THE SMOOTH MUSCLE CELL

III. Elastin Synthesis in Arterial Smooth Muscle Cell Culture

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ABSTRACT

Primate arterial smooth muscle cells and skin fibroblasts were examined for their
ability to synthesize elastin in culture. In the presence of the lathyrogen β-amino-
propionitrile, the smooth muscle cells incorporate [^{3}H]lysine into a lysyl oxidase
substrate that was present in the medium and associated with the cell layer. A
component having a mol wt of 72,000 and an electrophoretic mobility similar to
that of authentic tropoelastin was isolated from the labeled smooth muscle cells by
coacervation and fractionation with organic solvents. In the absence of β-amino-
propionitrile, long-term cultures of smooth muscle cells incorporated [^{14}C]lysine
into desmosine and isodesmosine, the cross-link amino acids unique to elastin. In
contrast, no desmosine formation occurred in the fibroblast cultures. These
characteristics demonstrate that arterial smooth muscle cells are capable of
synthesizing both soluble and cross-linked elastin in culture.

Our earlier studies in this series of investigations demonstrated that in many tissues the smooth muscle cell is a connective tissue synthetic cell similar to the fibroblast, the osteoblast, and the chondroblast (13). In the vascular system the arterial smooth muscle cell is principally responsible for the synthesis of the major connective tissue components of the vessel wall, namely the elastic fiber, collagen, and glycosaminoglycan (11, 20). We have previously shown that it is possible to grow arterial smooth muscle cells in homogeneous culture and that, under appropriate conditions, these cells synthesize collagen and elastic fiber microfibrils (10).

The elastic fiber contains at least two morphologically and chemically distinct proteins, the elastic fiber microfibril and elastin (4, 12). In this report, we present data demonstrating that arterial smooth muscle cells synthesize elastin in culture, in contrast to dermal fibroblasts.

MATERIALS AND METHODS

Primate arterial smooth muscle cells were derived from primary thoracic aorta medial explants of the pigtail monkey (*Macaca nemestrina*). They were grown in 250-ml Falcon flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a modified Dulbecco-Vogt medium supplemented with 10% heat-inactivated fetal calf serum or 5% heat-inactivated homologous serum as described elsewhere (10). Only flasks containing cells grown to confluence were used for each experiment.

Studies on Soluble Elastin Precursor

LABELING OF CELLS AND EXTRACTION: Cells were labeled in the absence of serum by medium lack-

ing lysine. They were preincubated for 1 h in 10 ml of Dulbecco-Vogt medium without lysine but supplemented with 50 μg/ml each of ascorbic acid and β-aminopropionitrile (BAPN). The medium was then replaced with fresh medium containing ascorbic acid, BAPN, and 2.5 μCi/ml of DL [6-^{3}H] or DL [4,5-^{3}H]lysine (New England Nuclear, Boston, Mass.) and incubated for 24 h at 37°C. After incubation the medium was separated. The cells were homogenized in 5.0 ml of 0.1 M NaH$_2$PO$_4$, 0.15 M NaCl, pH 7.5 (PBS) at 4°C, with a ground glass homogenizer, and centrifuged at 20,000 g for 10 min (8). The insoluble residue was re-extracted and the supernates were combined. The cell extract and the medium were then dialyzed extensively against water at 4°C to remove free radioactive lysine, and lyophilized.

PREPARATION OF LYSYL OXIDASE AND ASSAY OF SUBSTRATE: Lysyl oxidase was obtained from embryonic chick cartilage by the method of Siegel and Martin (16). The lyophilized medium or cell extract to be assayed as substrate to lysyl oxidase was dissolved in PBS to make 1 mg (approximately 1-2 million counts) per ml and dialyzed against PBS. The standard assay mixture consisted of 0.9 ml of the enzyme preparation, 0.5 ml of the substrate containing 0.5 ml of the lyophilized medium or cell extract in PBS, and 0.1 ml of PBS. After incubation at 37°C for 8 h, the water in the sample was collected by distillation (16) and 1.0 ml was counted with Beckman Readysolv VI counting solution (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) in a Packard model 3320 liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill). Control samples lacked enzyme or were supplemented with 100 μg of BAPN. The amount of substrate was calculated by subtracting the cpm tritium released in the presence of BAPN from that liberated in its absence. This difference represents the cpm inhibited by BAPN, which is a specific inhibitor of lysyl oxidase (the lysyl oxidase in the assay might arise from the enzyme or substrate preparation.)

