4,5). These observations suggest that, in addition to collagenase, lysosomal enzymes play an important role in the degradation of collagen during resorption of granulation tissue.

It was therefore of significant interest to us to ascertain the presence of partially degraded collagen fragments in the resorbing granulation tissue. We report here that pepsin-solubilized insoluble collagen contains small fragments of collagen and the amounts of these small fragments are much greater in

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the resorbing granulation tissues than in the growing tissues.

MATERIALS AND METHODS

A granuloma pouch was induced in male Sprague-Dawley rats weighing 130 - 160 g by subcutaneous injection of a 2 % solution of Seakem 202 carrageenin according to the procedure described previously (6). On day 8 or day 23 after carrageenin injection, granulation tissues were taken and homogenized in a Vir-Tis homogenizer with 5 volumes of 1 M NaCl - 0.05 M Tris·HCl (pH 7.5) at 1°C. The homogenate was stirred overnight at 1°C. NSC* was further extracted twice from the precipitate by stirring for 24 hr at 1°C with 5 volumes of 1 M NaCl - 0.05 M Tris·HCl (pH 7.5). ASC was then extracted 3 times repeatedly from the precipitate for 24 hr at L°C with 5 volumes of 0.1 M acetic acid. The remaining residue containing IC was homogenized in 0.01 M acetic acid and lyophilized.

Pepsin digestion of the lyophilized insoluble residue was performed according to the procedure of Epstein, Jr. (7). Briefly, the lyophilized insoluble residue (700 mg) was suspended in 140 ml of 0.4 M acetic acid containing 70 mg pepsin (Sigma, twice crystallized, 2760 units per mg protein) and stirred at 18°C for 22 hr. Subsequent operations for purification of pepsin-solubilized IC were performed at 4°C. The reaction mixture was centrifuged at 80,000xg for 60 min. The collagen was precipitated twice by dialyzing the supernatant against 0.02 M Na_2HPO_4 and then once by dialysis against 0.5 M acetic acid containing 0.86 M NaCl. Each precipitate was dissolved in 100 ml of 0.1 M acetic acid. The last precipitate was dissolved in 0.1 M acetic acid and then dialyzed extensively against 0.1 M acetic acid. The dialyzed collagen solution was lyophilized and referred to as pepsin-solubilized IC.

CM-cellulose chromatography: An aliquot (30 mg) of the pepsin-solubilized IC was dissolved in 10 ml of 0.1 M acetic acid and dialyzed exhaustively against 0.04 M sodium acetate (pH 4.8) containing 1 M urea at 4°C. The dialyzate was heated at 50°C for 30 min and chromatographed on a column (1 x 11 cm) of CM-cellulose (Whatman CM-52) at 42°C in 0.04 M sodium acetate (pH 4.8) containing 1 M urea. Elution was achieved with a linear gradient of NaCl from 0 to 0.1 M over a total volume of 200 ml at a flow rate of 60 ml per hr.

Disk electrophoresis: Samples were dialyzed against 0.01 M phosphate buffer (pH 7.4) containing 0.2 % SDS and 2 M urea, and then denatured at 50°C for 30 min. Electrophoresis of the denatured samples in SDS acrylamide was performed as described by Furthmayr and Timple (8) in 5 % gels for 5 hr at 5 mA per tube.

Gel filtration: A Sephadex G-100 column (1.6 x 95 cm) was equibrated and eluted with 0.04 M sodium acetate (pH 4.8) containing 1 M urea and 0.5 M NaCl. Lyophilized fractions from CM-cellulose column were dissolved in 1 ml of the eluting buffer and applied to the column at a flow rate of 6 ml per hr at room temperature.

RESULTS

Wet weight of granulation tissue reaches a maximum about day 8 after carrageenin injection and then decreased gradually, though some rats have a large size of granuloma pouch for more than 30 days (9). In the present

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*Abbreviations: NSC, neutral salt-soluble collagen; ASC, acid-soluble collagen; IC, insoluble collagen; SDS, sodium dodecyl sulfate.

