

# Elastin Synthesis by Ligamentum Nuchae Fibroblasts: Effects of Culture Conditions and Extracellular Matrix on Elastin Production

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**ABSTRACT** Fetal bovine ligamentum nuchae fibroblasts maintained in culture synthesized soluble elastin but were unable to form the insoluble elastic fiber. Secreted elastin precursors accumulated in culture medium and were measured using a radioimmunoassay for elastin. When elastin production was examined in ligament tissue from fetal calves of various gestational ages, cells from tissue taken during the last trimester of development produced significantly more elastin than did cells from younger fetal tissue, with maximal elastin synthesis occurring shortly before birth. Soluble elastin was not detected in ligament cells plated at low density until proliferation began to be density inhibited and the cells became quiescent. Also, soluble elastin production per cell declined with increasing population doubling or with age in culture.

Cells grown in the presence of 5% fetal calf serum produced approximately four times as much soluble elastin as cells grown in serum-free medium. The addition of dexamethasone (0.1  $\mu$ M) and bleomycin (1  $\mu$ g/ml) increased soluble elastin production by cultured cells 180% and 50%, respectively, whereas theophylline (5  $\mu$ g/ml) depressed production 50% and antagonized stimulation by dexamethasone. Ascorbate (50  $\mu$ g/ml), soybean trypsin inhibitor (1 mg/ml), insulin (100  $\mu$ U/ml), and aminoacetonitrile (50  $\mu$ g/ml) had no effect, but cycloheximide at  $10^{-4}$  M completely inhibited soluble elastin production.

In contrast to cells in culture, ligament tissue minces (ligament cells surrounded by *in vivo* extracellular matrix) efficiently incorporated soluble elastin precursors into insoluble, cross-linked elastin. In addition, soluble elastin production per cell (per microgram of DNA) was higher in tissue minces than elastin production by cells maintained on plastic. These results suggest a role for extracellular matrix in formation of the elastic fiber and in stabilizing elastin phenotypic expression by ligament fibroblasts. Fibroblasts from the bovine ligamentum nuchae present an excellent model for *in vitro* studies of elastin biosynthesis.

A central question in the analysis of elastin biosynthesis is the nature of the signals involved in activating and regulating elastogenesis throughout development of elastic tissues. Studies designed to investigate elastin biosynthesis have been impeded by the insolubility of elastin and the lack of applicable model systems to test the effects of physiologic or environmental stimuli on elastin metabolism. Although elastin is synthesized as a soluble precursor molecule (tropoelastin), *in vivo* cross-linking reactions quickly and covalently join soluble elastin chains into an insoluble, polymeric protein that cannot be

dissociated into component subunits (15). The constraints imposed by this process complicate both chemical evaluation of elastin precursor and description of the biological processes of elastin biosynthesis.

In this report, we examine elastin synthesis by cells from bovine fetal ligamentum nuchae. This tissue, in large grazing mammals such as cattle and sheep, has the highest content of elastin of any tissue and the relative ease with which ligament elastin can be purified makes it the best tissue for studying the biochemical and physical properties of elastin. The ligamentum

nuchae contains a single fibroblast cell type (12, 18) that is easily propagated in culture and provides an excellent model for studying elastin biosynthesis. In culture, ligament cells fail to covalently link soluble elastin precursor molecules into an insoluble polymeric elastic fiber. By measuring soluble elastin levels in cell culture medium, it is possible to use ligament cells to study elastin biosynthesis generally, to study interactions between the cell and its microenvironment, and to observe the effects of biological and pharmacological factors on elastin production by a cell specialized to secrete an extensive extracellular matrix of which elastin is the major component (9, 18, 37).

## MATERIALS AND METHODS

### Materials

Fetal calf serum and other cell culture supplies were obtained from Kansas City Biological, Kansas City, Mo.; EN<sup>3</sup>HANCE from New England Nuclear, Boston, Mass.; [<sup>3</sup>H]lysine (40 Ci/mmol), [<sup>3</sup>H]leucine (52 Ci/mmol), and methyl-<sup>3</sup>[H]thymidine (21 Ci/mmol) from Amersham Corp., Arlington Hgts., Ill.; [<sup>14</sup>C]lysine (312 mCi/mmol) from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.; Budget-solve scintillation cocktail and [<sup>3</sup>H]valine (1 Ci/mmol) from Research Products International Corp., Elk Grove Village, Ill.; Hexadrol phosphate (dexamethasone sodium phosphate) from Organon Inc., West Orange, N. J.; Illetin (insulin) from Eli Lilly and Co., Indianapolis, Ind.; Blenoxane (bleomycin sulfate) from Bristol Labs, Syracuse, N. Y.; and theophylline, cycloheximide,  $\beta$ -aminopropionitrile fumarate, aminoacetonitrile HCl, and ascorbic acid from Sigma Chemical Co., St. Louis, Mo.

