PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **181**, 387–391 (1986)

Synthesis of Crosslinked Elastin by a Mesothelial Cell Culture (42269)

J. O. CANTOR, M. WILLHITE, B. A. BRAY, S. KELLER, I. MANDL, AND G. M. TURINO

Columbia University, College of Physicians and Surgeons, New York, New York 10032

Abstract. Synthesis of crosslinked elastin was measured in cultures of pleural mesothelial cells. Results indicate that mesothelial cells are a rich source of crosslinked elastin and may therefore be useful for *in vitro* studies of this connective tissue component.

The development of a highly sensitive and specific assay for crosslinked elastin (1) has provided an opportunity to define the capacity of cells and tissues to synthesize even minute amounts of this connective tissue component. Using this procedure, we have previously demonstrated that cultured rat lung endothelium synthesizes crosslinked elastin (2). However, the ubiquity of elastic fibers in the lung suggests that other cell types are also capable of producing elastin. The present study characterizes crosslinked elastin synthesis by pleural mesothelial cells. Results indicate that mesothelial cells are an unusually rich source of crosslinked elastin, and may therefore serve as an excellent *in vitro* system for studying the biosynthesis of elastin.

Materials and Methods. *Preparation of cell cultures.* The cells used in this study were obtained from the American Type Culture Collection (Rockville, Md.) and represent an established line derived from rat visceral pleura (3). Cells were received at passage 11 and grown at 37°C in an atmosphere of 95% air and 5% CO_2, using F-12(K) medium supplemented with 15% fetal bovine serum and 1% L-glutamine. At passage 25, cultures were tested for elastin synthesis. They were seeded with 2.0×10^6 cells and grown to confluence in preparation for labeling studies.

Labeling of cell cultures. Cultures were incubated with 10 μCi [^{14}C]lysine (ICN Radiochemicals, Irvine, Calif.; sp act: 275 mCi/mmole) in 10 ml medium for 24, 48, and 96 hr in order to label the lysine-derived, elastin-specific crosslinks, desmosine and isodesmosine. Replicate flasks were used to determine cell density.

Isolation of the elastin-specific crosslinks. The labeled media were removed from the flasks, combined with two cell washes in Hanks' balanced salt solution, then filtered to remove cells. The cell layers were extracted in Hanks' balanced salt solution with a rubber-tipped rod. Cell and medium fractions were first dialyzed against unlabeled lysine and then extensively against water, lyophilized, and hydrolyzed in 6 *N* HCl for 24 hr at 110°C. The hydrolysates were evaporated to remove HCl, reconstituted in water, and filtered. An aliquot of each hydrolysate was measured for total [^{14}C]lysine incorporation in a liquid scintillation spectrometer. Samples were then spotted on Whatman 3MM filter paper and subjected to descending chromatography in a system of butanol, glacial acetic acid, and water (4:1:1). To increase the resolution of components with lower R_f values, the chromatograms were dried and reimmersed in the solvent system five additional times. The origins, containing the desmosine and isodesmosine crosslinks, were cut out and eluted twice in water. The eluates were concentrated and spotted on 20 × 20-cm cellulose-coated thin-layer plates (MN-300, Brinkmann Instruments, Westbury, N.Y.) and subjected to one-dimensional electrophoresis in a system of pyridine, glacial acetic acid, and water (1:10:189) at 600 V for 90 min (1). Additional samples were subjected to two-dimensional separation consisting of electrophoresis as just described and chromatography in butanol, acetic acid, and water (4:1:1) (1).

Identification of desmosine and isodesmosine. The thin-layer plates were sprayed with Enhance (New England Nuclear, Boston,