COLLAGENASE DIGESTION: For collagenase digestion, 0.95 mg of the cell extract in 0.95 ml of 0.05 M Tris buffer, pH 7.5 containing 0.005 M CaCl$_2$ and 0.001 M N-ethylmaleimide (the latter was added to inhibit nonspecific proteinases (9), was incubated with 125 U (50 μg) of purified collagenase (collagenase Form III obtained from Advance Biofactures Corp., Lynbrook, N. Y.). After 24 h at 37°C the incubation mixture was dialyzed against PBS and divided into two parts. One part was assayed with lysyl oxidase in the absence of BAPN and the other with BAPN.

PURIFICATION OF TROPOELASTIN[1]: The labeled cells were extracted in 5 ml/flask of cold 0.5 M acetic acid (7) and the extract was lyophilized.

[1] The uncross-linked precursor to elastin has been referred to as tropoelastin.

To the lyophilized material, 3-5 mg of purified pig tropoelastin carrier (obtained from copper-deficient pigs (14)) was added and the mixture dialyzed against 0.02 M NaH$_2$PO$_4$, 1.0 M NaCl, pH 8.0. The dialysate was incubated at 37°C for 1 h in order to coacervate the tropoelastin, which was subsequently separated by centrifugation at 42,000 g for 30 min (17). Further purification was achieved by fractionation with organic solvents (15). The coacervate was dialyzed extensively against 0.1 M ammonium formate buffer, pH 5.5; 1.5 vol of N-propanol and 2.5 vol of n-butanol were then added to the dialyzed material. After the precipitated material was removed by centrifugation, the supernate containing tropoelastin was lyophilized.

POLYACRYLAMIDE GEL ELECTRO-PHORESIS: Separation of tropoelastin was achieved in 7.5% gels in the presence of 6 M urea at pH 4.8 (17, 18). Authentic tropoelastin prepared from pig (14) or chicks (gift of Dr. R. B. Rucker, College of Agriculture, Davis, Calif.) was used for reference. Under these conditions, collagen and tropoelastin are conveniently separated from each other and other proteins. Molecular weight was determined by sodium dodecyl sulfate (SDS) gel electrophoresis in 5% gels according to the method of Weber and Osborn (19). For this purpose, bovine serum albumin, ovalbumin, and pepsin were used as standards. In both methods, the gels always contained carrier tropoelastin and after electrophoresis they were stained with Coomassie Blue. For monitoring the radioactivity, the gels were sliced, digested in 30% H$_2$O$_2$ at 60°C, and counted in Aquasol (New England Nuclear) (8).

Studies of Desmosine Cross-Link Formation

For these experiments, cells that remained at confluence for periods of 3, 4, 10, or 14 wk were used. The cells were labeled with 10 μCi/flask of L[U-^{14}C]lysine (New England Nuclear, Boston, Mass.) in the absence of BAPN. New isotope was added with each change of culture medium. Labeling was carried out for 24 h, 72 h, 7, 10, and 21 days. At the end of each labeling period, the medium was removed and lyophilized. The cells were trypsinized (5 ml of 1 mg/ml trypsin in 0.1 M NH$_4$HCO$_3$), the flask was washed with an additional 2 ml of trypsin solution and the wash was added to the cell suspension which was subsequently lyophilized. Half of each of the lyophilized suspensions was dissolved in 2 ml of constant boiling HCl and hydrolyzed under vacuum for 20 h at 110°C. The tubes were cooled, opened, and dried under a stream of nitrogen.

Control experiments were carried out with normal diploid monkey skin fibroblasts (obtained from the same donors that provided the smooth muscle cells) and L cells (courtesy of Dr. Charles Nabors, Department of Anatomy, University of Utah). Monkey skin fibroblasts were labeled for identical time periods as the smooth muscle cells, whereas the L cells were labeled for 24 h or 9 days.

Longer incubation of L cells was not possible because of cell detachment.

