BIOSYNTHESIS AND SECRETION OF TROPOELASTIN BY CHICK AORTA CELLS

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SUMMARY

Cells were isolated from the aortae of 17-day old chick embryos by digestion of the vessels with a combination of trypsin and collagenase. When these cells were incubated in suspension culture in Krebs-Ringer media containing pancreatic trypsin inhibitor and radioactive amino acids, they synthesized and secreted labeled proteins into the media. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the secreted proteins labeled with [^{14}C]proline revealed two major components. The larger component with an approximate molecular weight of 125,000 had a [^{14}C]hydroxyproline content consistent with a form of procollagen. The molecular weight of 70,000 and [^{14}C]hydroxyproline content of the second component was consistent with that previously reported for tropoelastin extracted from chick aortae. By following the kinetics and secretion of tropoelastin labeled with [^{3}H]valine, we have estimated that 17 minutes are required to synthesize and secrete the molecule under these experimental conditions.

In vertebrates,the protein elastin is a vital component of major blood vessels, imparting to them their elastic, rubber-like quality. Purified elastin is very inert and insoluble due to extensive crosslinking of the polypeptide chains by desmosines and other cross-linkages (1,2) derived from the oxidation of lysine residues by a copper requiring enzyme (3-5). Because of its insolubility, comparatively little was known concerning the chemical and physical structure of the elastin molecule until Sandberg, et al. (6) prepared a soluble protein from aortae of copper deficient pigs. This protein, called tropoelastin, consisted of a single polypeptide chain with an estimated molecular weight of about 70,000 and an amino acid composition which closely resembled that of insoluble elastin except for the absence of the desmosines. Rapid progress has been made in sequencing tropoelastin (7) and a model for cross-linked elastin has been proposed based on this data (8). A similar soluble protein has been isolated from the aortae of copper deficient or lathyritic chicks (9,10).

We have previously reported that isolated embryonic chick aortae synthesize a soluble protein whose labeling pattern with radioactive amino acids was suggestive of that of tropoelastin and which migrated close to serum albumin (68,000 daltons) during gel electrophoresis in sodium dodecyl sulfate (11). During pulse-chase experiments this soluble protein was incorporated into an insoluble residue. Smith and Carnes (12) have demonstrated that when aortae from normal newborn pigs were incubated in vitro, they synthesize a labeled protein which manifests many of the properties of added carrier tropoelastin. Cells isolated from the media of pig aortae can synthesize elastin after they have been in long tern culture (13).

None of the above biological systems have permitted ready manipulation of the cells synthesizing elastin or estimation of the time required to secrete elastin. In the present communication we report conditions under which cells isolated from embryonic chick aortae synthesize tropoelastin and estimate the time required to synthesize the molecule and secrete it into the surrounding media to be approximately 17 min.

MATERIALS AND METHODS

Isolation of Cells. Cells were isolated from 17-day chick embryos by modification of a technique previously described for isolation of fibroblasts from embryonic tendons (14). A section of the thoracic aorta extending from the root to a point just proximal to the entrance of the ductus arteriosus was dissected with sterile precautions. The innominate arteries to the point of their bifurcation into the subclavian and carotids were also included. Loose connective tissue was stripped from the aortae which were cut along their long axis and then minced. The small fragments were then resuspended in Eagles minimal media containing 2% fetal calf serum and 1 mg/ml purified collagenase (Worthington) and 2.5 mg/ml trypsin (Grand Island Biologicals). One ml of media equilibrated with 5% CO_2, 95% air was used per 12-15 aortae and the aortae were shaken in a water bath. At the end of 1 hour of digestion at 37°C the fragments were essentially completely solubilized. The suspension was passed through lens paper and the filtrate centrifuged at 1200 x g for 5 minutes. The cells were washed three times by resuspension and centrifugation in Krebs-Ringer containing 2% calf serum. Approximately 3 million cells were obtained per aorta.

Incubation of Isolated Cells. The cells were incubated at a concentration of 2 million cells per ml in Krebs-Ringer containing 2% fetal calf serum, 25 µg/ml of ascorbate and 200 µg/ml pancreatic trypsin inhibitor (Worthington) and radioactive amino acids (New England Nuclear) for variable periods of time. Details of particular experiments are given in the table and figures. The [^{14}C]hydroxyproline content of some samples labeled with [^{14}C]proline was measured by the method of Juva and Prockop (15).

Disc Gel Electrophoresis in Sodium Dodecyl Sulfate. Disc gel electrophoresis in sodium dodecyl sulfate was performed as previously described (11) using 5% acrylamide gels and one-half the standard amount of cross-linker. The gels were immediately chilled and cut into 1.5 mm fractions in order to minimize losses, and

the fractions were solubilized with 30% H_2O_2 and counted in a scintillation counter. Duplicate gels were run containing radioactive samples, purified α and β chains of rat tail collagen and bovine serum albumin. These gels were stained with Coomassie blue and the radioactive peaks were identical in the stained and unstained gels when corrected for gel swelling during destaining.

