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Kinetics of processing of type I and type III procollagens in fibroblast cultures

(pulse-chase/gel electrophoresis)

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ABSTRACT **The rates of conversion of types I and III procollagens to their respective collagens were compared in seven fibroblast culture systems of murine or human origin. During 24 hr of radioactive labeling or after shorter pulses followed by chases of 20–48 hr, no evidence was obtained for conversion of radiolabeled type III procollagen to insoluble collagen. In the same cultures and under the same conditions, native collagen was generated from radiolabeled type I procollagen. There was no evidence for proteolytic degradation of type III procollagen during the chase experiments. The results are ascribed to a lack of availability of the enzymes required for processing of type III procollagen.**

Soluble secreted precursors (procollagens) have been demonstrated for each of the four major genetic types of collagen (see refs. 1 and 2 for general reviews). Type I procollagen is converted to collagen by enzymatic excisions of nonhelical peptides from the amino and carboxy termini of the molecule (3, 4). It is presumed that type III procollagen is converted to collagen by analogous reactions, but it is not known if different enzymes are required for conversion of type I and III procollagens.

Cultured fibroblasts synthesize and secrete both type I and type III procollagens (5, 6). Accordingly, such cultures were used in experiments that compared the rates and modes of processing of the two precursors. Data from pulse-chase experiments showed that only type I procollagen was converted to insoluble collagen during the intervals used. The significance of these data for the *in vivo* metabolism of the two collagen types is discussed.

MATERIALS AND METHODS

Cells, Culture Conditions, and Radioactive Labeling. The following fibroblast lines or strains were used: mouse lines Balb/3T3, Swiss 3T3, and Swiss 3T6; three normal human adult skin fibroblast strains; and one human fetal skin fibroblast strain. The cells were grown in plastic petri dishes (100-mm diameter) in Dulbecco-Vogt medium (Grand Island Biologicals) supplemented with 10% fetal calf serum and sodium ascorbate (75 μg/ml). Experiments were performed 1 week after the cultures reached confluence. The cell layers were washed with serum-free medium and incubated for various intervals with fresh medium containing 1% fetal calf serum, ascorbate, 3-aminopropionitrile fumarate (50 μg/ml; Aldrich Chemical Co., Inc.), and 25 μCi each of [^{3}H]glycine and L-[^{3}H]proline (New England Nuclear).

Processing of Media and Cell Layers. All procedures were done at 4° unless otherwise noted. The culture medium was collected and any cell debris was removed by centrifugation. Acetic acid was added to 0.5 M, the solution was equally divided, and pepsin (100 μg/ml) was added to one sample. Both samples were incubated for 5 hr at 15°, neutralized with NaOH, exhaustively dialyzed against water, and lyophilized.

Cell layers were scraped into 10 ml of 0.5 M acetic acid and extracted on a rotary shaker for 18 hr. The suspension was centrifuged at 27,000 × *g* for 15 min. The supernate was divided and treated with pepsin as described above, and the samples were dialyzed and lyophilized. The pellet from the centrifuged cell layer extract was suspended in 0.5 M acetic acid and incubated with pepsin (200 μg/ml) at 4° for 18 hr and then at 15° for 3 hr. The suspension was centrifuged and the supernate was neutralized with NaOH, exhaustively dialyzed against water, and lyophilized.

Lyophilates were taken up in buffer (0.01 M phosphate, pH 7.2/1% sodium dodecyl sulfate/0.5 M urea) and heat denatured (60°, 1 hr) before application to cylindrical 5% polyacrylamide gels. For reduction, samples were made 2% in 2-mercaptoethanol before heat denaturation and application to gels. Electrophoresis and measurement of radioactivities in gel slices were performed as described (7). Electrophoresis in a 5–10% linear gradient polyacrylamide slab gel was performed by the method of Studier (8). The ^{3}H-labeled proteins in the slab gel were detected by the method of Bonner and Laskey (9).