### Explant and Cell Cultures

Ligamentum nuchae were obtained from fetal calves within 30 min after the death of the mother. Fetal ages were determined by measuring the forehead to rump length (5). Ligaments were cleaned of adherent fat and fascia, washed in warm Hanks' balanced salt solution, minced into pieces (1–3 mm<sup>3</sup>), and incubated directly as described below, or used as explants for starting primary cell cultures. 10–20 explant pieces in 2 ml of Dulbecco's modified Eagle's (DME) medium supplemented with nonessential amino acids, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and 10% fetal calf serum were dispersed over the bottom of a 25-mm plastic tissue culture flask and attached by gently standing the flask cap-side-up. After 30–40 min at 37°C, an additional 2 ml of DME medium was added to cover the explants, and the flasks were placed in a 5% CO<sub>2</sub>/95% air moist incubator at 37°C.

The explants were removed from the flasks after 10 d and reattached to new culture flasks, yielding a second batch of cells. Cells that grew from the explants were detached with 0.025% trypsin/0.02% EDTA and replated in 100-mm plastic culture dishes at a density of  $1.5 \times 10^6$  cells/dish. Culture medium was replaced twice weekly.

### Antibody Specificity—Immunoprecipitation of Tropoelastin

Dialyzed and lyophilized tissue extract from 270-d fetal ligament minces radiolabeled overnight with 10  $\mu$ Ci/ml [<sup>3</sup>H]leucine in the presence of 50  $\mu$ g/ml aminoacetonitrile was dissolved in 20 mM Tris, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 (21). Nonimmune rabbit serum (1:50 dilution) and goat anti-rabbit globulin (1:10 dilution) were added and the mixture was incubated overnight at 16°C. The immunoprecipitate was removed by centrifugation in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 2 min. Antiserum to bovine ligament alpha-elastin was added to the supernate to a dilution of 1:250, and the mixture was incubated overnight at 16°C. Immune complexes were precipitated by an overnight incubation at 16°C with a 1:5 dilution of goat anti-rabbit globulin. The precipitate was sedimented by centrifugation in a microfuge for 2 min, and the pellet was washed three times by repeated centrifugation in 0.05 M Tris buffer, pH 7.4, containing 0.15 M NaCl, 0.5% deoxycholate, 1.0% Tween-20, 10.0 mM EDTA, 1 mM *N*-ethylmaleimide, and 5 mM phenylmethanesulfonyl fluoride. The washed pellet, along with an aliquot of the initial tissue extract and the supernate from the immunoprecipitation, were boiled for 5 min in 0.5 M Tris, pH 6.8, containing 2.3% SDS/10 mg/ml dithiothreitol and analyzed using SDS polyacrylamide gel electrophoresis with a 4% stacking gel and 10% separating gel according to the method of Laemmli (26). Purified ligament tropoelastin and fetal calf skin collagen served as protein markers. After electrophoresis, the gel was fixed

overnight in 10% acetic acid/25% methanol, impregnated for 1 h with EN<sup>3</sup>HANCE, and washed for 1 h in water containing 1% glycerol to prevent gel cracking. The dried gel was exposed for 9 d at –70°C to Kodak XR-5 film prefogged to a background density of 0.15. The developed film was scanned using a Zeinh soft laser scanning densitometer (Biomed Instruments, Inc., Chicago, Ill.).

### Estimation of Soluble Elastin by Radioimmunoassay

Soluble elastin in medium and tissue or cell extracts was quantified by competitive protein binding radioimmunoassay (30) using antiserum to bovine ligament alpha-elastin (31). Briefly, an aliquot from the sample to be tested was incubated overnight at 4°C with antiserum and iodinated bovine ligament alpha-elastin. Antigen-antibody complexes were precipitated with heat-killed, formalinized *Staphylococcus aureus* cells (IG<sub>5</sub>SORB; The Enzyme Center, Inc., Boston, Mass.), and the bound radioactivity in the pellet was counted after several washings. Estimation of immunoreactive soluble elastin in the sample was determined from a competitive binding standard curve using known amounts of alpha-elastin as the competing antigen. Results are expressed as solubilized elastin equivalents.

### Effects of Serum on Total Protein and Soluble Elastin Production

Confluent ligament fibroblasts were incubated with Dulbecco's modified Eagle's (DME) medium containing 0, 2.5, 5.0, 7.5, and 10.0% fetal calf serum in the presence or absence of  $\beta$ -aminopropionitrile (BAPN). Media were removed after overnight incubation at 37°C and dialyzed after the addition of proteolysis inhibitors.<sup>1</sup> Control media (media as above incubated without cells) were treated similarly. Cell layers were washed three times with Hanks' balanced salt solution, scraped into 10 ml of neutral salt buffer (0.02 M phosphate, 0.05 M NaCl, pH 7.4) or HEPES-Triton buffer (0.05 M HEPES, 1.0 M NaCl, 0.1% (vol/vol) Triton X-100, pH 7.5) containing proteolysis inhibitors, and homogenized with a Brinkmann Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.) at high speed for 30 s. Cellular debris was removed by a 20-min centrifugation (16,000 g) and the supernate dialyzed against water. Dialyzed media and cell extract supernates were lyophilized, reconstituted with assay buffer to 2.5% of the original volume, and analyzed for soluble elastin by radioimmunoassay (30).