CHROMATOGRAPHY OF CROSS-LINK AMINO ACIDS: The hydrolysates were dried, defatted in 2 ml of chloroform, and dissolved in 5 ml of 0.15 M pyridine acetate, pH 4.5. 1 ml of the hydrolysate was mixed with a hydrolysate derived from 10 mg of cold elastin and separated on an Aminex A-5 column by a modified method of Green et al. (3). This modification consisted of an eight-chamber automatic gradient device substituted for the three-step manual procedure. The eight chambers were each loaded with 50 ml of buffer solution as follows: chambers 1, 2, and 3 contained pyridine-acetate, pH 4.5 (0.15 M pyridine); chambers 4, 5, and 6 contained pyridine-acetate, pH 4.5 (0.30 M pyridine); and chambers 7 and 8 contained pyridine-acetate, pH 5.0 (1.0 M pyridine). Fractions of 2.8 ml were collected and assayed for amino acids by the ninhydrin reaction, and for radioactivity by scintillation counting. The fractions of desmosine were counted twice, using 0.1 ml and 1.0 ml samples, respectively.

FIGURE 1 Release of tritium from cell extract of [4,5-^{3}H]lysine-labeled smooth muscle cells. The cell extract was dissolved in and dialyzed against PBS and assayed with lysyl oxidase as described under Materials and Methods. The assay was carried out without BAPN (●—●) and with 100 μg of BAPN (O- - -O).

RESULTS

Synthesis of Tropoelastin

The distribution of substrate for lysyl oxidase in the medium and extract of cells labeled for varying periods is given in Table I. Most of the substrate is associated with the cell extract (88%, 83%, and 72% after 4, 24, and 72 h of labeling, respectively), and only a minor portion is present in the medium. The release of tritium was proportional to the amount of substrate employed for assay and is inhibited by BAPN (Fig. 1). The amount of total substrate (medium plus cells) increases with labeling time (Table I).

The lyophilized medium and cell extract from cultures labeled for 24 h in the presence of BAPN were subjected to electrophoresis in 6 M urea at pH 4.8. This method is specific for tropoelastin, the soluble precursor of elastin, and separates it from collagen and other proteins (8, 17). The results demonstrate that the cell extract contains a radioactive peak that corresponds to tropoelastin (Fig. 2 A). This peak represents 3.4% of the radioactivity applied on the gel. In the medium, no significant peak is observed in this region (Fig. 2 B). Similar results were obtained with cells labeled for 6 h (not shown).

Because most of the lysyl oxidase substrate activity as well as the material electrophoretically migrating with tropoelastin is present in the cell extracts, these extracts were employed for further
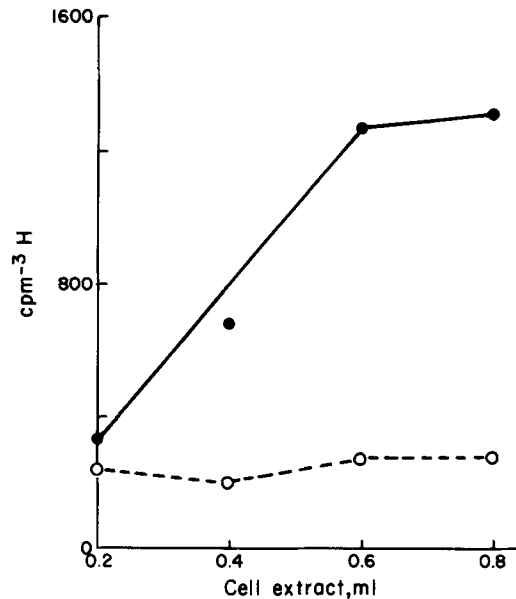
characterization of tropoelastin. Such extracts were derived from cells labeled for 24 h.

That the lysyl oxidase substrate associated with the cells is not due to collagen was confirmed by collagenase digestion, because the substrate action of collagen and not tropoelastin is destroyed by collagenase[2] (Table II). Table III demonstrates that after treatment with collagenase the lysyl oxidase substrate obtained from the cell extract was recovered completely.