RESULTS

In cultures of Balb/3T3 fibroblasts, approximately 75% of the collagen synthesized is type I; the remainder is type III (10). Soluble procollagens of both types accumulate in the culture medium. Chase experiments were performed to determine if these procollagens in the medium are converted to collagen at comparable rates. In a representative experiment, a confluent culture was incubated with L-[^{3}H]proline and [^{3}H]glycine for 18 hr; the medium was collected, supplemented with a 10,000-fold excess of unlabeled proline and glycine, and in-

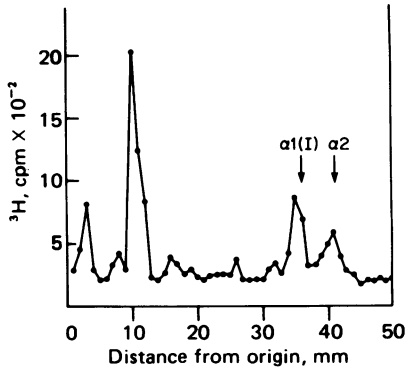

FIG. 1. Gel pattern of medium from Balb/3T3 cultures labeled for 18 hr with L-[^{3}H]proline and [^{3}H]glycine.

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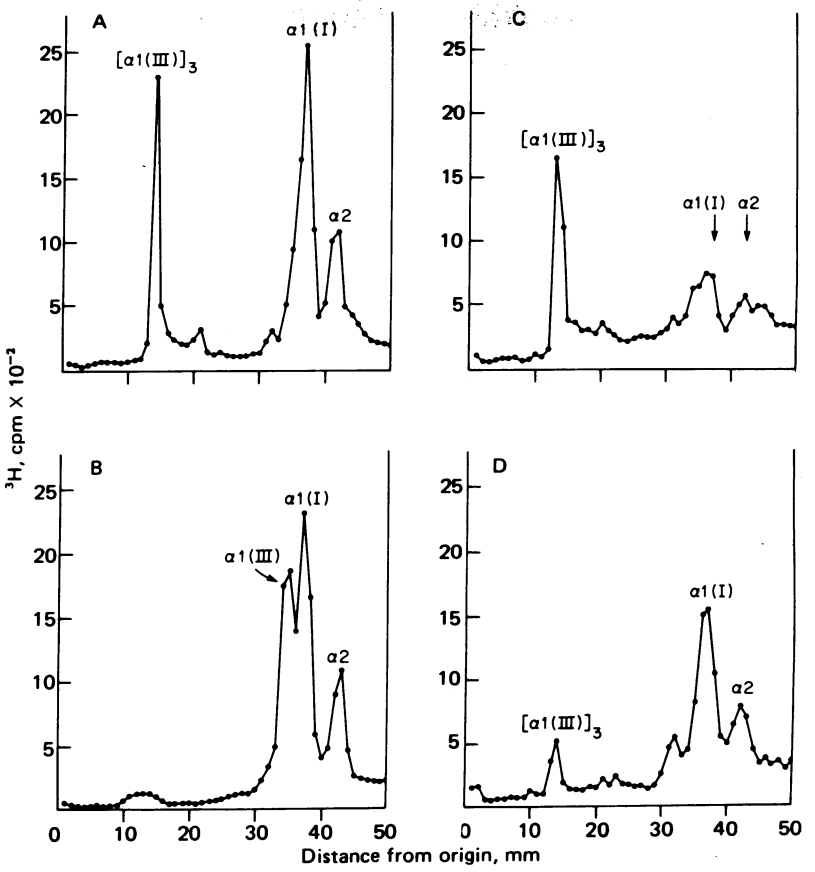

FIG. 2. Gel patterns of medium and cell layer extract during a 48-hr chase. Medium of Fig. 1 was supplemented with unlabeled proline and glycine and placed on unlabeled cell layer. Medium was sampled before (0 hr) and after the chase; cell layer was extracted with acid after the chase. All samples were subjected to limited digestion with pepsin. Equal amounts of radioactivity were applied to the gels. (*A*) Medium, 0 hr; (*B*) medium (reduced), 0 hr; (*C*) medium, 48 hr; (*D*) cell layer, 48 hr.

cubated for 48 hr with an unlabeled confluent layer of Balb/3T3 fibroblasts. Samples of medium and the acid extract of the cell layer were applied to sodium dodecyl sulfate/polyacrylamide gels before and after limited digestion with pepsin.