The effects of serum on total protein synthesis were examined by incubating confluent ligament cell cultures with leucine-deficient DME media supplemented with 1.5  $\mu$ Ci/ml [<sup>3</sup>H]leucine and fetal calf serum as above. After overnight incubation at 37°C, protein secreted into culture media was precipitated and washed with cold 5% TCA, and counted for radioactivity in a Beckman LS8000 scintillation counter using Budget-solve scintillation cocktail.

### Effects of Cell Density on Soluble Elastin Production

Ligament fibroblasts from a 220-d fetus were subcultivated into 20 100-mm tissue culture dishes at a density of  $3 \times 10^5$  cells/plate. Cells were grown in DME medium supplemented with 5% fetal calf serum, nonessential amino acids, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). Medium was harvested every 3 d, pooled, and dialyzed after the addition of proteolysis inhibitors. Dialyzed media were lyophilized and soluble elastin was determined by radioimmunoassay (30). Two plates at each feeding were trypsinized and the cell number per plate was determined using a hemocytometer. DNA was determined in cells as described by Hill and Whatley (20) and in tissue as described by Cookson and Adams (10). Thymidine incorporation was determined using a third plate essentially as described by Pardee (34) with 5 ml/plate growth medium containing 0.2  $\mu$ Ci/ml methyl-<sup>3</sup>[H]thymidine. Radioactivity in the sample was counted as described above.

### Comparison of Soluble Elastin Production by Cultured Ligament Cells and Ligament Tissue Minces

Minces from 185-, 218-, 266-, and 270-d fetal nuchal ligaments and first passage cells derived from tissues of similar ages were incubated for 24 h in

<sup>1</sup> Final concentration of proteolysis inhibitors added to extraction buffers and harvested cell or tissue culture media: phenylmethanesulfonyl fluoride, 5 mM;  $\epsilon$ -aminocaproic acid, 10 mM; EDTA, 5 mM; and *N*-ethylmaleimide, 5 mM.

valine-deficient DME media containing 3% fetal calf serum, 10.0  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-valine, and 100  $\mu\text{g/ml}$  aminoacetonitrile to inhibit cross-linking (14). Conditioned media were dialyzed after the addition of proteolysis inhibitors and lyophilized. Tissues were washed thoroughly with water and homogenized in 0.5 N acetic acid containing 1  $\mu\text{g/ml}$  pepstatin, using a Brinkmann Polytron, and the homogenate was stirred overnight in the cold (4°C). Extract supernates obtained after centrifugation at 10,000 g for 30 min were dialyzed against water and lyophilized. The dried samples were dissolved in phosphate-buffered saline and an aliquot was taken for radioimmunoassay. The remaining sample was boiled in 0.5 M Tris, pH 6.8, containing 2.3% SDS/10 mM dithiothreitol and analyzed using SDS polyacrylamide gel electrophoresis as described above.

### Estimation of Desmosine and Synthesis of Insoluble Elastin

20 100-mm culture dishes of 10-d confluent ligament fibroblasts were incubated for 7 d with three changes of DME medium containing one-half the normal lysine concentration, 5% fetal calf serum, and 1.0  $\mu\text{Ci/ml}$  [ $^{14}\text{C}$ ]lysine. Viable ligament minces were incubated for 24 h with the same medium. The washed cell layers and ligament tissue were then homogenized in distilled water and centrifuged at 16,000 g for 20 min. Elastin was isolated by resuspending the pellets in 10 ml of water and autoclaving twice at 28 psi ( $1.93 \times 10^5 \text{ N/m}^2$ ) for 20 min. The resultant insoluble residues were washed with water and hydrolyzed in constant boiling HCl at 110°C for 72 h.

Incorporation of radioisotope into insoluble elastin was quantified using a Beckman 119C amino acid analyzer with a modified program for resolving elastin cross-linking amino acids (29). Column effluent was collected in 1-ml fractions and radioactivity determined by counting in 15 ml of Budget-solve scintillation cocktail. Elution times for radioactive amino acids in unknown samples were compared with elution times established for radiolabeled lysine, desmosine, and isodesmosine standards.

### Effects of Pharmacologic Agents on Soluble Elastin Production

Confluent cultures of ligament cells in DME medium/5% fetal calf serum were incubated overnight with dexamethasone ( $10^{-7}$  M), bleomycin (1  $\mu\text{g/ml}$ ), theophylline (1 and 5  $\mu\text{g/ml}$ ), dexamethasone ( $10^{-7}$  M) plus theophylline (1  $\mu\text{g/ml}$ ), BAPN (50  $\mu\text{g/ml}$ ), ascorbate (50  $\mu\text{g/ml}$ ), insulin (100  $\mu\text{U/ml}$ ), or cycloheximide ( $10^{-4}$  M). Medium and cell layer extracts, prepared and treated as described above, were analyzed for soluble elastin by radioimmunoassay. Values were normalized to solubilized elastin equivalents per  $10^6$  cells and expressed as percent of control (untreated cells).

