The Nature of the Collagen Synthesized by Cultured Human Fibroblasts

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Communicated by Francis O. Schmitt, December 9, 1970

ABSTRACT The hydroxyproline-containing proteins (hyproproteins) synthesized by cultured human fibroblasts have been partially characterized. The hyproprotein extracted from the cell layer was found to be similar to the collagen extracted from skin in the ratio of hydroxyproline to proline, chain composition, solubility, and resistance to proteolytic digestion.

The hyproproteins isolated from the medium were different. About 20% of the peptide-bound hydroxyproline was found in randomly coiled chains. The α2 chains were present in considerable excess over the α1 chains, suggesting that the α2 chain may be synthesized in quantities greater than required to form a collagen molecule with a chain composition (α1)₂α2. The remaining medium hyproprotein appeared to be an unusual form of native collagen which, unlike typical native collagen, was soluble under physiological conditions. This hyproprotein did not yield α chains when denatured and contained material that had a molecular weight greater than α chains. A similar size distribution was observed in the protein synthesized in the presence of β-aminopropionitrile, a specific inhibitor of collagen cross-linking. After treatment with pepsin, typical α1 and α2 chains were obtained from the protein in a 2:1 ratio. Since the medium protein is soluble and has properties different from the typical collagen molecule, it may represent a modified form that functions in the transport of collagen from the cell to the fiber.

In earlier studies, the synthesis of collagen by fibroblasts and other cells in vitro was followed by measuring nondialyzable hydroxyproline, a relatively unique marker for collagen, and by observing extracellular collagen fibrils with the electron microscope (1–6). It has been reported that only about half of the nondialyzable hydroxyproline formed by human fibroblasts was deposited in the cell layer, while the remainder was found in the medium (4).

We have observed that most of the hydroxyproline-containing protein (hyproprotein) associated with the cell layer was insoluble in cold neutral salt solution. However, when hyproproteins were synthesized in the presence of β-aminopropionitrile, a specific inhibitor of collagen cross-linking, a hyproprotein could be readily extracted and characterized. Its properties have been compared with the hyproproteins secreted into the medium.

MATERIALS AND METHODS

Fibroblasts were obtained from biopsies of normal human skin by standard techniques and grown in plastic culture flasks containing 20 ml of Dulbecco-Vogt medium supplemented with 300 μg/ml of glutamine, 50 μg/ml of ascorbic acid, 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 10% fetal calf serum. Cultures of confluent fibroblasts were labeled with [U-14C] hydroxyproline (2 μCi/ml) or both [U-14C] hydroxyproline (1 μCi/ml) and [U-14C] glycine (2 μCi/ml) in 15 ml of Eagle’s basal medium. In addition, certain flasks received 50 μg/ml of β-aminopropionitrile·HCl (BAPN) to block the cross-linking of collagen. After 24 hr, the medium was decanted from the cell layer and dialyzed exhaustively against the appropriate buffers used for chromatography. The cell layer was rinsed with unlabeled medium, removed from the flask surface in 20 ml of 1 M NaCl-0.05 M Tris (pH 7.4), and extracted for 2 days at 4°C. The soluble proteins were separated from the insoluble material by centrifugation at 10,000 rpm for 10 min, then dialyzed against the appropriate buffers used for chromatography. Carrier collagen (20 mg) prepared from the skin of lathyritic rats (8), was added to the medium or cell-extract samples to serve as an internal standard for measuring the recoveries of the radioactive α1 and α2 chains, as well as to mark their position on elution. In certain experiments, medium or cell extract samples were brought to 0.5 M acetic acid and incubated with pepsin (9) (100 μg/ml) for 6 hr at 15°C. The pH of the samples was adjusted to 8.0 with 1 M NaOH and allowed to stand for at least 20 min to irreversibly inactivate pepsin (10). CM-cellulose chromatography was performed on the heat-denatured proteins as described by Pies et al. (8). Molecular-sieve chromatography was performed in 1 M CaCl₂-0.05 M Tris·HCl (pH 7.4) on Bio-Gel A-1.5, 112 × 1.5 cm columns (11). Radioactivity was assayed with a liquid scintillation spectrometer by standard counting techniques (12, 13). [14C] hydroxyproline and [14C] glycine were analyzed after hydrolysis in 6 N HCl at 110°C for 24 hr with an amino acid analyzer equipped for continuous monitoring of the radioactivity in the column effluent, or by chemical procedures (14).

