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RAPID DEGRADATION OF NEWLY SYNTHESIZED COLLAGEN BY PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

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SUMMARY

Greater than 95% of the total radioactive hydroxyproline synthesized by adult rat hepatocytes during the first 24 to 48 hours in monolayer culture is soluble in 5% TCA. During the next 24-hour culture period, the amount of TCA-soluble hydroxyproline is reduced to approximately 40%. Likewise, rat hepatoma cells (HTC) also produce a significant amount of TCA-soluble hydroxyproline (48%). These findings suggest that hepatocytes not only have the capacity to synthesize collagen, but also have the ability to degrade this fibrous protein.

Liver fibrosis is a pathologic condition characterized by accumulation of abnormal amounts of collagen and often associated with impaired hepatic function. Although it has been assumed that the exclusive source of hepatic collagen is liver fibroblasts (1) or their resting precursors (perisinusoidal cells of Ito) (2), we have suggested that the hepatic parenchymal cell may also synthesize collagen. We demonstrated that prolyl hydroxylase, a key enzyme in collagen synthesis is a constituent of freshly isolated adult rat hepatocytes and also of primary, non-proliferating monolayer cultures of these cells (3,4). In addition, these cultures synthesize collagen measured as proteins that contain hydroxyproline, are susceptible to digestion by purified bacterial collagenase and exhibit chromatographic profiles identical to carrier collagen on agarose A-15M (5). The net amount of collagen formed during the first 24 hours of incubation of the hepatocytes is low by comparison with the liver in vivo (3). We have investigated

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this discrepancy and now report that in hepatocyte cultures, newly synthesized collagen apparently undergoes rapid catabolism at rates over and above those observed in specialized collagen-producing cells.

MATERIALS AND METHODS

Primary cultures of rat hepatocytes were prepared from livers of male Sprague-Dawley rats (200-250 gm). In brief, the livers were perfused *in situ* with calcium-free salt solution followed by 0.03% crude bacterial collagenase (Type I, Sigma Chemical Company, St. Louis, MO) in modified Waymouth MB752 medium (3). The isolated hepatocytes were separated from non-parenchymal cells by repeated centrifugation and resuspension in fresh culture medium. The hepatocytes were suspended in culture medium (1×10^6 cells per ml) and 3 ml were placed in 60 mm plastic culture dishes precoated with rat tendon collagen. In one study, hepatocytes were isolated without the use of bacterial collagenase according to the procedure of Rachman et al. (6). In this experiment, a partial hepatectomy was performed four days before the liver was perfused with calcium-free Waymouth MB752 culture medium containing 27 mM sodium citrate and 1% bovine serum albumin. The cell suspension was filtered through a 200 μ mesh sieve and hepatocytes were isolated after several centrifugations and repeated washings with culture medium. For studies of rat hepatoma cultures, HTC cells (a gift of Dr. R. Ivorie, San Franciso CA) were maintained in monolayer culture using published methods (7).

For measurement of hydroxyproline synthesis, the culture medium was replaced with fresh medium supplemented with ferrous sulfate (1×10^{-6} M), ascorbic acid (1.4×10^{-3} M), 1×10^{-3} M proline and [^{14}C]-proline, (New England Nuclear, Boston, MA). To eliminate possible trace contamination of commercial preparations of [^{14}C]-proline by [^{14}C]-hydroxyproline, the isotope was subjected to chromatography on a 1 x 30 cm Dowex AG50W-X8 ion exchange column using 1N HCl as eluant (8). The culture dishes contained 3×10^6 hepatocytes and the cells were incubated with [^{14}C]-proline (5 μCi) for 4 to 24 hours depending upon the particular experiment. The incubation was terminated by rapidly freezing the cultures. Later the cells and medium were thawed, pooled and placed on ice. The samples were disrupted by brief sonication and radiolabeled protein was precipitated by addition of trichloroacetic acid (TCA) to give a 5% final concentration. The insoluble material was centrifuged and washed three times with 5% TCA in the cold. The precipitated proteins and the TCA washes (containing the non-protein radioactivity) were hydrolyzed separately in 6N HCl for 14 hours at 15 psi, 120°C. The L-4-[^{14}C]-hydroxyproline was cleanly separated from all other radiolabeled amino acids by the use of a JEOL 5AH amino acid analyzer equipped with stream splitter and fraction collector. The amount of radioactive hydroxyproline was then quantitated using a Beckman LS-9000 liquid scintillation spectrometer. A blank formed by adding [^{14}C]-proline to unlabelled 48-hour old cultures showed formation of less than 0.5% of the L-4-[^{14}C]-hydroxyproline formed in cultures incubated for 24 hours in the presence of the isotope. Results are reported as the fraction of total [^{14}C]-hydroxyproline present in a TCA-soluble form expressed as percent and total radioactivity per culture plate.