### Lysyl Oxidase Determination

Lysyl oxidase activity was determined as described by Starcher et al. (42), using fetal calf ligament substrate. Medium from ligament fibroblasts in two confluent 100-mm culture dishes was concentrated in a macrosolute concentrator (Minicon B-15, Amicon Corp., Lexington, Mass.) to a volume of 1 ml. A 0.5-ml aliquot was mixed for 4 h at 37°C with 0.1 ml of substrate (75,000 cpm) in 1 ml of phosphate buffer. Tritiated water was collected by distillation and quantified by liquid scintillation spectrometry. Enzyme activity was corrected for tritium release in the presence of 50  $\mu\text{g/ml}$  BAPN.

## RESULTS

Ligament fibroblasts readily grow from tissue explants, assuming polygonal morphology in early culture (23) and becoming increasingly bipolar with subsequent passage. Ultrastructurally, cultured cells contain fine filaments similar to the actin filaments noted in myofibroblasts by Gabbiani et al. (16). Early passage cells contain fewer filaments, have shorter division times, and become confluent at higher cell densities than older cells. Cells in early culture appear similar to those found in intact tissue and are ultrastructurally similar to fibroblasts not associated with elastogenesis (12).

### Specificity of Antisera

Antiserum to bovine ligament alpha-elastin specifically precipitated tropoelastin from bovine ligament tissue extracts (31) (Fig. 1). Fetal ligament cell cultures contain large amounts of

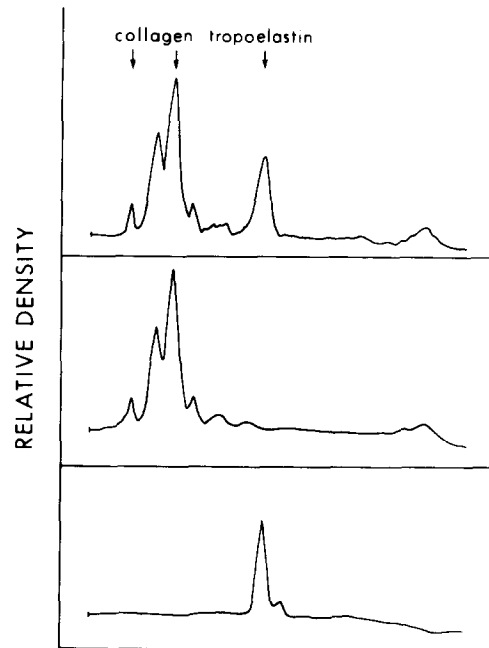


FIGURE 1 Reactivity of antiserum to bovine ligamentum nuchae alpha-elastin with tropoelastin was shown by immunoprecipitation of tissue extract from fetal ligament minces labeled overnight with [ $^3\text{H}$ ]leucine in the presence of aminoacetonitrile. Shown are densitometric tracings from 10% SDS gels of: (top) total tissue extract, (middle) tissue extract supernate after immunoprecipitation, and (bottom) immunoprecipitate. Indicated are the migration positions of fetal calf skin collagen and fetal ligament tropoelastin, which were included as markers.

fibronectin and other proteins that precipitate nonspecifically, contaminating the immunoprecipitate. Contaminating proteins can be largely removed by preincubating with nonimmune serum and goat anti-rabbit globulin and by extensively washing the immunoprecipitate with buffers containing detergents.

### Synthesis of Insoluble Elastin

Newly synthesized insoluble elastin in ligament cell culture or in ligament minces was identified by incorporation of [ $^{14}\text{C}$ ]lysine into desmosine, a lysine-derived cross-link unique to elastin. The sensitivity of radiolabeled desmosine detection by amino acid analysis was  $\sim 10$  ng (20 pmol), corresponding to 1  $\mu\text{g}$  of mature, ligament elastin.

Desmosine was identified in autoclave-insoluble protein isolated from ligament minces after a 24-h labeling period. In contrast, no radiolabeled desmosine was detected in cell layer hydrolysates, even after 7 d of continuous labeling. Soluble elastin was detected in labeling medium from cells but not in medium from ligament minces. Cell layer extracts contained  $<10\%$  of the soluble elastin measured in the medium. The possibility that lysine-derived cross-links other than desmosine joined elastin precursor chains is unlikely, because soluble elastin levels did not increase when BAPN was included in culture medium to inhibit cross-linking. BAPN did inhibit incorporation of [ $^{14}\text{C}$ ]lysine into desmosine in tissue minces. Soluble elastin, which did not diffuse into medium from the minces, was extracted with neutral salt buffer or 0.5 N acetic acid.

Table I shows lysyl oxidase activity in culture medium from ligament cells.

