BIOSYNTHESIS OF LYSINE-DERIVED ELASTIN CROSSLINKS IN AORTIC CELL CULTURE[1]

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SUMMARY

Culture of an established line of aortic medial cells in the presence of L-[^{14}C] lysine for 72 hours, beginning on the twenty-first day after transfer, has resulted in the incorporation of label into a residue, insoluble after autoclaving. Acid hydrolysates of this residue with or without reduction by $NaBH_4$ were subjected to ion exchange chromatography. Several radioactive lysine-derived residues were identified, by comparison to standards, as the distinctive crosslinks of elastin, isodesmosine, desmosine, merodesmosine and lysinonorleucine. This confirms the synthesis of elastin in aortic cell culture and establishes the formation of insoluble crosslinked elastin. Differences in the heights of the peaks in the reduced and nonreduced elastin indicate the probable occurrence of dehydromerodesmosine and dehydrolysinonorleucine as well and suggest that these may be intermediates in crosslink formation.

The biosynthesis of soluble elastin by aortic cells in culture has recently been reported in both short-term primary incubation (1) and in a long-term culture of medial cells (2). The incorporation of a radioactive valine label into the insoluble elastin residue of aortic tissue (1) suggested the rapid crosslinkage of newly synthesized elastin in the culture system. However, the nature of the bonds was not demonstrated. Similar incorporation of labeled valine into a minute insoluble residue of the cell culture (2) afforded the possibility of studying the development of crosslinks in a matrix-free system. The synthesis of several lysine derivatives has been demonstrated in the insoluble residue of young cultures by ion exchange chromatography after labeling with [^{14}C] lysine. Four of these derivatives have now been identified with characteristic crosslinks of elastin.

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MATERIALS AND METHODS

The culture used in this study is an established line of aortic medial cells isolated from newborn pig aorta (3) and maintained in Eagle's minimal essential medium (MEM) with 10% serum (equal parts of fetal bovine and porcine) containing penicillin and streptomycin in Falcon plastic flasks gassed with 10% CO_2 in air. Flasks were inoculated with 1 x 10^6 trypsinized cells which were allowed to grow to confluency and were held in stationary phase by feeding every three days. On the twenty-first day after transfer, the cells were fed a lysine-free medium, otherwise identical to MEM, supplemented with ascorbic acid (75µg/ml) and L-[^{14}C] lysine (2.5 µCi/ml) for 72 hours. The cells were then harvested, homogenized and extracted overnight with phosphate buffered 0.5M NaCl (pH 7.2). The supernatant was separated by centrifugation and the residue was repeatedly autoclaved in distilled water until no more radioactivity was eluted. The insoluble cell residue was washed with 100% ethanol and ether and then dried in air.

A portion of the insoluble residue was reduced with $NaBH_4$ at pH 9.3 with constant stirring for two hours (4). The pH was maintained by the occasional addition of 0.1N NaOH and the reaction was terminated by lowering the pH to 5 with glacial acetic acid. The suspension was centrifuged and the residue was washed with distilled water several times and air-dried. Both the reduced and the nonreduced insoluble residues were hydrolysed with 6N HCl for 72 hours at 110° in sealed evacuated tubes. The hydrolysates were evaporated *in vacuo*.

A standard elastin was prepared from mature pig aortas obtained from a slaughterhouse. The frozen tissue was trimmed free of fat and adventitia and cut into thin strips. The tissue was ground and sequentially extracted with 1M NaCl and 5M guanidine-hydrochloride containing 1% mercaptoethanol. The insoluble residue was autoclaved repeatedly in distilled water until the supernatant was free of protein. The residue was extracted with alcohol and ether and then air-dried. It was hydrolysed in 6N HCl as described above.

Lysine-derived crosslinks of the elastin hydrolysates were separated by ion exchange chromatography on an Aminex A-5 column by the method of Green *et al* (5) with some modifications. The column (1.5 x 15 cm) was maintained at 50° and a stepwise gradient of pyridine acetate buffers of pH 4.5 was used. The first buffer was 0.15M and the second was 0.225M. The column was finally washed with 1M pyridine acetate buffer at pH 5.0. A flow rate of 32 ml/hr was maintained and fractions of 3.3 ml were collected. Radioactive peaks were identified by counting aliquots of the fractions in a Packard Tri-Carb counter using a xylene-based commercial scintillation fluid (Amersham-Searle Corporation). Lysine-derived crosslinks of the aortic elastin were located by ninhydrin analysis of the fractions. The chromatogrpaphic column was calibrated by using standard samples of isodesmosine, desmosine, merodesmosine, lysinonorleucine, hydroxylysine and lysine.