RESULTS

The amount of radioactive proline incorporated into proteins (expressed as nondialyzable proline and hydroxyproline) by confluent cultures of fibroblasts is shown in Table 1. About half of the radioactive hydroxyproline formed in 24 hr was found in the medium. Of the peptide-bound hydroxyproline that precipitated with the cell layer, only about 20% could be extracted with cold neutral salt solution. However, 70–80% of the protein was extractable when synthesized in the presence of BAPN. The addition of BAPN to the cultures did not alter the amounts of peptide-bound...
[14C]hydroxyproline or [14C]proline formed or the distribution of radioactive peptide-bound amino acids between the cell layer and medium.

Nature of the hyproprotein extracted from the cell layer

The hyproprotein extracted from the cell layer was further characterized to determine its relationship to the collagen extracted from human skin. Typical radioactive profiles obtained by CM-cellulose chromatography of the salt-soluble hyproprotein extracted from the cell layer and the medium are shown in Fig. 1. As expected, in those cultures labeled in the absence of BAPN, only a small amount of the total radioactivity incorporated into proteins chromatographed as α1 and α2 (not shown). Little additional radioactive protein was extracted from the residue of the cell layer by a subsequent extraction with 0.5 M acetic acid (not shown). However, when fibroblasts were labeled in the presence of BAPN, two major radioactive peaks were obtained that chromatographed in the approximate positions of the α1 and α2 chains of the denatured carrier collagen. Measurement of the area under the radioactive α1 and α2 peaks indicate that, as with the denatured rat skin collagen, the radioactive α1 and α2 chains were present in a ratio of 2:1. Both radioactive peaks were desalted and examined by molecular sieve chromatography, and found to be identical in elution volume to authentic α chains obtained from rat skin collagen (Fig. 2, only α2 is shown). Proline and hydroxyproline radioactivity in the α1 and α2 peaks was determined to be in the ratio of 1.44 and 1.40, respectively. On the basis of these findings, and the similarity to other collagens, the radioactive peaks were identified as α1 and α2 chains.

While the labeled human α1 chain cochromatographed with the rat a1, the human α2 was eluted later than the rat α2 from CM-cellulose. However, both the labeled α1 and α2 peaks cochromatographed with human carrier α1 and α2 chains (not shown).

To determine whether the α chains extracted from the cell layer were present in the triple-helical collagen structure or as randomly coiled α chains, the cell extract was incubated with pepsin. Pepsin rapidly degrades denatured collagen while the native protein, with the exception of the nonhelical telopeptide regions, is resistant to such attack (9). No significant differences were noted in the radioactive profiles of the protein incubated with or without pepsin (not shown) under conditions in which the denatured protein would have been degraded to small dialyzable peptides. We conclude that the hydroxyproline-containing protein extracted from the cell layer was present as typical native molecules composed of two α1 and one α2 chains.

![Fig. 1. CM-cellulose elution patterns of the salt-soluble radioactive protein extracted from the cell layer, and a portion of the radioactive proteins isolated from the medium, of fibroblasts labeled in the presence of 50 μg/ml of BAPN (top). Pattern of absorbance of denatured rat skin collagen used to mark recoveries and position of the α1 and α2 chains (bottom).](image-url)
as carrier, were also dialyzed against 0.02 M NaHPO₄ adjusted to pH 7.4 with HCl. The precipitate was isolated by centrifugation, dissolved in acetate buffer, and chromatographed on CM-cellulose. The supernatant fluid was dialyzed against appropriate buffers and also chromatographed. Almost all of the carrier collagen from rat skin precipitated, as judged by chromatography of the denatured protein (not shown). However, less than 20% of the radioactive α chains that were eluted by the salt gradient in the original sample of medium precipitated. The remaining α chains were found in the supernatant fluid. These results indicate that randomly coiled α chains account for a portion of the hyproprotein in the medium.

