Extracellular Matrix-specific Induction of Elastogenic Differentiation and Maintenance of Phenotypic Stability in Bovine Ligament Fibroblasts

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ABSTRACT We studied the process of elastogenic differentiation in the bovine ligamentum nuchae to assess the mechanisms that regulate elastin gene expression during development. Undifferentiated (nonelastin-producing) ligament cells from early gestation animals initiate elastin synthesis when grown on an extracellular matrix (ECM) substratum prepared from late gestation ligamentum nuchae. ECM from ligaments of fetal calves younger than the time when elastin production occurs spontaneously in situ (i.e., beginning the last developmental trimester at ~180 d of gestation) does not stimulate elastin production in undifferentiated cells. Matrix-induced differentiation requires direct cell matrix interaction, is dependent upon cell proliferation after cell-matrix contact, and can be blocked selectively by incorporation of bromodeoxyuridine into the DNA of undifferentiated cells before (but not after) contact with inducing matrix. Quantitative analysis of elastin synthesis in young cells after matrix-induced differentiation indicates that the entire cell population is competent to respond to the matrix inducer, and continued synthesis of elastin after young cells are removed from the ECM substratum indicates that the phenotypic transition to elastin synthesis is stable and heritable. Although ligament cells do not require continuous contact with ECM to express the elastin phenotype, elastin synthesis is increased substantially when elastin-producing cells are grown on ligament matrix, suggesting that elastogenic differentiation is stabilized by ECM. The matrix substratum was also found to alter the distribution of tropoelastin between the medium and matrix cell layer. When grown on tissue culture plastic, ligament cells secrete >80% of newly synthesized tropoelastin into the culture medium. When cultured on ECM, however, 50–70% of the newly synthesized tropoelastin remains associated with the cell layer and is cross-linked to form insoluble elastin as shown by the incorporation of radiolabeled lysine into desmosine.

A fundamental concept of developmental biology is that embryonic development is under the influence of the microenvironment and that many developmental steps made by the cell are guided by environmental stimuli. It is now clear that the extracellular matrix (ECM)[1] plays an essential role during development of most vertebrate tissues and organs by providing signals that affect cellular differentiation (for pertinent reviews, see references 17, 38, 44).

The interaction between ECM and embryonic cells has been under intensive investigation and many examples have been presented of what Grobstein (14, 15) termed "embryonic induction" or tissue interaction, that is, the interaction of different but closely associated cell populations to generate new and diverse cell types during development. Most instances of inductive tissue interactions are heterotypic—involving more than one cell type—and can be classified according to the source of the inducing and responding tissue, respectively, as epithelial–epithelial, epithelial–mesenchymal, or mesenchymal–epithelial (16). There are few examples, however, of homotypic cell-matrix interactions wherein a

[1] *Abbreviations used in this paper*: BAPN, β-aminopropionitrile; BrdU, bromodeoxyuridine; DME, Dulbecco's modified Eagle's (medium); ECM, extracellular matrix; FCL, fetal calf ligament; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; RIA, radioimmunoassay; TCA, trichloroacetic acid.

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cell's own extracellular matrix is autocatalytic in initiating new gene expression or directing progressive differentiation (although it is well known that extracellular matrix can stabilize the differentiated phenotype beyond the time of final commitment [1, 32, 42]).

In this study, we investigated the mechanisms underlying the developmental transitions of a fibroblastlike mesenchymal cell in the developing bovine ligamentum nuchae. The ligament cell is specialized to secrete an extensive ECM of which elastin is the major component. The ease with which fetal calf ligament (FCL)[2] cells can be maintained in culture (20, 29) and the availability of sensitive analytical tools to study elastin synthesis make it possible to observe how changes in the cellular environment affect the developing phenotype. Our results show that differentiation and phenotypic stabilization of the ligamentum nuchae fibroblast, as expressed by elastin synthesis and secretion, is guided by inductive interactions between the cell and its own secreted ECM. The ligament cell does not require signals or products from other cell types for normal differentiation.

MATERIALS AND METHODS

Cell Culture: Ligamentum nuchae were obtained from fetal calves within 90 min after the death of the mother. Fetal ages were determined by measuring the forehead to rump length (4). Ligaments were cleaned of adherent muscle and fascia, washed in warm Hanks' balanced salt solution (HBSS), minced into small pieces with a scalpel, and used as explants for starting primary cell cultures. Cells were cultured in growth medium consisting of Dulbecco's modified Eagle (DME) medium containing high glucose and high bicarbonate and supplemented with nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (all fetal calf serum used in this study was from a single serum lot, Kansas City Biological, Lenexa, KS). Cells were subcultured with trypsin as described earlier (29).