The presence of tropoelastin in the cell extract was further investigated by the isolation and purification of the radioactive tropoelastin from the cell layer. For this purpose, the cells were extracted in 0.5 M acetic acid, because by this method tropoelastin is obtained in undegraded form (7). Nonradioactive carrier tropoelastin was added to the cell extract, and purification was achieved by standard procedures employed for tropoelastin, namely coacervation and fractionation with organic solvents (15, 17). The preparation so obtained had 0.9% of the radioactivity of

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[2]Narayanan, A. S., R. C. Page, and G. R. Martin. Unpublished data.

Distribution of Lysyl Oxidase Substrate in Medium and Cell Extract of Smooth Muscle Cells Labeled for Varying Periods

Labeling time	Incubations*	Tritium release	Substrate activity‡	Substrate/flask§	
				Tritium release	%
h		cpm		cpm	
4	Lysyl oxidase + cell extract	451	226	603	88
	Lysyl oxidase + cell extract + BAPN, 100 µg	225			
	Lysyl oxidase + medium	237	31	83	12
	Lysyl oxidase + medium + BAPN, 100 µg	206			
24	Lysyl oxidase + cell extract	1,446	776	2,795	83
	Lysyl oxidase + cell extract + BAPN, 100 µg	673			
	Lysyl oxidase + medium	621	253	577	17
	Lysyl oxidase + medium + BAPN, 100 µg	368			
72	Lysyl oxidase + cell extract	1,353	773	3,744	72
	Lysyl oxidase + cell extract + BAPN, 100 µg	580			
	Lysyl oxidase + medium	631	227	1,430	28
	Lysyl oxidase + medium + BAPN, 100 µg	404			

* Each incubation contained 0.9 ml of lysyl oxidase, 0.5 mg of lyophilized cell extract or medium in 0.5 ml of PBS in a total vol of 1.5 ml. 0.1 ml of BAPN solution in PBS containing 100 µg was added where indicated.
‡ The substrate activity was calculated by subtracting the cpm Tritium released in the presence of BAPN from that in the absence of BAPN and represents Tritium release from 0.5 mg of substrate.
§ Calculated for the total weight of lyophilized cell extract or medium per flask.

the original cell extract (in a typical experiment, 4.9×10^4 cpm were obtained from 5.4×10^6 cpm of the cell extract). That this material is tropoelastin was confirmed by electrophoresis at pH 4.8 in 6 M urea. From Fig. 3 it is observed that the chief radioactive component of the preparation possessed electrophoretic mobility identical to that of authentic pig tropoelastin. Similar results were observed by SDS gel electrophoresis (Fig. 4). By the latter method, the molecular weight of this radioactive fraction was calculated to be 72,000 ± 3,000; this value agrees well with that reported for pig and chick tropoelastin (7, 14, 17).

Since only a small percent of the total radioactivity is recovered in tropoelastin, it is likely that this fraction represents cellular proteins that are nonspecifically adsorbed by the carrier tropoelastin and which appear in the same region as the carrier during electrophoresis. In order to exclude this possibility, three confluent flasks of human gingiva fibroblasts that predominantly synthesize collagen and no tropoelastin were labeled similarly, carrier tropoelastin was added and purification was achieved by coacervation and organic solvent extraction as described above. By this method, some radioactivity was indeed adsorbed to tropoelastin (in a typical experiment 5.8×10^3 cpm (0.4%) were recovered from 1.4×10^6 cpm), but this preparation did not contain a radioactive tropoelastin peak when subjected to urea-gel electrophoresis (Fig. 3) or SDS-gel electrophoresis (Fig. 4).

Synthesis of Desmosine Cross-Links

The above studies, especially the isolation of a component that has the characteristics of tropoelastin, strongly indicate the presence of this protein in the cell extracts. Further proof of this possibility and of elastin formation was sought by an analysis for desmosine and isodesmosine, the cross-links unique to elastin. This study was performed in cultures labeled in the absence of BAPN. The presence of isotopically labeled desmosine and isodesmosine in smooth muscle cultures is illustrated in Fig. 5 and Table IV. These data represent distribution of counts of cell extracts since the medium contained no counts in the cross-link amino acids (now shown). Fig. 5 demonstrates a typical elution pattern of amino acids from a hydrolysate of a smooth muscle cell culture

system in which both desmosine and isodesmosine have been formed. The identification of desmosine and isodesmosine peaks was facilitated by the addition of a cold elastin hydrolysate which produced a recognizable amino acid pattern with ninhydrin that was used as an internal standard in each experiment. In order to qualify as the cross-links, two peaks of approximately equal radioactivity must correspond to the desmosine and isodesmosine of the internal standard, in our belief.[3] The validity of each of these peaks as desmosines in each experiment was confirmed by high voltage paper electrophoresis and Cadmium-ninhydrin stain (2). If necessary, the paper electrophoresis was carried out in duplicate. One paper strip was then cut into 1-cm strips, and radioactivity of each strip was measured by liquid scintillation counting. This was compared with the duplicate strip that was stained (Fig. 6).

