Vol. 90, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 12, 1979 Pages 240-246

ATTACHING HUMAN FIBROBLASTS SECRETE A TYPE I PROCOLLAGEN RICH IN 3-HYDROXYPROLINE

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Received July 30,1979

SUMMARY

The predominant collagenous protein secreted during the attachment of freshly trypsinized human foreskin fibroblasts was found to be Type I procollagen. Evidence is presented that both the α_1 and α_2 chains exhibit a 3-hydroxyproline/4-hydroxyproline ratio 4-5 fold higher than that of normal Type I collagen. These findings suggest that caution should be exercised in assigning an observed increase in the 3-hydroxyproline/4-hydroxyproline ratio to the synthesis of a basement membrane type collagen.

In an earlier study of the incorporation of radioactive proline into high molecular weight proteins secreted by attaching human foreskin fibroblasts it was (1) noted that the ratio of 3-hydroxyproline to 4-hydroxyproline was elevated over the value expected for Type I collagen (2) the predominant collagen secreted by such cells. In view of recent reports (3,4) that certain cells in culture secrete a specific collagen rich in 3-hydroxyproline, we have undertaken the determination of the nature of the collagenous product of the foreskin fibroblast which is responsible for the elevated 3-hydroxyproline/4-hydroxyproline ratio in secretory material from this cell line.

METHODS

Incubation Medium (Medium A): HEPES-buffered Hank's balanced salt solution, pH 7.3 was used for serum free incubation studies (5). This salt solution was further supplemented with D-glucose, 1.00 g/l; ascorbic acid, 50 mg/l and the essential and non-essential amino acids, except proline, at the concentration of Minimal Essential Media (Gibco, Incorporated).

Maintenance of Cells: Human diploid fibroblasts were derived from an explant of newborn foreskin (1) and cultures were routinely maintained in 15 cm Falcon tissue culture dishes (6) in Dulbecco's medium (Gibco Incorporated) supplemented with 10% (v/v) fetal calf serum (Medium B) at 37°C in a humidified atmosphere of 10% CO_2 90% air.

0006-291X/79/170240-07$01.00/0

Preparation of Labeled Medium Proteins: Human fibroblasts were harvested by trypsinization and collected by centrifugation at 400 xg for 5 min. They were resuspended twice in Medium A. Experimental cultures (10 cm culture dishes) were initiated at zero time with 3-4 x 10^6 cells plus 50 μCi of L-[5-^{3}H]-proline or 10 μCi of [U-^{14}C]-proline (New England Nuclear) in 10 ml of Medium A.

After incubation at 37°C for 4 hr, the medium was collected and chilled. Protease inhibitors were added to give the following final concentrations: NEM, 10 mM; EDTA, 10 mM and PMSF, 1 mM. Fifty μg of rat skin salt soluble collagen (RSSC) were added per 10 ml of media as a carrier. The medium was then dialyzed against 3% acetic acid at 4°C. Portions of the dialyzate were subjected to either hydroxyproline and proline analysis or SDS-polyacrylamide gel electrophoresis (PAGE). Other portions were subjected to pepsin digestion (24 hr at 4°) and then either fractionated by carboxymethyl (CM) cellulose chromatography, or analyzed by SDS-PAGE. Proteins eluted from the CM cellulose were further analyzed for hydroxyproline and proline content.

SDS-polyacrylamide Gel Electrophoresis: The SDS system of Laemmli (7) was used. Gels, 5% in acrylamide, 0.07% in methylenebisacrylamide, were run at 3 ma/gel. Reduced samples contained 1% (v/v) 2-mercaptoethanol. All samples were heat denatured in a boiling water bath for 2 min prior to electrophoresis.

Carboxymethyl Cellulose Chromatography: Samples of denatured, pepsin digested collagen were chromatographed on carboxymethyl cellulose as described by Miller (8). Briefly a jacketed 0.9 x 5 cm column of Whatman CM-52 was equilibrated in 0.2 M NaAc, 1 M urea pH 4.8 at 45 ml/hr at 41°C. Just prior to heating the sample in starting buffer at 45°C for 30 min, 0.1 ml of pepstatin (0.1% solution in dimethylformamide) was added per ml of sample to inactivate any residual pepsin. Elution of the component chains was achieved with a linear NaCl gradient (0 to 0.12 M) of 200 ml total volume. One mg of RSSC was used as a carrier. Fractions of 2.4 ml were collected and those found to contain the α_1 and α_2 chains were pooled, dialyzed against 3% acetic acid and lyophilyzed. The lyophilyzed chains were then subjected to analysis for radioactive hydroxyproline and proline.