Quantification of Soluble Elastin Production by Radioimmunoassay: Soluble elastin in medium and in tissue or cell extracts was quantified by competitive protein binding radioimmunoassay (RIA) using rabbit antiserum to bovine ligament α-elastin as described (26–28). Results are expressed as α-elastin equivalents normalized to cell number or to micrograms of DNA. DNA content was determined as described by Hill and Whatley (18). Maximum sensitivity for the RIA was approximately 5–10 ng of elastin per ml.

Preparation of Ligament ECM Substratum: To prepare acellular ligament ECM, fetal bovine ligamentum nuchae was subjected to repeated (10 times) freeze thawing. The loss of tissue viability was confirmed by incubating a section of the tissue for 6 h with [^{3}H]leucine in leucine-deficient DME medium–10% fetal calf serum and analyzing the medium for protein-associated trichloroacetic acid (TCA) insoluble radioactivity.

To prepare the ligament as a substratum for cell culture, nonviable tissue was serially sectioned tangential to the long axis of the ligament on a freezing microtome to produce 80-μm-thick slices. Tissue slices were spread over the bottom of tissue culture dishes and sterilized by rinsing with 70% alcohol and drying overnight under a germicidal lamp (GE No. 15T8, General Electric Co., Cleveland, OH). Freshly trypsinized FCL cells were then plated on the ligament slices and cultured as described above. Cell division was determined by [^{3}H]-thymidine incorporation as described by Pardee (33) using growth medium containing 0.2 μCi/ml methyl-[^{3}H] thymidine (sp act 2 Ci/mmol, New England Nuclear, Boston, MA).

Preparation and Testing of Conditioned Medium: 5 g of minced 270-d-old fetal ligament tissue (both viable and nonviable) and three 850-cm^2 roller bottles of confluent FCL-270 cells were incubated for 2 d with growth medium buffered with 30 mM HEPES at pH 7.4. The conditioned media (100 ml from tissue minces and 25 ml from each roller bottle) were filtered through 0.22-μm Nalgene filters (Nalge Co., Rochester, NY) and used directly or were stored frozen at −70°C. Late log phase FCL-129 and FCL-270 cells in 60-mm^2 dishes were re-fed with culture medium containing conditioned medium at concentrations from 20 to 80% (vol/vol). After 3 d, the media were removed and assayed for elastin by RIA. In all determinations, the results were corrected for elastin in the conditioned medium.

Radiolabeling and Immunoprecipitation of Tropoelastin: Proteins synthesized by confluent ligament cell cultures were radiolabeled overnight with 1 μCi/ml of [^{3}H]leucine (sp act 10 Ci/mmol, New England Nuclear) in 5 ml/plate of leucine-deficient DME medium containing 100 μg/ml D-penicillamine, 100 μg/ml β-aminopropionitrile (BAPN), 5% fetal calf serum, nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 30 mM HEPES, pH 7.4. At the end of the labeling period, 100 μl of medium was removed for determination of total protein synthesis and the remaining medium was dialyzed against water (four changes, 4 liters each) and lyophilized. The cell layers were scraped into 0.5 N acetic acid and extracted overnight at 4°C by end-over-end stirring. After removing cellular debris by centrifugation (3 min, Beckman model 12 microfuge, Beckman Instruments, Inc., Spinco Div., Palo Alto, CA), the supernatant was dialyzed against water as above and lyophilized.

Lyophilized samples were reconstituted in 200 μl of 20 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM EDTA, and then preabsorbed to reduce nonspecific precipitation by adding normal rabbit serum to a dilution of 1:20 followed by a five times excess (vol/vol) of a 10% suspension (wt/vol) of IgGSORB (The Enzyme Center, Boston, MA). The solution was made 0.5% in Triton X-100 and left at 4°C for 18–24 h. The samples were centrifuged for 3 min in the microfuge and the pellets were discarded. Immunoprecipitation of tropoelastin in the supernatant was as described previously (30).