TABLE I  
Lysyl Oxidase Activity in Cultures of Fetal Calf Ligament Fibroblasts \*

	Lysyl oxidase activity‡ cpm/mg protein
Fetal ligament fibroblasts	2392 ± 486
Mouse skin fibroblasts	1304 ± 171

\* Enzyme activity in two confluent 100-mm culture dishes was determined using tritiated fetal calf ligament substrate. Values are corrected for tritium release in the presence of  $\beta$ -aminopropionitrile. Mouse skin fibroblasts represent cells previously shown to synthesize lysyl oxidase and are included for comparison.

‡ Mean ± SD of duplicate determinations.

### Effects of Serum and Culture Conditions

To test the effects of fetal calf serum on total protein and soluble elastin production, cultured cells were incubated with serum-free medium and with identical medium at four different serum concentrations. Total protein synthesis was maximal in medium containing 5% fetal calf serum and remained constant in higher serum concentrations. Soluble elastin production was also elevated with 5% serum but could not be measured reliably at higher serum levels because of high background values in the radioimmunoassay. Background values could be appreciably reduced when fetal calf serum was preabsorbed with anti-alpha-elastin IgG bound to Sepharose. The presence of elastin antigens in serum is currently being investigated. Preabsorption of serum with elastin antibody did not alter the growth rate or rate of elastin production of cultured ligament cells.

Soluble elastin production by cultured cells decreased with serial subcultivation and age in culture. After six trypsinizations over a period of 8 wk, for example, soluble elastin production decreased 50–60% from the levels measured in primary culture. In addition, soluble elastin production by sixth-passage cells was much less sensitive to dexamethasone stimulation than was elastin production by first-passage cells (Fig. 2). When third-passage cells were maintained for 12 wk in culture, soluble elastin production dropped to 1.6 ng/10<sup>6</sup> cells from 32.4 ng/10<sup>6</sup> cells measured after the first week.

### Effects of Cell Density on Soluble Elastin Production

Fig. 3 shows the relation between cell density and elastin production. Soluble elastin was not detected in culture medium of proliferating cells until the cultures approached confluence (as indicated by a reduction in thymidine uptake and a shift from logarithmic growth). Elastin production was greatest during the late log phase and remained relatively constant in confluent cultures. It should be noted that the elastin radioimmunoassay was sufficiently sensitive to detect elastin production by rapidly proliferating cells had they been secreting elastin at the rate determined for cells in confluent cultures.

### Elastin Production Relative to Fetal Age

Soluble elastin production by ligament minces or cultured cells from fetal ligament of various gestational ages was examined by radioimmunoassay. Soluble elastin production was highest in ligaments taken during the last trimester of development, with maximal synthesis occurring shortly before birth. Cultured cells consistently produced less soluble elastin per microgram of DNA than did age-matched ligament minces (Table II). Pulse-labeling experiments of ligament minces in-

cubated with [<sup>14</sup>C]valine in the presence of aminoacetonitrile (Fig. 4) indicated a large increase in the proportion of tropoelastin synthesized in late gestational calves, substantiating the results obtained by radioimmunoassay.

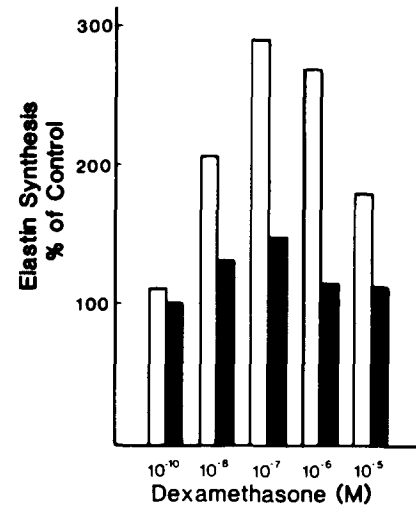


FIGURE 2 Comparison of dexamethasone stimulation of elastin production in ligament fibroblasts at first (open bars) and sixth (solid bars) passage. Confluent ligament fibroblasts were cultured overnight with DME medium supplemented with 5% fetal calf serum and dexamethasone at the concentrations indicated. Soluble elastin levels in media and cell layers were determined by radioimmunoassay. Cell number per plate was determined for each group by trypsinization of one plate and counting of viable cells with a hemocytometer after trypan blue dye exclusion. Values were normalized to soluble elastin equivalents per 10<sup>6</sup> cells and expressed as percent of control (cells not treated with dexamethasone).

