FURTHER EVIDENCE FOR A TRANSPORT FORM OF COLLAGEN. ITS EXTRUSION AND EXTRACELLULAR CONVERSION TO TROPOCOLLAGEN IN EMBRYONIC TENDON

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Received 14 June 1971
Revised version received 8 July 1971

1. Introduction

Several laboratories have recently reported that cells *in vitro* synthesize polypeptide chains of collagen which are larger than α-chains [1–6]. The larger form recovered from the medium of fibroblasts in tissue culture had significant solubility properties than either α-chains or α-chains in the native three-stranded conformation of tropocollagen, and it was converted to α-chains by limited digestion with pepsin [1, 2]. The medium of the culture system, however, also contained α-chains and therefore it was not clear whether the larger form of collagen was converted to α-chains intracellularly or extracellularly. Subsequently a similar larger form of collagen with a molecular weight of 140,000 was found in organ cultures of rat calcaria [3].

We now report that the collagen synthesized and extruded by tendon cells is a molecule with individual polypeptide chains of about 125,000 daltons and that the chains in the molecule are rapidly cleaved to the size of α-chains in the extracellular matrix.

2. Materials and methods

Cells were isolated from the leg tendons of 17-day old chick embryos by digesting the tissue with trypsin and purified bacterial collagenase as described previously [4, 5]. The cells were incubated with ^{14}C-proline (New England Nuclear Corp.) in Krebs medium at 37° and were separated from the medium by centrifuging at 1500 *g* for 20 min. For the experiments with intact tendons the embryos were made lathyritic by placing 20 mg of β-aminopropionitrile fumarate (BAPN) (General Biochemicals, Chagrin Falls, Ohio, USA) on the chorio-allantoic membrane the day before the experiment, and the incubation medium contained 50 μg per ml of BAPN.

The media from the two systems were adjusted to 1% sodium dodecyl sulfate (SDS), 1% mercaptoethanol and 0.1 M sodium phosphate buffer, pH 7.4, by adding one-tenth volume of a concentrated stock solution. The tendons were vigorously homogenized in 4.0 ml of 1% SDS, 1% mercaptoethanol and 0.1 M sodium phosphate buffer, pH 7.4. The samples were further denatured by incubating at 37° for 15 hr, dialyzed against 0.1% SDS in 0.1 M sodium phosphate buffer, pH 7.4, for 24 hr at room temperature and then centrifuged at 15,000 *g* for 30 min before gel filtration in 0.1% SDS and 0.1 M sodium phosphate [7]. Aliquots of 0.2 ml were assayed directly for ^{14}C and the rest of the fractions was hydrolyzed in 6 N HCl for assay of ^{14}C-hydroxyproline [8]. Values shown are corrected for the aliquots. The recovery of non-dialyzable ^{14}C in the SDS extracts and from the agarose column was over 90%.

β-Chains of collagen (190,000 daltons) were purified from rat skin by acetic acid extraction, NaCl and ethanol precipitation [9] and then CM-cellulose

chromatography [10]. α-Chains of collagen (95,000) were obtained by acetic acid extraction of the skin of lathyritic chicks. Phosphorylase a (94,000), bovine serum albumin (BSA) (68,000) and pepsin (35,000) were obtained from Sigma Chemical Co. (St. Louis, Mo. USA). Reduced and carboxymethylated collagen from the cuticle of *Ascaris lumbricoides* (RCM) (60,000) was prepared by the procedure of McBride and Harrington [11]. The void volume (V_0) of the SDS-agarose column was determined with Dextran Blue 2000 (Pharmacia) and total volume (V_t) with ^3H_2O.

3. Results and discussion

About 70% of the ^{14}C-proline incorporated into protein by isolated tendon cells is in collagen, and about 87% of the ^{14}C-collagen synthesized by the system is recovered in the medium [4, 5]. The ^{14}C-collagen in the medium is essentially pure in that there is less than 10% contamination by other ^{14}C-labelled proteins [5].