Analysis of Radioactive Proline and Hydroxyproline: For determination of radioactive hydroxyproline and proline, samples were hydrolyzed in 6 N HCl, at 110° for 24 hrs in evacuated sealed vials. After hydrolysis, the samples were analyzed on a Beckman 120 amino acid analyzer by the method of Chung and Miller with the following modifications. The initial column temperature of 30°C was shifted to 51°C after 60 min. The second buffer was initiated at this time and the run was terminated after 130 min. Ninhydrin addition was omitted and one ml fractions were collected and counted in 10 ml of ACS scintillation fluid. Control experiments showed no interference from any SDS which may have been in the sample.

RESULTS

Chemical Characterization of Media Collagens Synthesized by 4 Hr Attaching Fibroblasts: As observed previously (1) fibroblasts do incorporate radioactive proline into secreted protein during the 4 hr period which includes initial attachment of cells to the substrate.

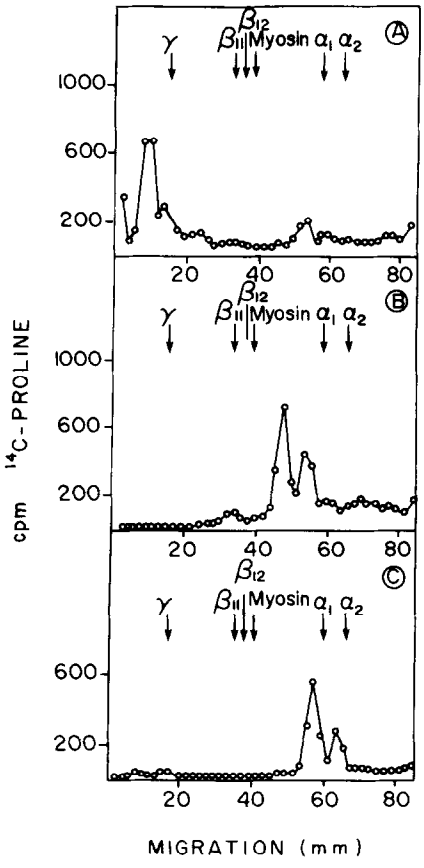

FIGURE 1 SDS polyacrylamide gel electrophoresis of [^{14}C]-proline labeled protein in the extracellular medium from 4 hr attaching fibroblasts

A. Whole medium
B. Reduced medium
C. Pepsin digested media

Myosin and rat skin salt soluble collagen were employed as molecular weight markers. They were run on parallel gels and were fixed and stained in Coomassie blue.

SDS-PAGE of the non-dialyzable media fraction indicated that the major portion of the radioactivity was associated with high molecular weight component(s). The reduced sample, however, exhibited the two lower molecular weight components, pro α_1 and pro α_2, in an approximate 2:1 ratio and with the appropriate gel position (Figure 1B) expected when the major soluble extracellular protein in Type I procollagen (10). Gel electrophoresis of pepsin digested media confirmed this, since essentially

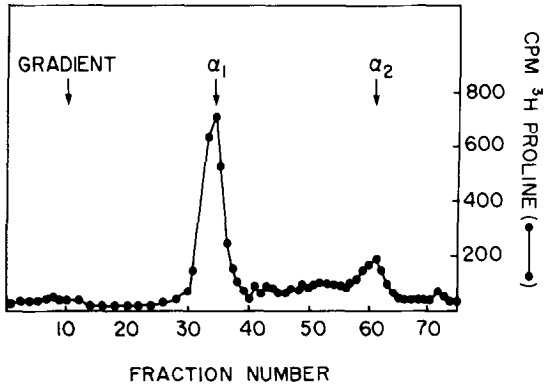

FIGURE 2 Chromatography of pepsin digested [^{3}H]-proline labeled medium of 4 hr attaching fibroblasts on carboxymethyl cellulose (Whatman CM-52)

The column was equilibrated with 0.2M (Na^+) sodium acetate, 1M urea pH 4.8. Elution was achieved with a linear salt gradient (0-0.12M NaCl) in 200 ml total volume. Fractions of 2.4 ml were collected.