Only the smooth muscle experiment gave activity corresponding precisely with the cross-links. Monkey skin fibroblast and L-cell control cultures
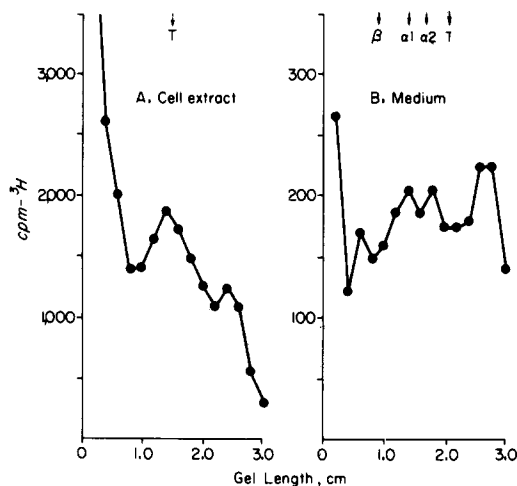

FIGURE 2 Polyacrylamide gel electrophoresis in 6 M urea of medium and cell extract obtained from labeled arterial smooth muscle cells. 2,898 cpm of the medium and 52,597 cpm of the cell extract were separated. Radioactivity of each 2-mm slice is indicated. The migration of β, $\alpha 1$ and, $\alpha 2$ chains of collagen and chick tropoelastin (T) are shown. The tropoelastin peak of the cell extract (1,786 cpm) represented 3.4% of the loaded radioactivity. The migration of pig tropoelastin is identical to that of the chick preparation.

[3] Narayanan, A. S., and R. C. Page. Manuscript in preparation.

TABLE II
Action of Collagenase on Chick Aorta Tropoelastin

| Treatment | Lysyl oxidase substrate | |
	Tritium release	%
	cpm	
Control	1,215	100
Collagenase digested*	954	77

[4,5[3]H]labeled tropoelastin was obtained from embryonic chick aortas as described in Narayanan, Page and Martin, B. B. Acta 351:126 (1974). About 22% of the lysyl oxidase substrate in this preparation is due to collagen (above ref.).
* Digested with 450 U of Collagenase III (Advance Biofactures Corp., Lynnbrook, N. Y.) in the presence of 5 mM Ca^{++} and 1 mM N-ethylmaleimide for 24 h at 37°. Under these conditions, 23% of the radioactivity was released in acid-soluble form.

for identical time points were uniformaly negative with respect to desmosine and isodesmosine (not shown).

Primate arterial smooth muscle cells and skin fibroblasts in culture yielded several other areas of radioactivity which have not yet been fully characterized. These are designated as X and Y in Fig. 5. Lysinonorleucine and hydroxylysine are seen as part of peak Y. In contrast, in labeled cultures of L cells (24-h and 9-day incubations), none of the polyfunctional amino acids were visualized. The baseline was absolutely flat even in the X and Y areas, and lysine was the only peak of radioactivity in this portion of the chromatogram (not shown). From these chromatograms, it appears that the monkey skin fibroblasts and aortic smooth muscle cells are both producing connective tissue elements containing lysinonorleucine, whereas L cells are definitely not oriented towards this capability. In contrast, desmosines were found associated only with smooth muscle cell products and not with dermal fibroblasts.