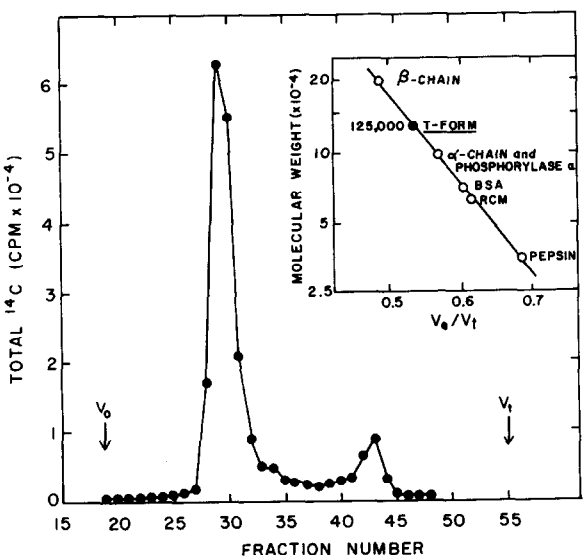

Fig. 1. Gel filtration on SDS-agarose of the medium of isolated tendon cells incubated for 120 min with ^{14}C-proline. Gel filtration was carried out at room temperature in a 1.5 × 90 cm column of 6% agarose (Bio-Gel A-5m, 200–400 mesh, Bio-Rad). The sample volume was 2.0 ml, the flow rate was about 10 ml per hr, and the fraction size was 2.0 ml. *Inset*: Calibration of the SDS-agarose column. Standard proteins were obtained as indicated in Methods.

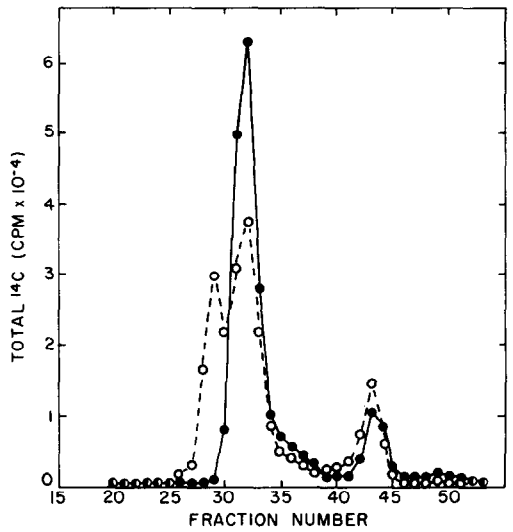

Fig. 2. Gel filtration on SDS-agarose of medium from the isolated cells after limited digestion by pepsin. The medium from 10 million cells incubated for 2 hr with 5 μCi of ^{14}C-proline was adjusted to pH 2.6 with glacial acetic acid and 100 μg per ml of pepsin (Sigma Chemical Co.) was added [2]. The sample was then dialyzed against 0.5 N acetic acid for 15 hr at 4° and then 3 hr at 15°. The samples were then treated with SDS and mercaptoethanol as described in Methods. The fraction numbers were adjusted slightly so that the V_t is directly comparable to that shown in fig. 1. Symbols: elution pattern of medium treated with pepsin (●——●); elution pattern of a mixture of about three parts of medium treated with pepsin and two parts of untreated medium (o- - -o).

Ten million tendon cells were incubated for 120 or 240 min with 2 μCi of ^{14}C-proline. Gel filtration of the medium on the SDS-agarose column indicated that about 80% of the non-dialyzable ^{14}C was eluted in a sharp, symmetrical peak about 10 fractions after the void volume of the column (fig. 1). The ratio of ^{14}C-hydroxyproline to total ^{14}C in the major peak was 51.7%. Adding 50 μg per ml of BAPN to the incubation medium had no effect on the ^{14}C incorporation or on the elution pattern.

A semi-logarithmic plot of elution volume versus molecular weight for 6 standard proteins was linear (see inset to fig. 1). The values for the globular proteins fell on the same line as the various collagens, probably because all proteins form rodlike complexes with SDS [7]. The standard curve indicated that the molecular weight of the major peak in the medium

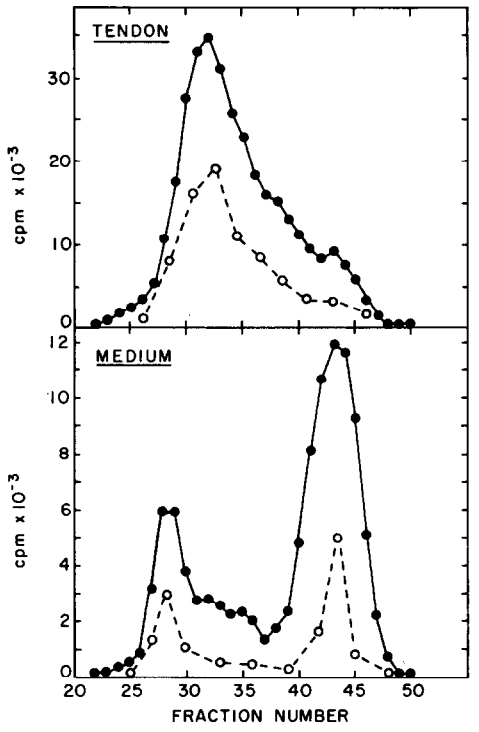

Fig. 3. Gel filtration on SDS-agarose of tendon homogenate and medium from the same incubation vessel. Incubation conditions for the tendons were as described in the text. Conditions for gel filtration were the same as in figs. 1 and 2. Symbols: Total ^{14}C (●—●); ^{14}C-hydroxyproline (○- - - -○).

was about 125,000. The same value ±5000 was obtained when the gel filtration was carried out with a coarser mesh 6% agarose (Bio-Gel A-5m, 100–200 mesh, Bio-Rad) and with Sepharose 4 B (Pharmacia).