Fig. 1 shows the gel pattern of the radiolabeled prechase medium before limited digestion with pepsin. The labeled material between 4 and 18 mm represents intact procollagens and procollagens that have undergone partial enzymatic excision of nonhelical NH_2- and COOH-terminal peptides (3, 4, 11). Limited digestion with pepsin excises terminal nonhelical peptides from procollagens, and the helical collagen molecules generated may be identified by their mobilities and chain compositions in the gels. Thus, when the prechase medium of Fig. 1 was treated with pepsin, two types of collagen molecules were resolved in the gel (Fig. 2*A*). Seventy-four percent of the collagen was type I, identified by its position and its characteristic chain composition of $[\alpha 1(I)]_2\alpha 2$. The other protein was identified as type III collagen (chain composition: $[\alpha 1(III)]_3$) by reference to standards and by its dissociation after treatment with 2-mercaptoethanol (12). After reduction (Fig. 2*B*), the radioactivity in type III collagen was quantitatively recovered in $\alpha 1$(III) chains which moved with a slightly slower mobility than the $\alpha 1$(I) chains. As expected, the disposition of type I collagen in the gel was unchanged by reduction.

After the chase, the medium contained mostly type III collagen (Fig. 2*C*). Comparison of Fig. 2 *A* and *C* shows that the chase did not significantly change the amount of type III protein in the medium but was associated with the loss of 70% of the type I molecules from the medium. Samples of postchase medium, with and without prior pepsin digestion, were reduced with 2-mercaptoethanol and applied to a 5–10% linear gradient polyacrylamide slab gel (Fig. 3). The pepsin-treated sample yielded mostly $\alpha 1$(III) chains, whereas the radioactivity in the undigested medium was in higher molecular weight p$\alpha 1$(III)* chains. Thus, type III molecules in the medium were still in a precursor form at the end of the chase.

The radioactive type I molecules lost from the medium were quantitatively recovered as native collagen in the pepsinized acid extract of the cell layer (Fig. 2*D*). A small amount of radioactive type III collagen was also identified in this extract. Gel electrophoresis of a sample of the extract that had not been treated with pepsin still showed recovery of type I collagen molecules as in Fig. 2*D*, but the radioactivity in type III collagen was now in a procollagen molecule of slower mobility (data not shown). The type III procollagen in the cell layer extract probably had its source in medium entrapped within the cell layer. From these data, I conclude that only type I procollagen in the medium was converted to native collagen and chased as such into the cell layer. Qualitatively similar results were obtained in experiments in which the cultures were pulsed for 4 and 6 hr and chased for 20 and 45 hr, respectively.

Different strains or lines of cultured fibroblasts may convert type I procollagen to collagen at different rates, and such con-

* By current usage, "p$\alpha 1$(III)" refers to constituent chains of type III procollagen that lack some of the nonhelical terminal segments of intact precursor chains ("pro α chains"). Given the long pulse and chase of this experiment, I presume that the chains in question had undergone some enzymatic shortening.

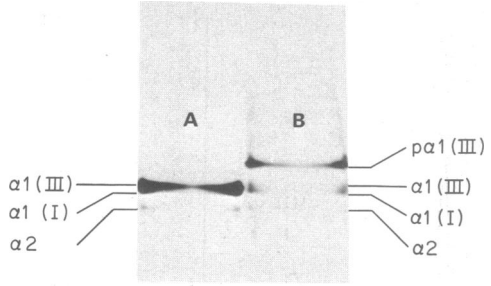

FIG. 3. Pattern of postchase medium when applied to 5–10% linear gradient polyacrylamide slab gel. Medium samples with (*A*) and without (*B*) limited digestion with pepsin were reduced with 2-mercaptoethanol and applied to the gel. Fluorography was performed by the method of Bonner and Laskey (9).