DISCUSSION

Although smooth muscle cells have been said to be responsible for connective tissue formation, until recently there has been relatively little data to demonstrate that these cells synthesize collagen, both elastic fiber proteins, and glycosaminoglycans (10, 11, 13, 20). In a series of in vivo observations in the developing rat aorta, we were able to demonstrate, by autoradiographic exami-

TABLE III

Collagenase Treatment of Lysyl Oxidase Substrate obtained from Cell Extract

			Substrate	
Treatment	Incubation	Tritium release	Tritium release	%
		cpm	cpm	
Control	Lysyl oxidase + untreated cell extract	1,093	663	100
	Lysyl oxidase + untreated cell extract + BAPN, 100 μg	430		
Collagenase digestion*	Lysyl oxidase + digested cell extract	886	726	110
	Lysyl oxidase + digested cell extract + BAPN, 100 μg	160		

* Both treatments contained equal amounts of cell extract. 0.95 mg of lyophilized cell extract was digested with 125 U (50 μg) of collagenase III for 24 h at 37°C. Reaction was stopped by adding EDTA to 10 mM, and the digest was dialyzed against PBS before assay with lysyl oxidase. Assay conditions are the same as those described under Table I.
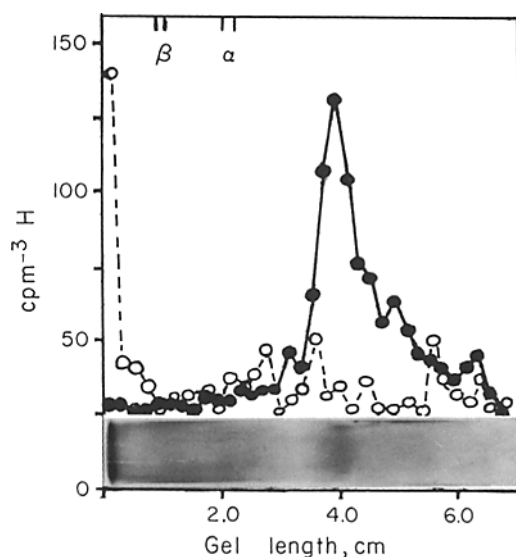

FIGURE 3 Urea gel electrophoresis of radioactive tropoelastin fraction purified from cell extracts of smooth muscle cells and human gingiva fibroblasts. The conditions were identical to those of Fig. 1. The migration of authentic pig tropoelastin is shown by a gel (with 50 μg protein) run under identical conditions. 459 counts obtained from smooth muscle cells (●—●) and 617 counts from a preparation from human gingiva fibroblasts (O- - -O) were loaded on the gel. Counts of each 1-mm slice are shown.
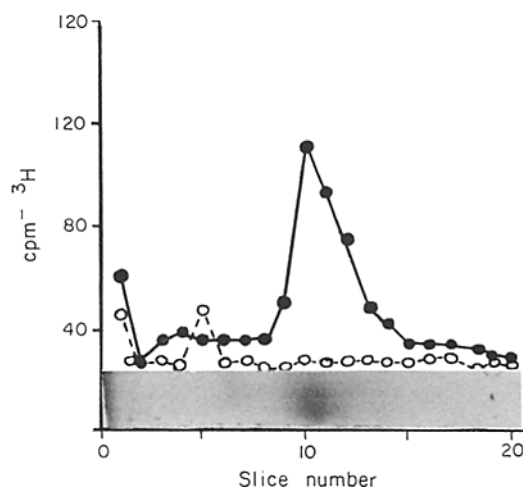

FIGURE 4 SDS gel electrophoresis of radioactive tropoelastin fraction purified from cell extracts of smooth muscle cells and human gingiva fibroblasts. Migration of authentic pig tropoelastin is shown with a gel that contained the smooth muscle cell preparation and carrier and run under identical conditions. Radioactivity of each 2-mm slice is shown. Approximately equal amounts of radioactive proteins from smooth muscle cells (●—●) and gingiva fibroblasts (O- - -O) were separated.

nation of [³H]proline incorporation, that smooth muscle cells incorporate proline into collagen and elastic fibers during growth and development (13).

The ability to grow arterial smooth muscle cells in culture presented the opportunity to study the growth characteristics of these cells, and their ability to form connective tissue matrix proteins in vitro. The factors that control protein formation and elaboration are not yet well understood. These investigations have been pursued to develop a system for studying the formation of tropoelastin, the soluble precursor to elastin, the isolation of this protein, and the factors that control its synthesis and secretion, as well as the formation of extracellular desmosine cross-links.