(·--·) cpm [^{3}H]-proline. 0.2 ml of each fraction was counted.

complete conversion of the procollagen material to α_1 and α_2 chains characteristic of Type I collagen is a ratio of about 2:1 (Figure 1C) was obtained. In Figure 1C the very small amount of material seen near the area of γ chain migration is probably Type III collagen as it is known to be synthesized by human fibroblasts yet remains cross linked by disulfide bridges after pepsin digestion (9,11,12). Analysis of the collagen chains isolated by CM-cellulose chromatography (Figure 2) showed that both the α_1 and α_2 chains exhibited a ratio of 3-hydroxyproline/4-hydroxyproline similar to that of the whole media (Table 1), which is higher than the value of 0.01 expected for Type I skin collagen (2). Quantitation of the recovery of radioactivity during all the procedures indicated that the Type I procollagen accounts for at least 50% of the radioactive proline incorporated into secreted proteins in the media.

DISCUSSION

Freshly trypsinized human foreskin fibroblasts will attach readily to plastic surfaces in the absence of serum and within 4 hrs assume

TABLE 1

DISTRIBUTION OF INCORPORATED L-[5-^{3}H]-PROLINE AMONG THE α CHAINS OF THE TYPE I COLLAGEN SYNTHESIZED BY 4 HR. CULTURES

Sample	3-Hyp	4-Hyp	Pro	$\frac{3\text{-Hyp}}{4\text{-Hyp}}$	$\frac{\text{Hyp}}{\text{Pro}}$
Whole Media	390 ± 20	8340 ± 420	20750 ± 1040	0.05	0.42
α_1	260 ± 13	7310 ± 370	9580 ± 480	0.04	0.79
α_2	120 ± 6	2380 ± 120	2700 ± 135	0.05	0.93

a characteristic spread morphology. Electron microscopy of these cells (data not shown) confirms the observations of Witkowski and Brighton (13) that although the cells are well spread in the absence of serum, the morphology is less regular than that seen in the presence of serum. The cells survive for periods of at least 24 hrs. in the absence of serum and actively secrete macromolecules into the medium (1,5,6).

The predominant collagenous component present in the medium after 4 hrs was found to be Type I procollagen. Smaller amounts of Type III procollagen may also be present during this time interval. The higher ratio of α_1 to α_2 chains observed in chromatography as compared with electrophoresis is primarily due to relatively higher retention of α_2 on the CM-cellulose. The total yield of α_1 and α_2 chain from CM-cellulose is usually observed to be somewhat low (8). The presence of $(\alpha_1)_3$ as a substantial product of these fibroblasts does not appear likely on the basis of the α_1/α_2 ratio evident from electrophoresis (Figure 1).

These conditions, attaching cells, are somewhat different from those usually employed for studies of collagen synthesis, and it is of interest that the general pattern is similar to that observed under other conditions (10,14). The presence of the elevated levels

of 3-hydroxyproline observed previously (1) has now been shown to result in these fibroblasts, from an increased 3-hydroxylation of both the α_1 and the α_2 chain of the predominant Type I procollagen during attachment. Slightly elevated levels of 4-hydroxylation are common in cell culture systems, but elevated levels of 3-hydroxylation have not been reported in a Type I collagen. The basis for increased hydroxylation is not known, but our present understanding of collagen biosynthesis would suggest that any delay in formation of triple helix would permit further 4-hydroxylase activity, which in turn favors increased action of the 3-hydroxylase (15). The structural and functional role of 3-hydroxyproline is unknown. In any case, this observation suggests caution in assigning an observed increase in the 3-hydroxyproline/4-hydroxyproline ratio of secreted cellular proteins to the synthesis of a basement membrane type collagen (16) without specific characterization of the collagen type.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Janet Covington, Mrs. Sharon Tollefson and Mrs. Laura Ann Sanders for their excellent technical assistance. The advice of Dr. Kenneth Lembach on cell culture procedures is gratefully acknowledged. This investigation was supported by an NIH Research Grant AM 19606.

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