A molecular form of collagen soluble under physiological conditions

As mentioned earlier, only about 20–30% of the nondialyzable hydroxyproline in the medium could be accounted for as α1 and α2 chains. The remaining radioactive, nondialyzable, hydroxyproline was not eluted from the CM-cellulose column by the salt gradient, but was removed by 0.4 M NaOH–1 M NaCl.

To determine the size and heterogeneity of this hydroxyproline-containing material, medium from cells labeled for 24 hr was lyophilized, redissolved in 3 ml of 1 M CaCl₂–0.05 M Tris·HCl (pH 7.4), and chromatographed with 5 mg of carrier collagen on 8% agarose. A considerable portion of the radioactive material emerged before the α chain marker (Fig. 3, top). [¹⁴C]hydroxyproline analysis of the desalted, hydrolyzed fractions indicated that about 40–50% of the nondialyzable hydroxyproline was present in high molecular weight material that emerged before the α chain marker (Fig. 3, top). Another portion of the medium was treated with pepsin under conditions in which the native structure is not attacked. Fractionation of the sample on the same agarose column after incubation with pepsin demonstrated that most of the radioactive hydroxyproline now emerged with the α chain marker (not shown, but similar to chromatogram of cell extract). Since most of the radioactive hydroxyproline-containing protein in the medium was not degraded to small peptides, it must have been present in native molecules.

Another portion of the medium was chromatographed on CM-cellulose to determine the relative amounts of the α1 and α2 chains obtained after proteolytic digestion with pepsin. The amount of radioactivity chromatographing on CM-cellulose as α chains was found to be markedly increased after pepsin treatment. The ratio of α1 to α2 was the same (Fig. 4) as that observed with the collagen extracted from the cell layer. Less than 10% of the nondialyzable hydroxyproline remained bound to the CM-cellulose column. The radioactive proteins in the α1 and α2 peaks were found to migrate as α chains on a molecular sieve. Moreover, the ratio of proline to hydroxyproline in the α chains was similar to that found for human α chains.

The possibility that cross-linking or nonspecifically-associated mucopolysaccharides altered the properties of the collagen molecules secreted into the medium was investigated. Cross-linking via lysine-derived aldehydes cannot account for the unusual chromatographic properties of the collagen that is present in the medium in soluble form, since the yield of labeled α chains was not increased when the soluble collagen was synthesized in the presence of BAPN. Hyaluronidase or

Nature of the hydroxyproline-containing proteins in the medium

The CM-cellulose elution pattern of the radioactive proteins from the medium of fibroblasts labeled in the presence of BAPN is also shown in Fig. 1. Again two peaks were eluted from the ion-exchange column in the approximate positions of the α1 and α2 chains of the carrier collagen, but an α1:α2 ratio of 2.1 was not observed. Usually the radioactivity in the α2 peak exceeded that in the α1 peak by 2–3-fold, although occasionally the amounts of radioactivity in these peaks were found to be about equal. The radioactive material in each peak was desalted and examined by electrophoresis and molecular-sieve chromatography. By these techniques, this material was indistinguishable from the α1 and α2 chains of denatured skin collagen. The ratio of radioactive proline to hydroxyproline in the α1 and α2 chains was found to be 1.33 and 1.52, respectively. However, only about 20–30% of the nondialyzable [¹⁴C]hydroxyproline in the medium could be accounted for in these peaks. The remaining hydroxyproline-containing protein was not eluted from the CM-cellulose column with the salt gradient (see below).

To determine whether these α chains were present in the medium in the triple-stranded collagen structure or as randomly coiled polypeptide chains, an additional method was devised to distinguish native from denatured collagen. Although native collagen in solution precipitates when dialyzed against 0.02 M NaHPO₄ (15), we have found that denatured collagen (gelatin) does not precipitate under similar conditions. As judged by this test, native collagen was found not to denature when incubated in the medium used for cell culture for periods of up to 24 hr at 37°C. Under the same conditions, no renaturation of denatured collagen was found to occur.