RESULTS

We have previously reported that in hepatocyte cultures incubated for 24 hours with [^{14}C]-proline, 90 to 95% of the total radioactive hydroxy-

TABLE 1. Distribution of radioactive hydroxyproline in hepatocyte and hepatoma cultures.

Cultures Tested	^{14}C-Hydroxyproline dpm/culture plate*	% of Total
I. Hepatocyte Monolayers (24-48 hours)		
TCA soluble	12,636	97
TCA insoluble	403	3
II. Non-Enzymatically Prepared Hepatocytes (0-24 hours)		
TCA soluble	6,400	>95
TCA insoluble	<100	-
III. Hepatocyte Monolayers (48-72 hours)		
TCA soluble	2,360	37
TCA insoluble	4,140	63
IV. Hepatoma Cells (HTC)		
TCA soluble	2,170	48
TCA insoluble	3,434	52

*The values represent total ^{14}C-hydroxyproline per 60 mm culture plate. In experiment I, 11 plates were pooled and analyzed; II, 10 plates; III, 6 plates; and IV, 2-75 cm^2 flasks, equivalent to 5.3 - 60 mm plates.

proline produced was present as dialyzable or TCA-soluble material (3). Examining this phenomenon in greater detail, we found that when hepatocyte cultures were incubated for the first 24 hours in standard medium followed by a second 24-hour period in medium containing [^{14}C]-proline, approximately 97% of the total [^{14}C]-hydroxyproline formed was soluble in 5% TCA (Table 1, I). In contrast, when the hepatocytes were permitted to adapt to conditions of culture for 48 hours, and then exposed to ^{14}C-proline for a subsequent 24-hour period, the percent of soluble radioactive hydroxyproline was reduced to 37% (Table 1, III). It is possible that retention by the cultures

of the bacterial collagenase used to prepare isolated hepatocytes (9) may account for the higher proportion of soluble hydroxyproline production in younger cultures. Nevertheless, hepatocytes isolated without the use of proteolytic enzymes also degraded more than 95% of the collagen (Table 1, II). Moreover, in rat hepatoma cells (HTC) maintained in monolayer culture and never exposed to bacterial collagenase, approximately half of the hydroxyproline produced was soluble (Table 1, IV), a value similar to that measured in 48-hour-old primary hepatocyte cultures. The total ^{14}C-hydroxyproline formed between 24 to 48 hours of incubation of enzymatically prepared hepatocytes in culture (Table 1, I) was approximately two times higher than that formed in non-enzymatically prepared hepatocytes (Table 1, II) or in standard cultures between 48 to 72 hours of incubation (Table 1, III). This difference may be due to changes in isotope uptake or in amino acid pool sizes associated with the bacterial collagenase treatment of hepatocytes because altered membrane transport characteristics for amino acids and other small molecules have been reported in hepatocytes during the initial periods following isolation and establishment in monolayer culture (10-12).

The time course of rapid production of newly synthesized hydroxyproline was examined by adding ^{14}C-proline to 48-hour-old hepatocyte cultures. Total ^{14}C-hydroxyproline was formed in direct proportion to time during the next 24 hours of incubation (Figure 1A). However, during the first 8 hours following the addition of [^{14}C]-proline to the cultures, all of the [^{14}C]-hydroxyproline formed was soluble in 5% TCA and none could be detected in the precipitated proteins (Figure 1B). With longer periods of exposure of the cells to the isotope, the proportion of total radioactive hydroxyproline present in a TCA-soluble form gradually declined to 50% and lower (Figure 1B).

DISCUSSION

Several laboratories have suggested that newly synthesized collagen is degraded far more rapidly than is mature extracellular collagen (13-19). For