RESULTS AND DISCUSSION

Preliminary experiments in which the cells were incubated with radioactive amino acids revealed that a variable amount of labeled protein secreted into the media had a molecular weight of approximately 70,000 as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. However, a large fraction of these labeled secreted proteins were of lower molecular weight. Because the newly synthesized tropoelastin contains a relatively large amount of lysine we would expect it to be rather sensitive to any residual trypsin remaining from the cell isolation procedure. A number of specific trypsin inhibitors were, therefore, added individually during the incubation of the cells with labeled amino acids to test their ability to increase the yield of the 70,000 molecular weight component. The inhibitors which were tested over a range of concentrations were p-tosyl-L-lysine (Sigma), and trypsin inhibitors from lima bean, soy bean and bovine pancreas (Worthington). Only the inhibitor from bovine pancreas proved effective and did not inhibit synthesis of labeled proteins. A concentration of 200 µg/ml proved optimal since at lower concentrations the yield of the 70,000 molecular weight component was slightly less and at higher concentrations such as 400 µg/ml there was a 10% inhibition of total incorporation and no appreciable increase in yield of the 70,000 dalton component. Pancreatic trypsin inhibitor was, therefore, included in all incubations and a typical experiment is illustrated in Fig. 1 using [^{14}C]proline. This experiment demonstrated that the cells continued to synthesize labeled proteins at a linear rate for at least 4 hr and that a progressively larger fraction was found in the media so that at 4 hr approximately 50% of the incorporated label was in secreted proteins. In order to characterize these labeled proteins with respect to molecular weight, they were subjected to electrophoresis on polyacryl amide gels in sodium dodecyl sulfate. Fig. 2A illustrates the pattern obtained from the labeled intracellular and secreted proteins after incubation of the cells for 1

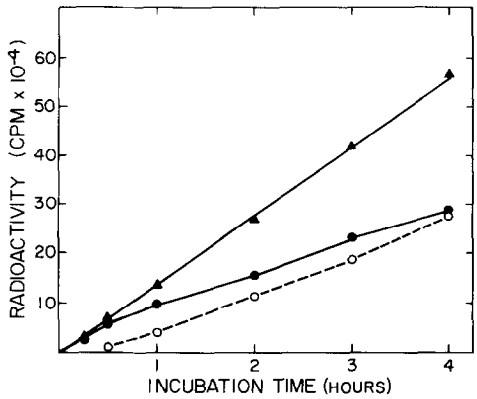

Fig. 1 Synthesis and secretion of [^{14}C]proline labeled proteins by isolated embryonic chick aorta cells. The cells were isolated as described in materials and methods and then incubated in Krebs-Ringer (2 x 10^6cells/ml) containing 3 μCi/ml [^{14}C]proline. One ml aliquots of the cell suspension were taken at the times indicated and centrifuged. The cells were resuspended in 1 ml of 0.01 M phosphate buffer, pH 7.4, and sodium dodecyl sulfate and mercaptoethanol were added to final concentrations of 1% to the cells and media. The samples were then placed in a boiling water bath for 2 minutes and dialyzed against 0.01 m phosphate buffer, pH 7.4, containing 0.1% sodium dodecyl sulfate and mercaptoethanol. After dialysis, aliquots were counted in a scintillation counter. Intracellular (●–●–●); extracellular (o-o-o); intracellular + extracellular (▲–▲–▲).

hr. Similar patterns were obtained at later time points when a greater fraction of proteins were secreted. Two major peaks were obtained in the secreted material along with a small amount of lower molecular weight components. The larger molecular weight component was found in a position corresponding to that previously described for procollagen synthesized by embryonic tendon cells, and it migrated somewhat more slowly than the α chain collagen standard. The second component migrated to a position nearly identical with that of the serum albumin marker. The [^{14}C]hydroxyproline content of the separated secreted proteins was determined by analysis of the hydrolyzed proteins obtained from the appropriate fractions of the polyacrylamide gels. Table I summarizes the results which show that the higher molecular weight protein had a [^{14}C]hydroxyproline content of 31.7%, suggesting that it is largely a form of procollagen. The [^{14}C]hydroxyproline content of 12.8% of the 70,000 molecular weight component is slightly greater than the value reported for chick aorta tropoelastin but less than the value reported for control chick insoluble elastin (10).