Samples of radioactive medium from cultures of confluent fibroblasts, to which 20 mg of rat skin collagen had been added
chondroitinase ABC treatment of the medium, under conditions where these enzymes degrade the known mucopolysaccharides synthesized by cultured fibroblasts, did not increase the yield of α chains.

DISCUSSION

It has been shown that cultured human fibroblasts synthesize a number of forms of collagen. Most of the collagen that precipitates with the cell layer, presumably as fibrils, is largely insoluble in solvents used to extract collagen from skin. This insolubility is due to cross-linking, via lysine-derived cross-links, since the extractability of the collagen synthesized in the presence of BAPN, a specific inhibitor of cross-linking, was markedly increased. Although high concentrations of BAPN (4–5 mM) have been reported to be cytotoxic or to enhance collagen synthesis (16, 17), the concentration of BAPN used in this study (about 0.5 mM) did not affect the amount of collagen synthesized or general protein synthesis (as judged by peptide-bound [14C]proline and [14C]hydroxyproline formation). Moreover, the distribution of the peptide-bound hydroxyproline between the cell layer and the medium was not significantly altered. Since current evidence (18) indicates that BAPN inhibits cross-linking by inactivating lysyl oxidase (the enzyme responsible for the formation of the lysine-derived aldehydes), it is concluded that cultured human fibroblasts also cross-link collagen.

The collagen extracted from the cell layer was found to be similar to the typical collagen extracted from skin. We presume that this collagen was polymerized and deposited as extracellular fibrils between the fibroblasts in the cell layer.

The fibroblasts were also found to secrete collagen into the medium. A portion existed in the medium as randomly-coiled α chains rather than in the triple-helical collagen structure. Interestingly, more α2 than α1 chains were recovered in this fraction. Although human skin collagen has a chain ratio of (α1)2α2, the relative amounts in which the α1 and α2 chains are synthesized is not known. It is possible that the α2 chains are synthesized in quantities greater than are required to form the typical collagen molecule.

The remaining hyproprotein in the medium was found to be resistant to proteolytic digestion. This would indicate that it has a triple-helical collagen structure. Unlike the collagen extracted from the cell layer, this collagen was soluble under culture conditions and did not yield α chains when denatured. However, after incubation with pepsin, the protein was found to chromatograph as α chains. The chain ratio and proline to hydroxyproline content of this collagen from the medium was similar to that of the collagen extracted from the cell layer.

![Graph](image-url)
This form of collagen was only found in significant amounts in the medium, since pepsin treatment of the cell extract did not increase the yield of $\alpha$ chains.

Since proteolytic digestion with pepsin or other proteases is known to cleave the nonhelical amino-terminal end of the collagen molecule that contains the intramolecular cross-links (9, 19, 20), these studies would suggest that the medium-soluble collagen was intramolecularly cross-linked. However, it is unlikely that the behavior of this collagen on a molecular sieve column can be attributed to lysine-derived intramolecular cross-links, since the chromatographic behavior of the protein was the same whether synthesized in the presence or absence of BAPN. Treatment designed to remove any associated mucopolysaccharides did not change the chromatographic properties of the collagen in the medium, indicating that a protein–polysaccharide complex is not involved.

On the basis of the insolubility of collagen under physiological conditions, Schmitt (21) postulated that collagen was secreted from the cell as a soluble precursor macromolecule which was converted by an enzyme to a form capable of polymerizing and aggregating into typical collagen fibrils. Although such an enzyme has not yet been found, there is evidence that a soluble hydroxyproline-containing protein, with a molecular weight greater than 150,000, occurs in blood in a structure resistant to digestion by proteases but not collagenase (22). Extracts of collagen from guinea pig skin have also been found to contain a small amount of collagen which is soluble under physiological conditions (23). It is possible that these soluble forms of collagen function in the transport of collagen from cell to fiber. Under in vitro conditions the utilization of the protein may be inefficient, thus accounting for its accumulation in the medium. The nature of the transport form has yet to be identified.

This work represents a partial fulfillment of the requirement for the Ph.D. degree at George Washington University and has appeared in abstract form [Fed. Proc., 29, 668 (1970)].