0037-9727/86 $1.50

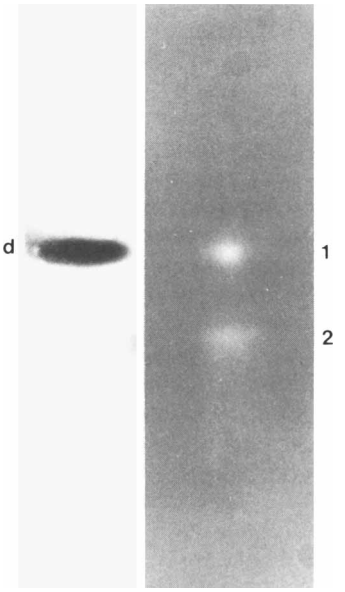

FIG. 1. Autoradiograph (right side; positive print) of one-dimensional, thin-layer electrophoresis of radiolabeled components previously eluted from origin of paper chromatogram (see Materials and Methods). Component 1 is identified as desmosine/isodesmosine, based on comparison with coelectrophoresed standard (left side (d); ninhydrin stained). Component 2 is an uncharacterized, lysine-derived component of elastin (see Fig. 2). (Direction of electrophoresis: bottom to top.)

Mass.) and overlaid with Kodak X-Omat AR-2 film (Eastman Kodak, Rochester, N.Y.). Following 1- to 2-day incubations, the films were developed. The identities of the labeled compounds were determined by comparing their exposure markings with coelectrophoresed human lung and ligamentum nuchae elastin hydrolysates and desmosine standards, stained with ninhydrin. The labeled components were then scraped off the plates and their respective radioactivities were measured in a liquid scintillation spectrometer.

Additional studies. In order to ascertain the specificity of the isolation procedures for crosslinks, additional cultures were incubated with [^{14}C]lysine in the presence of 50 μg/ml β-aminopropionitrile (4) and assayed as described above.

To eliminate any collagen crosslinks, labeled cell layers were incubated overnight at 37°C with 50 U/mg purified bacterial collagenase (Form III, Advance Biofactures, Lynbrook, N.Y.) in 0.05 *M* Tris buffer, pH 7.5, supplemented with 0.005 *M* $CaCl_2$ and 0.001 *M* *N*-ethylmaleimide (5). Following dialysis, the samples were assayed as described above. To determine the effectiveness of collagenase digestion, residual-labeled hydroxylysine was measured following paper chromatography.

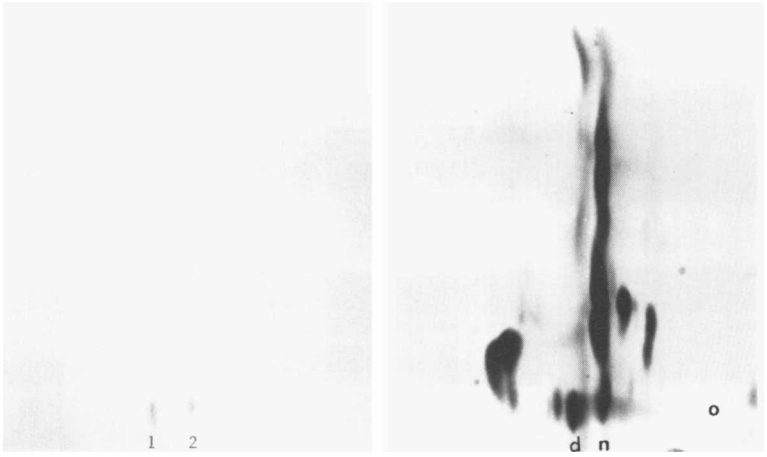

FIG. 2. Autoradiograph (left side) of two-dimensional, thin-layer separation of radiolabeled components previously eluted from origin of paper chromatogram (see Materials and Methods). Following comparison with ninhydrin-stained, two-dimensional separation of human lung elastin hydrolysate (right side), two components are identified: [1] desmosine/isodesmosine (d) and [2] a lysine-derived compound migrating to the region of the neutral amino acids (n), which may be a new crosslink. (Origin at lower right (o); direction of electrophoresis: right to left; direction of chromatography: bottom to top.)

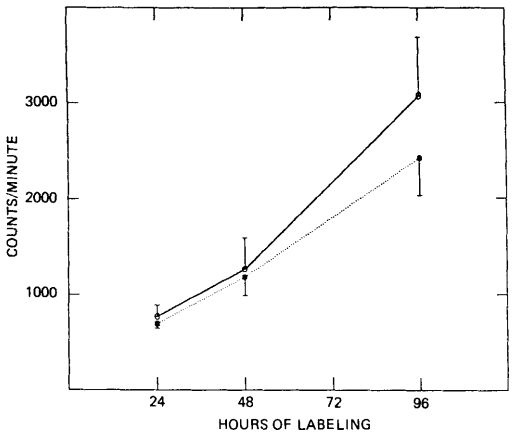

FIG. 3. Graph showing incorporation of [^{14}C]lysine (per 10^6 cells) into desmosine/isodesmosine (○) and the neutral compound (●). T bars: SD.