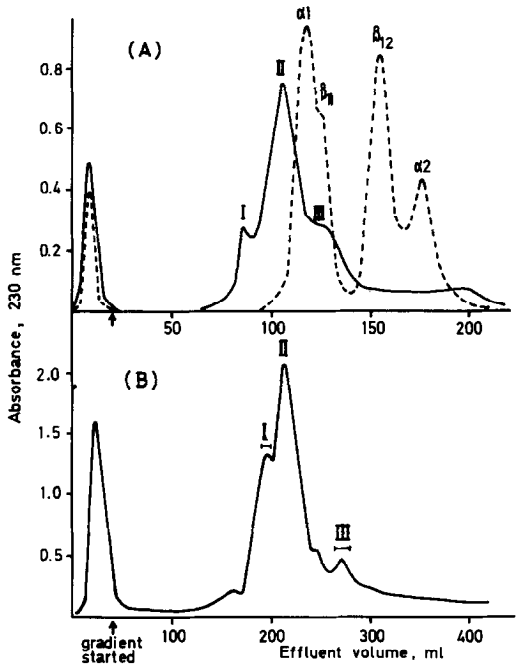

Fig. 1. CM-cellulose chromatograms of rat tail tendon collagen (A; - - - -) and pepsin-solubilized IC from 8-day-old (A; ——) and 23-day-old resorbing granulation tissues (B; ——). Pepsin-solubilized IC (145 mg) obtained from the 23-day-old resorbing granulation tissues was chromatographed on a column (1.6 x 13.5 cm) of CM-cellulose with a linear gradient of NaCl from 0 to 0.1 M over a total volume of 400 ml. Other experimental conditions are described in the text. The bars indicate fractions which were pooled for subsequent analysis (gel filtration).

experiments, wet weight of granulation tissue was 6.42 ± 0.18 g (the mean ± S.E. of 14 granulomas) on day 8, while that of the resorbing tissue was 2.90 ± 0.23 g on day 23. Granulation tissue collagen was almost insoluble, since the proportions of soluble collagens (NSC + ASC) extracted from 8- and 23-day-old granulation tissues were less than 14 % of the respective total tissue collagen contents.

The proportion of IC recovered as soluble collagen by pepsin digestion was about 30 % of IC. As shown in Fig. 1, CM-cellulose column chromatography of pepsin-solubilized IC revealed only one major peak, slightly preceding the position of the α1 peak from rat tail tendon collagen. Although the CM-cellulose chromatogram of pepsin-solubilized IC obtained from 23-day-old resorbing

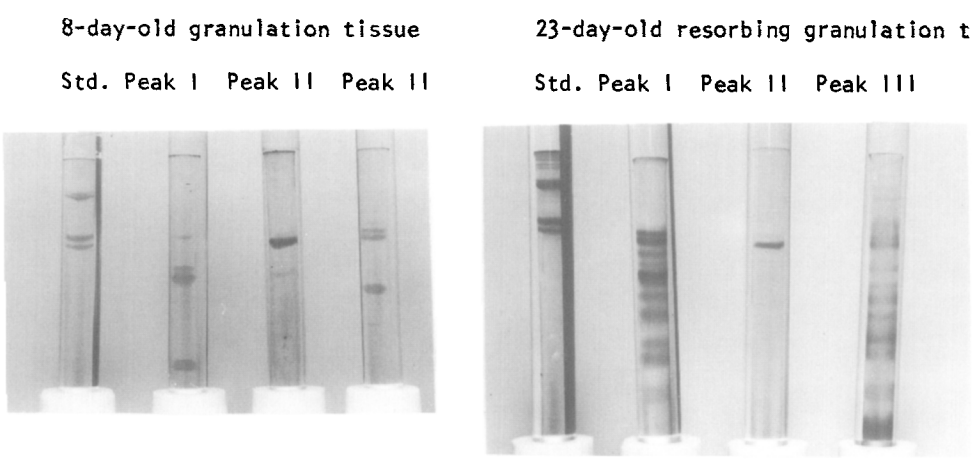

Fig. 2. SDS polyacrylamide gel electrophoresis of the materials in the peaks I, II and III isolated by CM-cellulose chromatography of pepsin-solubilized IC from 8-day-old and 23-day-old granulation tissues (Fig. 1). The standard (purified rat tail tendon collagen) is on the left.

tissue was similar to that from 8-day-old granulation tissue, SDS disk electrophoresis of each peak (peaks I - III) isolated by CM-cellulose chromatography in Fig. 1 gave different patterns (Fig. 2). As shown in Fig. 2, the main peak (peak II) was comprised of mainly one α chain, whereas shoulders (peaks I and III) contained several substances which migrated faster than α chains in SDS disk electrophoresis.