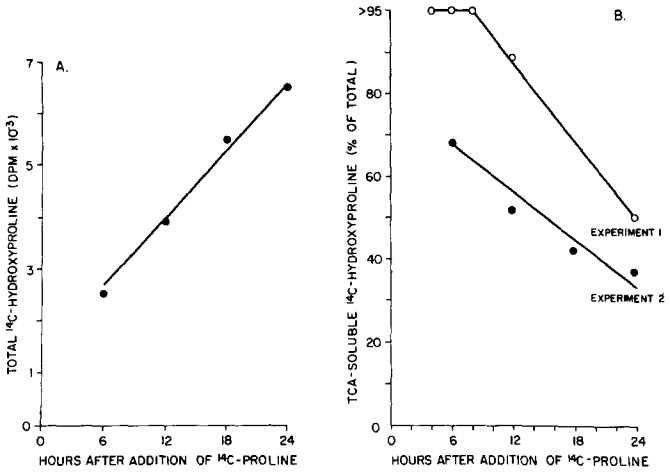

Fig. 1. A: ^{14}C-Hydroxyproline Formation in Hepatocyte Monolayers. ^{14}C-proline was added to 48-hour hepatocyte cultures and the total ^{14}C-hydroxyproline was determined at 6, 12, 18 and 24 hours; 5-60 mm confluent plates were analyzed at each time point and the results are expressed as total ^{14}C-hydroxyproline per culture plate. B: Production of TCA-Soluble ^{14}C-Hydroxyproline. ^{14}C-proline was added to 48-hour hepatocyte cultures and the percent TCA-soluble ^{14}C-hydroxyproline was determined at the indicated time points. In experiment 1, 6 plates were analyzed at each point and in experiment 2, 5 plates were analyzed at each point.

example, within minutes after addition of ^{14}C-proline, approximately 30% of the ^{14}C-hydroxyproline synthesized by rabbit lung explants or by human fetal lung fibroblasts is present as the free amino acid or in small peptides (13,14). Similar observations have been made with human normal skin and osteogenesis imperfecta fibroblasts (15), 3T6 mouse fibroblasts (16), embryonic lens (17), skin (18) and limb buds (19). The formation of TCA-soluble hydroxyproline is maintained at a constant fraction of the total hydroxyproline for as long as 24 hours after the isotopic is added to these systems. It does not appear that rapid degradation of newly synthesized collagen is an artifact of explant and cell culture systems because animals (19,20) and humans (21) injected with radioactive proline promptly produce labeled hydroxyproline in the form of small peptides. The exact mechanisms involved in the rapid formation of the hydroxyproline-containing peptides are not known. It has been argued that this reflects

rapid collagen degradation occurring intracellularly because extracellular collagenase activity was not detected (13,14). If this concept is correct, then the rapid degradation process might involve incomplete or incorrectly translated collagen chains or extra chains which did not participate in triple helix formation of the completed collagen molecule as suggested by Bienkowski, et al. (13).

If it is assumed that at least some of the soluble hydroxyproline arises from collagen, then our studies show that the liver parenchymal cell, like specialized collagen-forming systems, has the capacity to degrade newly synthesized collagen. Several differences between the connective tissue systems described above and the hepatocyte in culture should be noted. First, the extent of apparent collagen degradation did not remain constant but, instead, declined with increasing age of the hepatocyte cultures (Table 1). Although it is possible that excessive collagen degradation during the initial period of hepatocyte culture is due to residual bacterial collagenase, we have been unable to detect this enzyme after the first 24 hours of incubation (9). Moreover, hepatocyte cultures prepared without the use of proteolytic enzymes (Table 1, II) and HTC hepatoma cultures never exposed to the bacterial collagenase (Table 1, IV) produce significant quantities of TCA-soluble hydroxyproline, whereas only 30% of the total hydroxyproline formed by fibroblasts is soluble (13,14). If rapid collagen degradation provides "quality control" to ensure the production of structural and functional collagen, then the hepatocyte may synthesize increased amounts of "defective" collagen. Alternatively, the hepatocyte may synthesize a form or forms of collagen more susceptible to rapid intracellular or extracellular degradation than are the species produced by fibroblasts.

A second difference is that fibroblasts produce soluble hydroxyproline at a constant rate (approximately 30%) throughout an incubation period of 24 hours (13,14). In contrast, hepatocytes in culture produce TCA-soluble ^{14}C-hydroxyproline at a progressively decreasing percent of the total

hydroxyproline formed (Figure 1B). This phenomenon may reflect compartmentalization of the isotope preferentially into pools serving synthesis of rapidly appearing soluble hydroxyproline. An alternative possibility is that rapid degradation of collagen may be affected by changes in the composition of the culture medium due to cell metabolism. If this is correct, then this culture system may be most useful to study the mechanism involved in the rapid release of hydroxyproline-containing peptides and the metabolic factors which control this process.

Rapid degradation of collagen by the hepatocyte may be relevant to the pathogenesis of hepatic fibrosis. It is possible that under normal conditions there accumulates only a small amount of hepatocyte-derived collagen required for maintaining the micro-environment of these cells. If, following hepatocyte injury, local metabolic conditions reduce collagen degradation, then increased collagen deposition would occur. We are currently testing this idea in hepatocyte cultures by examining the effects of such therapeutic agents as steroids on collagen degradation.

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