version may occur more slowly in the medium than in the extracellular space of the cell layer (13). The failure to observe complete enzymatic conversion of type III procollagen molecules in the chase medium of the Balb/3T3 system might therefore have represented a special case. Accordingly, Balb/3T3, two other mouse fibroblast lines, and four human fibroblast strains were used in experiments that measured processing of collagenous molecules in the cell layers. Cultures were incubated with isotopes for 24 hr, and both the media and acid extracts of the cell layers were characterized in gels. Radiolabeled types I and III procollagens were identified in the media of all of the fibroblast cultures, and these precursors could also be detected in lesser amounts in the nonpepsinized acid extracts of the cell layers. Type I, but not type III, collagen molecules were detected in the gels of the nonpepsinized cell layer extracts. These acid extracts contained essentially all of the collagenous protein of the cell layers, because pepsin digestion of the residues from the acid extractions (see *Materials and Methods*) failed to release any additional radiolabeled type I or type III collagen. Thus, no evidence was obtained for conversion of type III procollagen to collagen in the cell layers of seven fibroblast cultures of diverse origin. The experiment with 3T6 fibroblasts was particularly informative. We have reported (13) that in this line type I procollagen is processed very rapidly, that type I collagen fibers are formed mostly from collagen molecules generated from procollagen in the extracellular space of the cell layer, and that relatively little type I procollagen accumulates in the culture medium. Accordingly, when a 3T6 culture was incubated with isotopes for 24 hr, only 25% of the total collagenous radioactivity was recovered in the medium. Type III collagen accounted for 6% of the radioactive collagenous species in the medium and type I for the remainder (data not shown). Fig. 4 shows the gel patterns of the acid extract of the cell layer before and after limited digestion with pepsin. Type I collagen was recovered in both instances but there was no significant recovery of radiolabeled type III molecules. If, as in the medium, type III procollagen had represented 6% of the procollagens in the extracellular space of the cell layer, and if type III procollagen had been processed as rapidly as type I procollagen, radioactive type III collagen should have been detected in the gels of Fig. 4.

DISCUSSION

Conversion of type III procollagen to collagen was not observed in the media or cell layers of fibroblast cultures after 24 hr of isotopic labeling or when labeling was followed by 48 hr of chase. By contrast, significant conversion of type I procollagen to collagen occurred in the same cultures during these intervals.

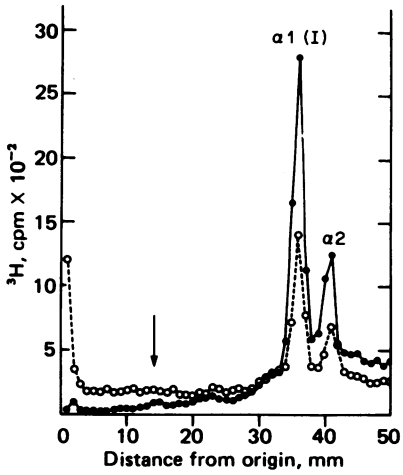

FIG. 4. Gel patterns of an acid extract of cell layer of 3T6 culture labeled for 24 hr with L-[^{3}H]proline and [^{3}H]glycine. ○, Without pepsin digestion; ●, after limited digestion with pepsin. Arrow indicates expected position for type III collagen.

Preferential proteolytic degradation of type III procollagen cannot explain the results because the precursor was quantitatively recovered in the media even after 48 hr of chase. The data are consistent with the view that the two procollagen types are converted by different enzyme systems and that the enzymatic activities for conversion of type III procollagen are in relatively greater deficit in fibroblast cultures. An alternative explanation is that only one rate-limiting enzyme system is required for processing of both precursor molecules and that type I procollagen preferentially competes for one or more of the shared enzymes.

The concentrations of types I and III procollagens in the medium depend upon their respective rates of synthesis and secretion by the fibroblasts. The reported data emphasize that the extracellular concentrations of the soluble precursors are also a function of the rate at which each molecule is converted to insoluble collagen. The relatively greater deficit in processing of type III procollagen in fibroblast cultures favors its disproportionate accumulation in the medium. Accordingly, measurements of the amounts of types I and III collagens synthesized by fibroblast cultures should be based upon recoveries from both the cell layer and the medium.

It is important to know whether the kinetics of processing of types I and III procollagens in tissue culture reflect the situation *in vivo*. Native type III collagen molecules have been recovered from neutral salt extracts of rat and bovine skin (14–18), indicating that some complete processing of type III procollagen occurs *in vivo*. However, the same salt extracts contained greater amounts of type III procollagen than type I procollagen, even though type I molecules (precursor and collagen) were in the majority. The extraction of a disproportionately large pool of type III procollagen from tissue is consistent with slower processing for this precursor molecule *in vivo*.

The physiologic implications of different rates of processing for types I and III procollagens are presently obscure. Indeed, unique physiologic roles for these two collagen types have not yet been established. They are usually found in the same tissues, generally with type I collagen in greater abundance (1, 19). However, the proportion of type III molecules is relatively elevated in fetal skin (20) and in healing wounds (21, 22), suggesting that the type III precursor may have some unique function at sites of accelerated collagen fibrillogenesis and remodeling.

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