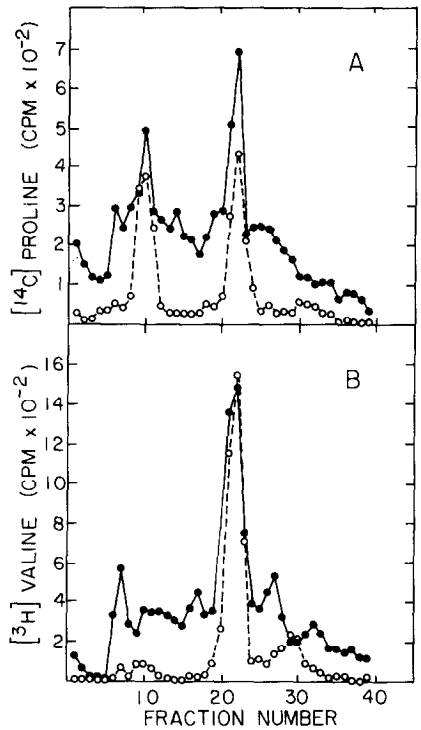

Fig. 2 Polyacrylamide disc gel electrophoresis of intracellular and extracellular labeled proteins synthesized by aorta cells. Aliquots of cells which had been incubated for 1 hour with either 3 μCi/ml [^{14}C]proline (A) or 10 μCi/ml [^{3}H]valine (B) were treated as described in Fig. 1. Electrophoresis and counting of the gels were then carried out as described in Materials and Methods. Marker β chains of rat tail collagen were located at fraction 8, α chains of rat tail collagen at fraction 13 and bovine serum albumin at fraction 22-23. Intracellular (●-●-●); extracellular (o-o-o).

Since the valine content of chick aorta tropoelastin is high (175 residues/1000) compared to collagen (20 residues/1000), [^{3}H] valine was used to preferentially label the tropoelastin. Fig. 2B illustrates the distribution of radioactivity after polyacrylamide gel electrophoresis of samples prepared from cells incubated for 1 hr. A prominent single peak of radioactivity was found in a position characteristic of tropoelastin and identical in position to that previously described for samples labeled with [^{14}C]proline. The rate of secretion of tropoelastin was studied by following the time course of appearance in the media of the 70,000 molecular weight peak labeled with [^{3}H]valine. The amount of labeled tropoelastin was estimated by summing the radioactivity in the appropriate peak fractions of the

Table I

[^{14}C]Hydroxyproline content of fractions obtained by polyacrylamide gel electrophoresis of labeled secreted protein.

Fractions Pooled	Total ^{14}C	[^{14}C]Hypro	Degree of hydroxylation*
	dpm x 10^{-3}	dpm x 10^{-3}	%
9-11	10.5	3.3	31.7
21-24	11.7	1.5	12.8

Chick aorta cells were incubated with 5 μCi/ml [^{14}C]proline for 2 hours as described in Fig. 1 and the labeled secreted proteins subjected to electrophoresis on polyacrylamide gels as described in Fig. 2. The gels were fractionated and one-third of each individual slice was dissolved and counted. The fractions containing the radioactive peaks were then pooled and hydrolyzed in sealed tubes with 6N HCl at 110° for 24 hours. The polyacrylamide was not appreciably solubilized by this procedure and the hydrolysate was filtered and the proteins analyzed for their [^{14}C]hydroxyproline content by a specific chemical procedure (15).

* Values are 100 times [^{14}C]hyroxyproline per total [^{14}C].

gels. Fig. 3 illustrates the results of these determinations. By projection onto the time axis of the straight line obtained for the secreted values, we estimate that there is a lag of about 17 minutes from the time of initiation of labeling until the first appearance of labeled tropoelastin in the media. By a similar analysis of the [^{14}C]proline labeled samples, we estimate a lag of 23 minutes before the appearance of labeled procollagen in the media (data not shown). This value agrees well with that previously reported for procollagen synthesized by isolated embryonic chick tendon fibroblasts (16).

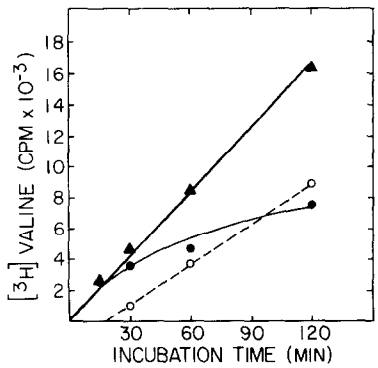

Fig. 3 Synthesis and secretion of tropoelastin. Aorta cells were incubated with 10 μCi/ml [^{3}H]valine and at the times indicated 1 ml aliquots of the cell suspension were centrifuged and the labeled intracellular and extracellular proteins prepared for electrophoresis as described in Fig. 1 and then subjected to polyacrylamide disc gel electrophoresis as in Fig. 2. By summation of the counts in fractions 20-24, an estimate of the radioactivity in intracellular and extracellular tropoelastin was made. These measurements were then plotted as a function of incubation time. Intracellular tropoelastin (●-●-●); extracellular tropoelastin (o-o-o); intracellular + extracellular (▲-▲-▲).

These results demonstrate that freshly isolated cells of chick aortae will maintain their differentiated function with respect to the synthesis and secretion of procollagen and tropoelastin even when incubated in suspension culture. Although further characterization of the cells must be carried out and we do not know at present whether each cell is simultaneously synthesizing both proteins, this system should prove useful in further studies designed to elucidate the mechanism of tropoelastin secretion.

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