After limited digestion with pepsin [2] the major peak recovered from the medium was eluted at the same position as α-chains (fig. 2). Since the chains were not cleaved to a size smaller than α-chains, the results suggest that three-quarters of the molecule was triple-helical during the pepsin digestion. The pepsin treatment did not significantly change the ^{14}C-hydroxyproline to total ^{14}C in the major peak. Gel filtration of a mixture of the pepsin-treated medium and untreated medium further demonstrated the shift in elution pattern.

Intact tendons from 4 lathyritic embryos were incubated for 10 min with 10 μCi of ^{14}C-proline and 50 μg per ml of BAPN, and then the label was chased by transferring the tissues to fresh medium containing 50 μg per ml of ^{12}C-proline and 50 μg per ml of BAPN. When the tissue was pulse-labeled for 10 min and chased for 240 min, most of the non-dialyzable ^{14}C and ^{14}C-hydroxyproline were eluted in the position of α-chains and no significant amounts were recovered in earlier fractions (fig. 3). The medium from the same incubation vessel, however, contained a significant amount of ^{14}C which eluted in a sharp peak with a molecular weight of about 125,000. The ratio of ^{14}C-hydroxyproline to total ^{14}C in this peak was 47.4% or about the same as in the major peak from the medium of the isolated cell system (see fig. 1). The peak of small molecular weight contained 25.0% ^{14}C-hydroxyproline and may represent degradation products.

The elution pattern of the ^{14}C in the tendons was essentially the same when the tissue was pulse-labeled for 10 min and chased for only 30 min (not shown).

Previous observations with the isolated cell system indicated that the ^{14}C-collagen in the medium was soluble under physiological conditions and that after denaturation its polypeptide chains were somewhat larger than α-chains [4, 5]. The results here demonstrate that essentially all the collagen synthesized and extruded by the isolated cells is in a molecular form in which the polypeptide chains are about 25% larger than α-chains. If the cells are left *in situ* in the tendons and the intact tendons are incubated under the same conditions, the medium contains a small amount of the larger form but most of the collagen is found in the tissue and is comprised of α-chains. These observations indicate that collagen is in the larger form as it is transported out of the cell. The conversion to tropocollagen presumably occurs in the matrix itself through cleavage by an extracellular enzyme. Since about 20 min is required for tendon cells to begin to extrude collagen [4], the observation that little of the larger form is present in tendons after 40 min, suggests that the extracellular cleavage occurs at a relatively rapid rate. Our results support the earlier suggestion [2] that the larger type of collagen can be regarded as a transport form of the molecule.

Acknowledgements

We wish to thank Miss Judy Parris and Mrs. Carolyn Rizzo for technical assistance. The work was supported in part by N.I.H. research grants FR-107 and AM-14,526 from the U.S. Publich Health Service.

References

[1] D.L. Layman, E.B. McGoodwin and G.R. Martin, Federal Proc. 29 (1970) 668.
[2] D.L. Layman, E.B. McGoodwin and G.R. Martin, Proc. Natl. Acad. Sci. U.S. 68 (1971) 454.
[3] G. Bellamy and P. Bornstein, Federal Proc. 30 (1971) 1195 Abs.
[4] P. Dehm and D.J. Prockop, Federal Proc. 30 (1971) 1196 Abs.
[5] P. Dehm and D.J. Prockop, Biochim. Biophys. Acta, in press.
[6] P. Ramaley and J. Rosenbloom, FEBS Letters, in press.
[7] J.A. Reynolds and C. Tanford, J. Biol. Chem. 245 (1970) 5161.
[8] K. Juva and D.J. Prockop, Anal. Biochem. 15 (1966) 77.
[9] D.S. Jackson and E.G. Clearly, Methods in Biochemical Analysis 15 (1967) 26.
[10] K.A. Piez, E.A. Eigner and M.S. Lewis, Biochemistry 2 (1963) 58.
[11] O.W. McBride and W.F. Harrington, Biochemistry 6 (1967) 1484.