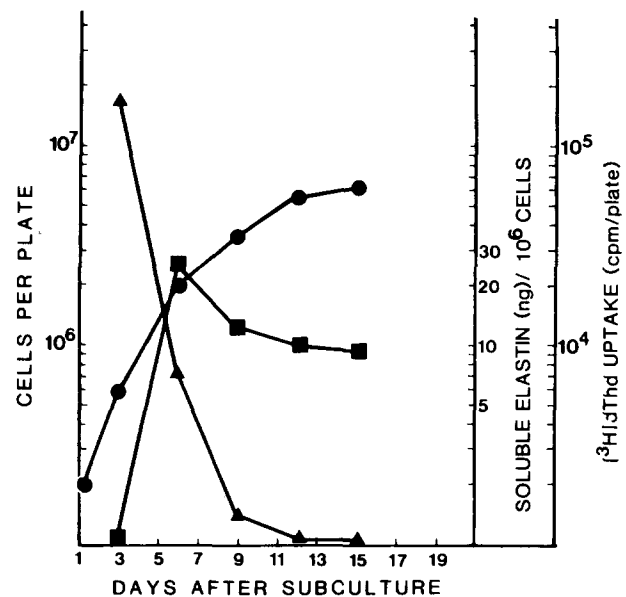


FIGURE 3 Effects of cell density on elastin production. Soluble elastin production was monitored by radioimmunoassay as fetal ligament cells were grown from sparse density ( $3 \times 10^5$  cells/100-mm plate, 20 plates total) to confluence ( $\sim 7 \times 10^6$  cells per plate). Soluble elastin (■) was not detected until cells approached confluence, indicated by reduction in growth. Cell counts (●) represent the mean of replicate determination. ▲, [<sup>3</sup>H]dThd uptake.

TABLE II

Changes in Elastin Synthesis in Bovine Ligament Nuchae during Development: Comparison of Elastin Production by Cultured Ligament Cells and Age-matched Ligament Minces

Fetal age <i>d</i>	Tissue minces		Cultured cells
	$\mu\text{g}$ DNA/g wet weight	Solubilized elas- tin equivalents per $\mu\text{g}$ DNA	Solubilized elas- tin equivalents per $\mu\text{g}$ DNA*
110	ND	ND	$0.09 \pm 0.01$
142	1,407.1	ND	ND
185	1,164.9	$0.84 \pm 0.13$	$0.33 \pm 0.09$
218	1,024.1	$1.28 \pm 0.08$	$0.46 \pm 0.10$
266	876.6	$3.89 \pm 0.10$	$1.02 \pm 0.06$
270	698.9	$4.06 \pm 0.25$	$1.11 \pm 0.18$

ND, not determined.

\*  $\mu\text{g}$  DNA/plate =  $38.3 \pm 1.31$ ;  $n = 5$

### Effects of Pharmacologic Agents on Elastin Production

The addition of  $10^{-7}$  M dexamethasone or 1  $\mu\text{g}/\text{ml}$  bleomycin increased soluble elastin production about 180 and 50%, respectively, over control (untreated) cells (Fig. 5). Theophylline (5  $\mu\text{g}/\text{ml}$ ), however, inhibited elastin production by 50% and was antagonistic to dexamethasone stimulation. Complete inhibition was obtained with  $10^{-4}$  M cycloheximide. Insulin (100  $\mu\text{U}/\text{ml}$ ), BAPN (50 or 100  $\mu\text{g}/\text{ml}$ ), or soybean trypsin inhibitor (1 mg/ml) had no effect, as did ascorbic acid (50  $\mu\text{g}/\text{ml}$ ), although ascorbate-fed cultures were noticeably more resistant to trypsinization and disruption. In no instance did these agents increase cell layer-associated elastin relative to control cells.

### DISCUSSION

Cultured ligamentum nuchae fibroblasts provide a useful model for studying elastin biosynthesis. In this report, we have used the ligament cell model to study: (a) the effects of culture conditions upon elastin production by ligament cells, (b) the effects of biological and pharmacological agents on elastin production, (c) the role of extracellular matrix in stabilizing the elastin phenotype, and (d) changes in elastin synthesis in the ligamentum nuchae during development.

As a tool for studying elastin biosynthesis, ligament cells offer the advantage over other model systems of not forming insoluble elastin in culture. Hence, measurement of soluble elastin released into culture medium is a reliable estimate of total elastin production. The reasons why ligament cells fail to form insoluble elastin in vitro are obscure. Although ligament cells in culture synthesize all known components of the elastic fiber (microfibrillar protein [38], elastin precursors, and lysyl oxidase), it is clear that synthesis of fiber constituents is not sufficient in itself to ensure proper assembly in the extracellular space of a functional elastic unit. One possible explanation for the observation that no insoluble elastin is formed by ligament cells in vitro is that elastic fiber formation is dependent upon still undefined matrix components that are missing in culture. This possibility is consistent with our finding that insoluble elastin formation can be reinitiated in cultured cells by providing the proper extracellular matrix, e.g., growing cultured cells on dead ligament tissue (28).