Total Protein Synthesis and Specific Activity of Intracellular Amino Acid Pools: Total protein synthesis was determined by TCA precipitation of radiolabeled proteins in medium from radiolabeling experiments described above. 100 μl of medium was made 10% in TCA and left on ice for 1 h. The insoluble proteins were pelleted by centrifugation for 3 min in the microfuge, washed twice with cold 10% TCA, redissolved in 0.1 N NaOH, neutralized with 0.5 N HCl, and counted for radioactivity in a Beckman LS8000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

In all radiolabeling experiments, values for total protein synthesis were adjusted for changes in the specific activity of the intracellular amino acid pool. For pool size determination, the cell layer was washed three times rapidly with cold HBSS, scraped from the plate in 2 ml of 0.01 N HCl, and sonicated for 1 min using a Fisher model 300 sonic dismembrator with microprobe. Cellular debris was removed by centrifugation (1,000 *g*, 10 min) and the supernatant was evaporated using a Speedvac Concentrator (Savant Instruments, Inc., Hicksville, NY). The dried pellet was suspended in 1 ml of analyzer sample dilution buffer, and total leucine in a 700-μl aliquot was determined by amino acid analysis with a Beckman 119C amino acid analyzer (Beckman Instruments, Inc., Fullerton, CA). [^{3}H]Leucine was determined by collecting the analyzer column effluent and counting each fraction for radioactivity. The ratio of [^{3}H]-leucine counts per minute to total free leucine was designated the pool size factor and was used for comparison between experiments.

In Vitro Radiolabeling of Desmosine: Ligament cells grown on tissue culture plastic or on nonviable ligament substratum were incubated for 7 d with three changes of DME medium containing one-third the normal lysine concentration, 5% fetal calf serum, and 1.0 μCi/ml [^{14}C]lysine. At the end of the labeling period, the cell layers were scraped in distilled water and homogenized using a Brinkmann Polytron with a PT-20 generator (Brinkmann Instruments, Inc., Westbury, NY). The pellets obtained after centrifugation at 16,000 *g* for 20 min were washed with water by repeated centrifugation until radioactivity in the supernatants was <100 cpm/ml. The pellets were then hydrolyzed in constant boiling HCl at 110°C for 48 h. Incorporation of radioisotope into desmosine and isodesmosine was quantified by collecting column effluent from the amino acid analyzer and counting the collected fractions for radioactivity by scintillation spectrometry. The analyzer column was developed with a modified program for resolving elastin cross-links (28). Elution times for desmosine and isodesmosine were determined using authentic radiolabeled standards kindly provided by Dr. Barry C. Starcher (University of Texas at Austin).

Immunofluorescence of Elastin and Fibronectin in Cell Culture: FCL cells were subcultured (using trypsin) into tissue culture chamber/slides (Lab Tek Div., Miles Laboratories Inc., Naperville, IL) at 5 × 10^4 cells per well. After 4 d the cells were fixed for 30 min at room temperature with 1% paraformaldehyde–5% sucrose in phosphate-buffered saline (PBS) and stained for elastin or fibronectin as described (30). Antifibronectin IgG was a gift from Dr. John A. McDonald, Jewish Hospital at Washington University Medical Center, St. Louis, MO.

Bromodeoxyuridine: FCL-132 and FCL-270 cells were passaged from explant culture at 1 × 10^6 cells in 100-mm^2 culture dishes. Shortly before reaching confluency one-half of the cultures were treated for 48 h with 30 μM

[2] Fetal calf ligament cell strains are identified by the abbreviation FCL followed by the age of the fetus from which the cells were derived. For example, FCL-135 indicates fetal calf ligament cells from a 135-day fetus.

bromodeoxyuridine (BrdU, Sigma Chemical Co., St. Louis, MO), removed from the plates using trypsin, and were seeded at subconfluent density on culture dishes or onto FCL-270 ligament substratum in the presence or absence of 30 μM BrdU. After 72 h the cultures were sampled for elastin production or cells were trypsinized and seeded into tissue culture/chamber slides for immunofluorescence microscopy. In a parallel set of experiments, BrdU was added to the cultures after the cells had been plated on the ligament substratum for at least 48 h. To determine if the effects of BrdU were reversible, medium containing 150 mM thymidine was added to cells 72 h after plating. After 48 h of thymidine exposure, the cultures were assayed for elastin production.

The extent of incorporation of BrdU into DNA was determined by incubating cultures with [^{3}H]BrdU (2 μCi/ml, sp act 26 Ci/mmol, New England Nuclear, Boston, MA). At the end of each incubation period, the cells were collected by trypsinization and solubilized with 2% Na_2CO_3 in 0.1 N NaOH (wt/vol). Nucleic acids were precipitated with 10% TCA and counted for tritium radioactivity.