In order to characterize the nature of these substances, the fractions indicated by the bars of peaks I and III in Fig. 1 were subjected to molecular sieve chromatography. The results are shown in Fig. 3. By gel filtration on Sephadex G-100 the peaks I and III were separated into three and four peaks, respectively. Amino acid analysis was performed on each peak except for peak III-3 isolated by gel filtration in order to investigate whether these substances were derived from collagen. All the peaks contained large amounts of Hyp and Hly, and one-third of their amino acid residues were accounted for Gly, indicating that these substances were derived from granulation tissue collagen.

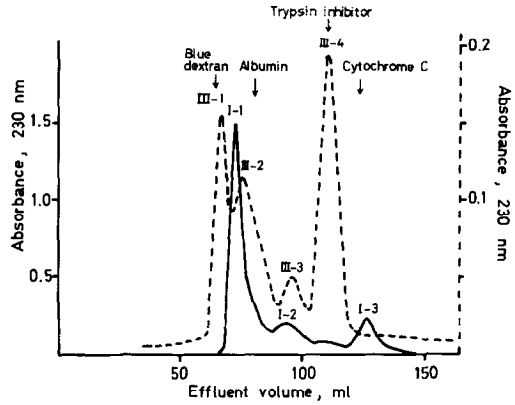

Fig. 3. Sephadex G-100 chromatograms of peaks I (——) and III (----) obtained from the CM-cellulose chromatography of pepsin-solubilized IC from 23-day-old resorbing granulation tissues (Fig. 1, B).

DISCUSSION

Pepsin-solubilized IC was purified and CM-cellulose chromatography of the pepsin -solubilized IC revealed only one major peak (one α chain) (Figs. 1 and 2), whereas SDS disk electrophoreses of soluble collagens revealed α1, α2, β_{11} and β_{12} chains (Fig. 4). It has been reported that both the types I and III collagens are present in granulation tissue (10, 11, 12) and type III collagen is less stable than type I collagen as judged by experiments with trypsin (13, 14). The possibility to be considered is that collagen composed of three identical chains (type III collagen) or α2 chain of type I collagen, or both, are selectively degraded by pepsin digestion, resulting in the production of one α chain.

Pepsin digestion of IC was done at a lower temperature (7°C) for 7 days and similar results to those shown in Figs 1 and 2 were obtained except for a decrease in the proportion of IC recovered as soluble collagen (about 18 % of IC was solubilized). As shown in Fig. 4, NSC and ASC of 23-day-old resorbing granulation tissue contained several bands other than α and β chains, on the other hand, those of 8-day-old growing tissue did not contain. These bands may be derived from degradation products of collagen, since collagen

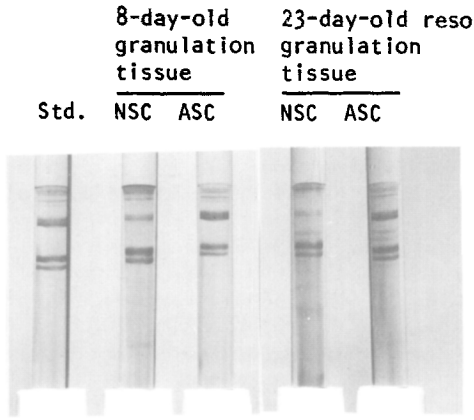

Fig. 4. SDS polyacrylamide gel electrophoresis of NSC and ASC from 8-day-old and 23-day-old granulation tissues.

synthetic activity is markedly decreased in 23-day-old granulation tissue (9). It may be argued, therefore, that the degradation products of collagen in peaks I and III (Fig. 1) are not formed during pepsin treatment, but accumulate in granulation tissue in the course of resorption of the tissue collagen fibers. This assumption was supported by the finding that the amounts of small fragments of collagen were much greater in the resorbing granulation tissues on day 23 than in the growing tissues on day 8 (Fig. 2).

Pardo and Tamayo (15) reported that collagenolytic activity was detected in partially purified normal skin collagen preparations after soluble collagen samples were kept in solution for several days or weeks at 4°C. In the present studies, however, the possibility may be ruled out that the activation of collagen degrading enzyme systems which might have been bound to the pepsin-solubilized IC occurrs during the process of extraction and purification of granulation tissue collagen, since soluble collagens (NSC and ASC) were removed from granulation tissues and the pepsin-solubilized IC was purified by the procedure described in the text.

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