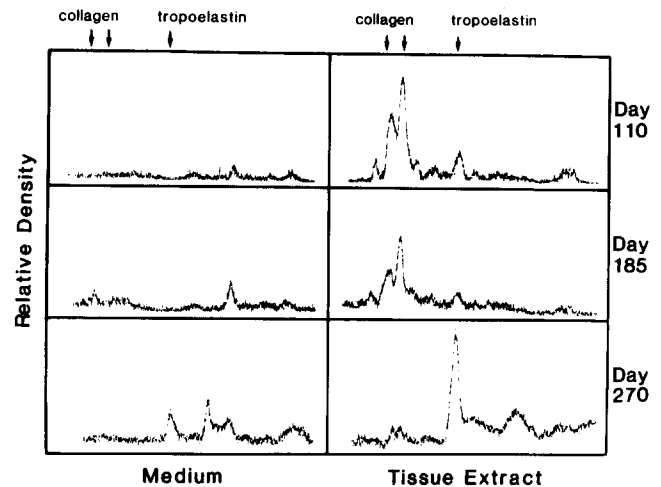


FIGURE 4 Qualitative changes in protein synthesis by ligamentum nuchae during development. Densitometric tracings of  $^3\text{H}$ -labeled proteins synthesized by ligament tissue of different gestational ages and analyzed on SDS 10% polyacrylamide gels. Tissue minces were incubated overnight with [ $^3\text{H}$ ]valine and 100  $\mu\text{g}/\text{ml}$  aminoacetonitrile. 100,000 cpm from concentrated, dialyzed culture media and tissue extracts were loaded on gels. Arrows indicate the migration positions of purified, unlabeled fetal calf skin collagen and fetal ligament tropoelastin, which were included as markers.

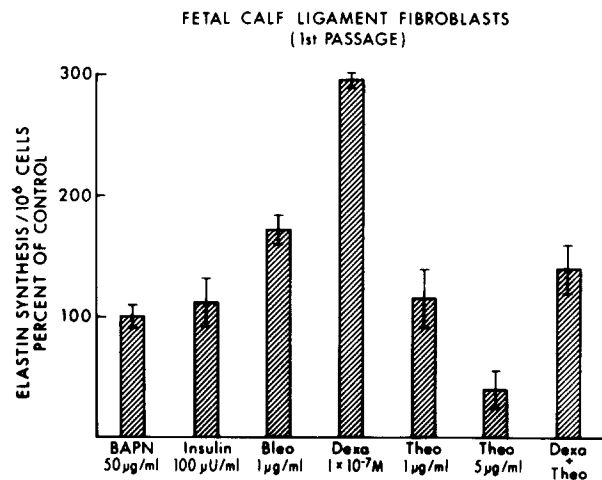


FIGURE 5 Effects of pharmacological and biological agents on soluble elastin production by cultured fetal ligament cells. BAPN, insulin, bleomycin (bleo), dexamethasone (dexa), theophylline (theo), or dexamethasone ( $10^{-7}$  M) plus theophylline (1  $\mu\text{g}/\text{ml}$ ) were incubated overnight with confluent cells in DME medium/5% fetal calf serum. Control cells were incubated with DME medium containing 5% fetal calf serum only. Soluble elastin released into culture medium was quantified by radioimmunoassay. Shown is the mean  $\pm$  SD of triplicate determinations.

Elastin production by ligament cells is highly dependent upon culture conditions. Optimal elastin synthesis requires adequate concentrations of fetal calf serum (>5%) and is highest in cultures shortly after they reach visual confluence. Total protein synthesis parallels elastin production, suggesting a coordination of the two processes. A similar correlation between total protein synthesis and collagen production by fibroblasts has been noted (35, 39).

Changes in the rate of elastin production with cell density suggest that elastin synthesis is not constant throughout all

phases of the cell cycle. Ligament cells produced no detectable soluble elastin until the late log phase when cells became density inhibited and quiescent. Histological studies of developing ligament have shown a correlation between maximal elastin deposition *in vivo* and diminution of cell growth. Thus, there may be an association between elastin synthesis and lengthening of the G1 phase of the cell cycle that occurs during density inhibition and quiescence. Cell density has also been shown to affect production of other connective tissue proteins: Goldberg and Green (17) observed that, during exponential growth, 3T6 fibroblasts secrete only small amounts of collagen with no formation of collagen fibers. In human and guinea pig fibroblasts, the ratio of type I and type III procollagen changes as a function of cell density (1). Hynes and Bye (22) showed that expression of extracellular fibronectin is cell density dependent, and Chen et al. (7) have shown that cell-cell contact stimulates synthesis of fibronectin. The relative importance of cell density effects, cell-cell interactions, and cell-matrix interactions (19, 32) in regulating elastin synthesis, however, is not yet known.

Previous studies have shown that many connective tissue-synthesizing cells lose differentiated functions when cultured *in vitro*. For example, embryonic chick chondrocytes lose the ability to accumulate cartilage-specific proteoglycan and type II collagen when subcultured at low density (4). Similarly, the collagen phenotype of cloned chick chondrocytes grown to senescence changes from type II procollagen to type I (27). In our studies, soluble elastin production by cultured ligament cells declined with long-term culture and repeated subcultivation. Elastin production decreased 50–70% in the interval of five subcultures, and late passage cells were less responsive to hormonal stimulation of elastin synthesis than early passage cells. Although one explanation for this observation could be loss of a differentiated function by elastin-producing cells, we cannot exclude the possibility that primary cultures contain two populations of cells, one composed of rapidly dividing fibroblasts not synthesizing elastin, and a second elastin-synthesizing population with a lower doubling rate. The percentage of rapidly dividing cells would increase with repeated culture passage, producing a dilution effect on total elastin production per culture. Further studies with cloned ligament cells might resolve this question.