RESULTS

In the elution profile of the standard aortic elastin hydrolysate on the Aminex A-5 column, isodesmosine, desmosine, merodesmosine, lysinonorleucine and lysine were identified by comparison to the standards. Several other peaks remain to be identified. When fractions 39 through 109 were pooled and rechromatographed on the long column of the Beckman 120B Amino Acid Analyzer,

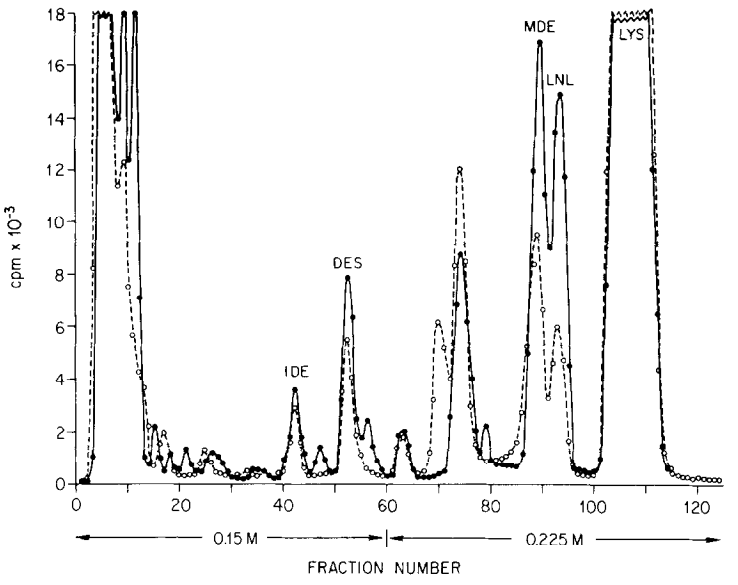

Fig. 1. Elution profile of lysine-derived crosslinks of an acid hydrolysate of insoluble culture residue on an Aminex A-5 column using a stepwise gradient of pyridine-acetate buffers (pH 4.5). The culture residue was hydrolysed either after reduction with $NaBH_4$ (—— ● ——) or without reduction (- - 0 - -). Among the radioactive peaks, isodesmosine, desmosine, merodesmosine, lysinonorleucine and lysine were identified by reference to standards.

Table 1. Relative Radioactivity of Lysine and its Derivatives in Labeled Culture Residue

	Isodesmosine	Desmosine	Merodesmosine	Lysinonorleucine	Lysine
Reduced	0.217	0.510	1.493	1.270	93.312
Non Reduced	0.192	0.324	0.911	0.461	93.962

the identities of the four lysine-derived crosslinks were confirmed. No hydroxylysine was detected.

Fig. 1 shows a composite chromatogram of the acid hydrolysate of insoluble residue of the cell culture after reduction with $NaBH_4$ and without reduction. Among the several radioactive peaks isodesmosine, desmosine, merodesmosine and lysinonorleucine were identified by the positions of the peaks on the calibrated column. A considerable amount of radioactivity was present in the region (fractions 3-13) where neutral and acidic amino acids elute.

Reduction of the insoluble cell residue with $NaBH_4$ resulted in enhancement of the radioactive peaks representing merodesmosine and lysinonorleucine with concomitant lowering of the major unidentified peak (fractions 71-78). Among the peaks which were identified by known standards, merodesmosine was the largest, followed by lysinonorleucine, desmosine and isodesmosine, in that order.

Table 1 shows the percentage distribution of labeled lysine and its derivatives in the insoluble culture residue. Nearly 94% of the total eluted activity was contained in the lysine peak (Table 1). Considerable loss of lysinonorleucine and merodesmosine occurred in the nonreduced hydrolysate as expected.

DISCUSSION

The identification of elastin crosslinks in the insoluble residue is evidence of the synthesis of insoluble elastin in an aortic medial cell culture. This is consistent with the hypothesis that the soluble protein (2), of closely related structure, is a precursor of insoluble, crosslinked elastin. This hypothesis is strengthened by the low ratio of crosslinks to lysine in the newly synthesized insoluble protein. In mature, insoluble pig aortic elastin, desmosine, isodesmosine and lysine occur in approximately equivalent amounts (6). The low ratio of the desmosines to lysine in this cell culture elastin indicates that crosslinkage is incomplete in the 72 hours of this experiment. This culture, therefore, offers a system for studying the order of appearance of intermediates in crosslink formation.

The increment in the reduced merodesmosine and lysinonorleucine peaks

over the nonreduced peaks indicates the presence of dehydromerodesmosine and dehydrolysinonorleucine, and suggests that both products may serve as intermediates in crosslink formation (7,8,9). This possibility will have to be proven in kinetic experiments. The substantial radioactivity eluted in the region of the acidic and neutral amino acids may represent the aldehydes and aldol condensation products and their decomposition products.

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