The initial evidence for the synthesis of soluble

elastin in these studies has been the incorporation of [³H]lysine into a lysyl oxidase substrate that is present in both the medium and cells but largely associated with the latter. The fact that most of this activity was associated with the cell layer even in the presence of the lathyrogen BAPN suggested that the material does not diffuse away from the cells as readily as does collagen in culture (5). Howe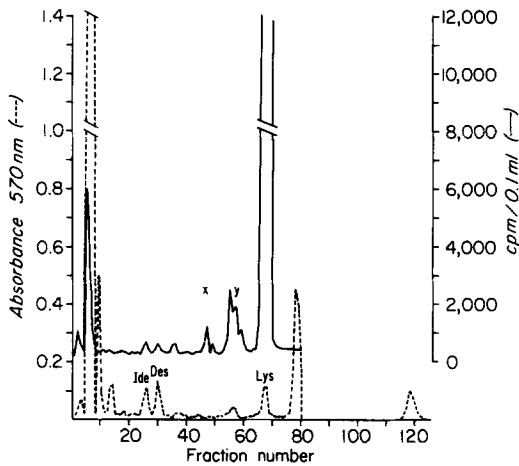ver, it is not clear as to whether this material is intracellular, associated with the cell surface, or bound to the extracellular matrix.

The material serving as a substrate for lysyl oxidase and obtained from the cells appeared to be noncollagenous as indicated by its resistance to collagenase. The electrophoresis data of the crude

FIGURE 5 Elution pattern of isodesmosine (Ide), Desmosine (Des), Lysine (Lys), and several unidentified elastin-related substances (peaks *x* and *y*) from Aminex A-5 ion-exchange resin. The sample consisted of a hydrolysate of monkey smooth muscle cells which had been incubated for 21 days with 10 μCi of L[U¹⁴C]lysine. After drying, the sample was mixed with 10 mg of a cold elastin hydrolysate in 0.15 M pyridine-acetate pH 4.5 and applied to the column. Amino acids were detected by the ninhydrin color reaction (broken line), and radioactivity by scintillation counting (solid line).
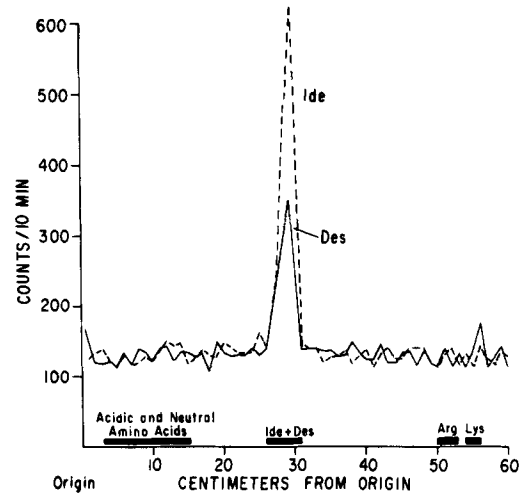
FIGURE 6 Radioactivity pattern of isodesmosine and desmosine peaks from the experiment described in Fig. 5. Each peak was subjected to high voltage paper electrophoresis. Radioactivity was detected by liquid scintillation counting of 1-cm portions. Thus, four points comprise each peak (27–30 cm). Both polyfunctional amino acids can be seen to electrophorese as a single peak. Both have identical mobilities well separated from those of other elastin-containing amino acids, the mobilities of which were determined from a standard electrophoresed simultaneously. Only two-thirds of the desmosine peak was loaded. Conditions of electrophoresis are identical to those described in ref. 3.

TABLE IV

Summary of Lysine-Desmosine Conversion Experiments with Monkey Aortic Smooth Muscle Cell in Culture

| Incubation time with ¹⁴C lysine | Radioactivity expressed as percent of total | | | | Lysine activity × 10⁻⁸* |
	Isodesmosine & Desmosine	Peak X	Y area	Lysine	
	%	%	%	%	
24 h	0	14.3	2.8	76.5	29.6
72 h	0.02	1.8	2.0	92.8	60.1
7 days	0.08	2.1	3.7	88.4	104.8
10 days‡	0	0.8	0.6	95.9	54.0
21 days	0.23	0.2	1.7	94.8	39.6