These results indicate that precise studies of elastin biosynthesis require careful control of culture conditions. Under conditions optimal for elastin production, the effects of various biological or pharmacological agents on early passage ligament cells were studied (Fig. 5). These agents were selected to provide insights into potentially important mechanisms for regulation of elastin biosynthesis *in vivo*. For example, the addition of dexamethasone (0.1  $\mu$ M) to first-passage cells increased soluble elastin production 180% over controls. This effect of steroid in cell culture is similar to stimulation by glucocorticoids of elastin biosynthesis in embryonic chick aorta reported by Eichner and Rosenbloom (11) and suggests the potential for hormonal regulation of elastogenesis during development. Theophylline (5  $\mu$ g/ml) decreased elastin production in cultured cells by 50% and at 1  $\mu$ g/ml reduced the level of dexamethasone stimulation. Theophylline is known to increase intracellular cAMP levels by inhibiting cAMP phosphodiesterase. Several lines of evidence indicate that intracellular cAMP levels have a regulatory influence on synthesis of other connective tissue macromolecules. For example, agents that elevate intracellular cAMP levels decrease collagen production

dramatically in human newborn foreskin fibroblasts (3). Also, high intracellular cAMP levels block spontaneous somite chondrogenesis and inhibit the ability of somites to respond to the inductive influences of notochord (25). Although theophylline may affect elastin production by mechanisms unrelated to cAMP, recent studies in our laboratory show a correlation between intracellular cyclic nucleotide levels and soluble elastin production in fibroblasts (our unpublished results).

Elastin production increased when bleomycin was added to cell culture medium. Bleomycin, a glycopeptide antibiotic, has been used as an antineoplastic agent in the treatment of various lymphomas and carcinomas. Its effect on elastin biosynthesis was examined because interstitial pulmonary fibrosis is reported to be an unfortunate side effect of bleomycin chemotherapy in some patients. Animal models of pulmonary fibrosis (40) show increased collagen and elastin content in lungs after intratracheal injection of bleomycin (41). Similarly, bleomycin added to lung slices (36) and cultured lung and skin fibroblasts (8) markedly stimulated procollagen biosynthesis. Results from the present study show that bleomycin stimulates elastin production in cultured ligament cells and, thus, may potentiate elastin production in lung cells *in vivo* as well.

Ascorbate is an important cofactor in hydroxylation of proline amino acids in collagen and elastin. Inhibition of proline hydroxylation by ascorbate deficiency greatly reduced secretion of procollagen (13, 43), whereas our results and those of others (2, 33, 43) show that elastin secretion remains unchanged. Hydroxyproline plays an essential role in stabilizing the helical structure of collagen. Underhydroxylated collagen (procollagen) will not fold into the triple-helical conformation at 37°C and is not secreted from the cell. It is unlikely that hydroxyproline serves a similar function in elastin since underhydroxylation does not alter elastin secretion.

In terms of elastin biosynthesis, there are three clear differences between ligament cells grown on tissue culture plastic and cells in viable ligament tissue:

(a) Cells surrounded by extracellular matrix (cells in ligament minces) rapidly incorporate soluble elastin into an insoluble polymer, whereas elastin precursors synthesized by cultured cells remain soluble.

(b) Cells in tissue minces synthesize more elastin per cell (per microgram of DNA) than do ligament cells in culture.

(c) When tissue minces are incubated with BAPN to inhibit cross-linking, soluble elastin is not released into the culture medium but remains associated with the matrix. In contrast, >90% of the soluble elastin synthesized by ligament cells in culture is released into the medium.

These observations suggest that extracellular matrix contributes to the specific differentiated properties of elastin-synthesizing cells and participates in organizing the elastic fiber. Further investigation will require both the identification and isolation of macromolecules that interact in this system before we can understand the molecular basis of elastin-matrix interactions or control of elastin gene expression.

It is now generally accepted that elastin deposition in elastic tissues begins late in fetal development (6, 11, 24). Fahrenbach et al. (12) and Cleary et al. (9) described an abrupt increase in the rate of insoluble elastin deposition in the developing bovine ligament beginning at the fetal age of 180–200 d (beginning the last trimester), rising to a maximum at 235 d, and persisting to the end of the first postnatal month. Our results using ligament cells in culture are in agreement with these histological observations; elastin synthesis is highest in ligament cells

shortly before birth. An intriguing question raised by these findings is the nature of inductive influences on elastin gene expression during progressive tissue differentiation. Although little is known about the biochemical evolution of the ligament fibroblast during development, the bovine ligamentum nuchae promises to be a useful model for studying biochemical and physiological mechanisms that regulate synthesis of elastin gene products.

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