The location of this amino acid on the chromatogram was determined by ninhydrin staining of a similarly chromatographed standard.

Results. *Radioautography.* As seen in Figs. 1 and 2, the autoradiographs of one- and two-dimensional thin-layer separations of components whose $R_f = 0$ revealed two exposure markings. When compared to coelectrophoresed standards, the labeled bands corresponded to desmosine and isodesmosine (component 1) which comigrate, and an unidentified compound (component 2) which is located in the region of the neutral amino acids. This latter material has been noted in similar labeling studies of rat pulmonary endothelial cells (2) and of whole hamster lung (6) and may represent another lysine-derived crosslink.

Measurement of crosslink synthesis. Incorporation of [^{14}C]lysine into both desmosine/isodesmosine and the neutral component was linear over a 96-hr period (Fig. 3). In contrast, total [^{14}C]lysine incorporation into cell proteins leveled off with time (Table I). Nearly all of the labeling of the crosslinks was associated with the cell layer (Table I).

Addition of β-aminopropionitrile to the

TABLE I. SYNTHESIS OF ELASTIN COMPONENTS BY MESOTHELIAL CELLS

Hours of labeling	No. samples tested	Total [^{14}C]lysine incorporation (cpm/10^6 cells)[a]	[^{14}C]des/ides (cpm/10^6 cells)	[^{14}C]neutral compound (cpm/10^6 cells)
			Cells	
24	2	5.61 ± 0.11	767 ± 121 (0.14)	694 ± 48 (0.12)
48	4	7.34 ± 1.02	1265 ± 326 (0.17)	1179 ± 197 (0.16)
Collagenase[b]	4	6.83 ± 0.29	1326 ± 340 (0.19)	1064 ± 212 (0.16)
β-APN[c]	2	5.95 ± 0.07	61 ± 4 (0.01)	78 ± 10 (0.01)
96	2	8.24 ± 1.03	3062 ± 619 (0.37)	2408 ± 387 (0.29)
			Media	
48	4	0.83 ± 0.12	29 ± 4	—
β-APN[c]	2	0.85 ± 0.02	20 ± 1	—

Note. Values are means ± SD. Figures in parentheses are percentages of total [^{14}C]lysine incorporation into cell layer.

[a] All values in this column × 10^{-5}.

[b] Collagenase digestions were performed on cell layers labeled for 48 hr.

[c] β-Aminopropionitrile (β-APN) was added to cell cultures labeled for 48 hr.

cultures abolished over 90% of the labeling of desmosine/isodesmosine and the neutral compound (Table I). Digestion of the cell layers with collagenase, in contrast, did not remove these labeled components, ruling out their presence in collagen (Table I). The effectiveness of collagenase digestion is indicated by loss of the collagen-specific amino acid, hydroxylysine (Fig. 4).

Discussion. Rennard *et al.* (7) first described formation of crosslinked elastin in cultures of pleural mesothelial cells. The present study further characterizes elastin crosslink synthesis by such cells using a highly sensitive assay for desmosine and isodesmosine. Confirmation of the specificity of the procedure is demonstrated by treatment of the cells with β-aminopropionitrile and collagenase.

Several differences between mesothelial cell cultures and other elastin-producing cells emerge from these measurements. First, despite the fact that desmosine and isodesmosine are known to be synthesized at relatively slow rates in other systems, labeling of these crosslinks was seen as early as 24 hr after incubation of the mesothelial cells with [^{14}C]lysine. These findings contrast with those of other studies involving smooth muscle cell cultures which indicate a much slower accumulation of crosslinks (4, 8, 9). Second, the presence of an uncharacterized, lysine-derived elastin component (or components) was observed. Like desmosine and isodesmosine, it has an R_f value of 0 on paper chromatography and is inhibited by β-aminopropionitrile. It is synthesized in parallel with desmosine and isodesmosine, in amounts comparable to these known crosslinks. Further characterization of this new compound is now in progress. Preliminary studies indicate that it has a molecular weight of approximately 730, which is greater than that of desmosine. Third, the cells maintain their ability to synthesize crosslinked elastin at passage 25, providing an unusually stable source of this connective tissue component in contrast to other cell types which may lose their elastin phenotype after several culture passages. Mesothelial cell cultures can therefore serve as a useful tool for studying elastin, especially the factors which modulate its synthesis.