* Expressed as cpm per 0.1 ml of all column fractions containing lysine. (see Fig. 4). Total cpm of lysine in each hydrolysate would be 280 times this. Counting efficiency was 80%.
‡ Incubated with 5% adult monkey serum rather than 10% fetal calf serum.

cell extract is indicative of the presence of tropoe-
lastin-like material in the cell extract. However,
the radioactivity present under the peak of tropo-
elastin represented only 3.4% of the total. This and
the presence of additional radioactive material in
the origin (Fig. 2 A) indicate that tropoelastin is
not the only component synthesized by the smooth
muscle cells. In similar experiments, Abraham et
al. have recently observed that only 7.2% of the
radioactive proteins synthesized by pig aorta cells
is elastin (1) (calculated from their data).

Confirmation of the presence of tropoelastin-
like material in the cell extract was achieved by
purification. Owing to the high content of hydro-
phobic amino acids, tropoelastin possesses charac-
teristic properties of coacervability above 20°C
and solubility in organic solvents. Only a protein
with similar amino acid composition will be ex-
pected to exhibit these properties. The presence of
a protein with similar properties strongly indicates
the presence of tropoelastin-like material in
smooth muscle cell cultures. That this protein is
indeed tropoelastin and not some nonspecific
protein that is adsorbed by carrier tropoelastin is
shown by the absence of radioactive tropoelastin
peaks in preparations obtained from fibroblasts.
The tropoelastin from smooth muscle cells is
similar to authentic tropoelastin in electrophoretic
behavior in 6 M urea and SDS gels, and has a mol
wt of 72,000, a value which agrees well with that
reported for pig and chick tropoelastion.[4]

These observations are strongly suggestive of the
potential of arterial smooth muscle cells to synthe-
size elastin components in culture. In order to
clearly establish that this is so, we performed a
separate series of investigations and examined the
incorporation of [14C]lysine into the cross-link
amino acids, desmosine, and isodesmosine, that
are unique to elastin. Experiments were performed
on relatively long-term cultures in the absence of
lathyrogen. It was of particular interest that these
culture experiments carried out in the presence of
10% fetal calf serum, revealed an increasing accu-
mulation of radioactivity in the desmosine and
isodesmosine areas (Fig. 5 and Table IV) as well as
a significant but variable amount of label in the
lysinonorleucine area (Y) and in an uncharacter-
ized peak (X). The radioactivity of desmosine and
isodesmosine represents 0.23% of the total after 21
days in culture. Although this suggests some

inefficiency in the formation of cross-links in vitro
it is not greatly removed from an "ideal result", in
that the cell extract after 24 h of labeling contained
only 3.4% of radioactivity in the tropoelastin
fraction (Fig. 2 A).

Although the activity of the cross-links is small,
we consider it to be significant. We have carefully
evaluated these amino acids by high voltage paper
electrophoresis to ascertain that they are indeed
the desmosine cross-links (Fig. 6). We therefore
believe that the presence of the desmosine cross-
links, which are unique to elastin, proves conclu-
sively that elastin is being synthesized in culture.
Repeated feeding of lysine to the cultures and the
known delay of incorporation of lysine into desmo-
sine (6) no doubt account for some of the dispro-
portionately high activity in lysine. Pulse labeling
would give a truer picture, and such studies are
currently underway. In the absence of the la-
thyrogens, it is presumed that the elastin becomes
insoluble due to cross-link formation, and it is
suggested that earlier observations of "amorphous
material" in longterm cultures of guinea pig
smooth muscle cells probably represented insolu-
ble elastin formation (10).

One of the observations made during this series
of studies was that relatively little lysine was in-
corporated into soluble elastin when the cells were
grown in the presence of homologous adult serum
rather than fetal calf serum (Table IV). We do not
yet know whether the role of fetal calf serum is
due to the fact that the serum was derived from
fetal vs. adult blood, or whether it is related to
factors present or absent in the two different classes
of donors. Studies are in process to examine the
role of fetal primate serum and to determine
whether substances are present in fetal serum that
may enhance desmosine formation and that may,
therefore, be necessary for cross-linking and the
formation of elastin fibers during growth. This
interesting aspect of these observations requires
further examination and elucidation.

[4]Narayanan, A. S., G. R. Martin, and R. C. Page.
Unpublished data.

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