In vivo, mesothelial cells may play a role in regulating pleural mechanics in normal and diseased lungs. In bleomycin-induced experimental pulmonary fibrosis, for example, a tangle of filamentous elastic fibers is observed histologically in regions of pleural reaction (10). The present findings suggest the possibility that deposition of this network of fibers may be a response of mesothelial cells to injury.

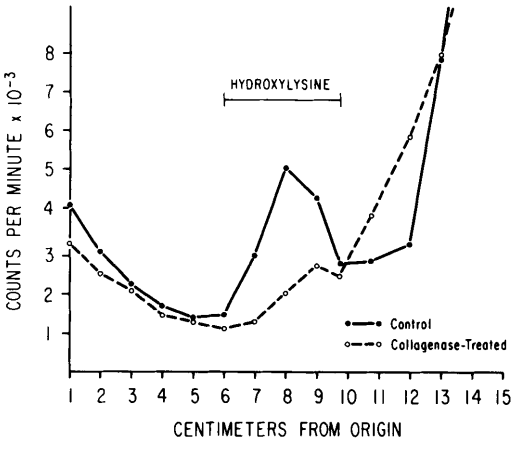

FIG. 4. Graph showing markedly reduced amount of [^{14}C]hydroxylysine on paper chromatogram following collagenase digestion of mesothelial cell layer.

This work was supported in part by USPHS Program Project HL-15832.

1. Keller S, Turino GM, Mandl I. The separation of elastin components by thin layer chromatography and electrophoresis. Connect Tissue Res **8**:251–254, 1981.
2. Cantor JO, Keller S, Parshley MS, Darnule TV, Darnule AT, Cerreta JM, Mandl I, Turino GM. Synthesis of crosslinked elastin by an endothelial cell culture. Biochem Biophys Res Commun **95**:1381–1386, 1980.
3. Aronson JF, Cristofalo VJ. Culture of epithelial cells from the rat pleura. In Vitro **17**:61–70, 1981.
4. Narayanan AS, Sandberg LB, Ross R, Layman DL. The smooth muscle cell. III. Elastin synthesis in arterial smooth muscle cell culture. J Cell Biol **68**:411–419, 1976.
5. Peterkofsky B, Diegelmann R. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry **10**:988–994, 1971.
6. Cantor JO, Osman M, Keller S, Cerreta JM, Mandl

I, Turino GM. Measurement of crosslinked elastin synthesis in bleomycin-induced pulmonary fibrosis using a highly sensitive assay for desmosine and isodesmosine. J Lab Clin Med **103**:384–392, 1984.
7. Rennard SI, Jaurand M-C, Bignon J, Kawanami O, Ferrans VJ, Davidson J, Crystal RG. Role of pleural mesothelial cells in the production of the submesothelial connective tissue matrix of the lung. Amer Rev Respir Dis **130**:267–274, 1984.
8. Faris B, Salcedo LL, Cook V, Johnson C, Foster JA, Franzblau C. The synthesis of connective tissue protein in smooth muscle cells. Biochim Biophys Acta **418**: 93–103, 1976.
9. Snider R, Faris B, Verbitzki V, Moscaritolo R, Salcedo LL, Franzblau C. Elastin biosynthesis and cross-link formation in rabbit aortic smooth muscle cell cultures. Biochemistry **20**:2614–2618, 1981.
10. Starcher BC, Kuhn C, Overton JE. Increased elastin and collagen content in the lungs of hamsters receiving an intratracheal injection of bleomycin. Amer Rev Respir Dis **117**:299–305, 1978.

Received September 4, 1985. P.S.E.B.M. 1986. Vol. 181.
Accepted November 7, 1985.