Because of the increased photosensitivity of BrdU-treated cells, all tissue culture work and experiments with BrdU were conducted in a room equipped with gold fluorescent bulbs (GE No. F40G0, General Electric Co.).

Fibroblast Elastase: Preparation of Cell Extracts, Plasma Membranes, and Conditioned Medium: 40 100-mm culture dishes of confluent 245-d FCL cells were washed with HBSS and one-half of the plates were incubated overnight with 5 ml/plate of DME medium plus 10% fetal bovine serum. The remaining 20 plates were incubated for the same period with 5 ml/plate of serum-free DME medium. Conditioned media with and without serum were pooled separately, dialyzed for 48 h against water using Spectrapor No. 1 dialysis tubing (molecular weight cutoff 6,000–8,000, Spectrum Medical Industries, Inc., Los Angeles, CA), and lyophilized. The cell layer in each group was washed with HBSS, scraped from the plates in 1 ml of PBS, pooled, and disrupted by freeze-thawing three times followed by sonication for 1 min. Cellular debris was removed by centrifugation at 10,000 *g* for 10 min and the supernatant (PBS extract) was assayed for elastase as described below.

A detergent extract was prepared by washing FCL-245 cells in four, 870-cm^2 roller bottles with 85 mM Tris, pH 7.8, 30 mM NaCl, 10 mM glucose, 1 mM EDTA, 1% wt/vol octyl-β-glucopyranoside (Sigma Chemical Company, St. Louis, MO). The detergent extraction buffer was added to the first bottle in each age group and cells were scraped from the walls with a rubber policeman. The slurry was then transferred to the next bottle. After the final extraction, the cell slurry was centrifuged at 10,000 *g* for 30 min. The pellet was discarded and the supernatant was frozen until further analysis.

Plasma membranes from FCL-245 cells were prepared using a modification of the procedure of Thom et al. (40). Four confluent 850-cm^2 roller bottles (~7 × 10^7 cells per bottle) were rinsed once with ice-cold PBS and the cells were scraped into 10 ml per bottle of cold PBS. The slurries were pooled and the cells were pelleted by centrifugation at 500 *g* for 10 min. The cell pellet was resuspended to a total of 5 ml in cold PBS and added dropwise to 500 ml of 20 mM Na borate, 0.2 mM EDTA, pH 10.2, to lyse cells. After stirring for 10 min, twice the volume of 0.5 M Na borate, pH 10.2, was added with continued stirring for 5 min at room temperature. The solution was filtered through nylon gauze and the filtrate was centrifuged at 500 *g* for 5 min at 4°C. The supernatant was removed from a loose pellet and recentrifuged at 12,000 *g* for 2 h at 4°C. The plasma membrane-rich pellet was resuspended in PBS by dounce homogenization and layered on 4.5 ml of 35% (wt/wt) sucrose solution in PBS. The plasma membrane-enriched fraction was removed from the top of the sucrose after centrifugation at 150,000 *g* for 30 min, resuspended in a convenient volume of PBS and collected by centrifugation at 10,000 *g* for 30 min at 40°C. Membranes were stored frozen in PBS.

Solubilization of elastin peptides from purified, ^{14}C-labeled insoluble bovine elastin (2) or hydrolysis of succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide (Bachem, Inc., Torrence, CA) as described by Bieth et al. (3) was used to assay for elastase activity in conditioned medium, cell extracts, and plasma membrane-enriched fractions.

RESULTS

Quantitative Changes in Elastin Production in Developing Ligamentum Nuchae

Changes in the level of elastin synthesis by ligament cells in culture or in minces of fetal ligament tissue at different gestational ages are shown in Fig. 1. Elastin production showed a progressive increase from low levels in 140-d tissue to a maximum value reached shortly before birth. Production of elastin by ligament cells in culture followed closely that of the tissue, although total elastin synthesis by cultured cells was less than that of age-matched tissue minces. These results are in agreement with the studies of Fahrenbach et al. (7) that describe an intense period of elastogenesis occurring in fetal ligament during the last developmental trimester.

Is the Switch to Elastin Production the Result of an Intrinsic Developmental Clock?

Long-term culture was used to examine if the onset of elastin production in ligament cells is governed by an intrinsic developmental program that activates the elastin phenotype at a precise time or after a specific number of cell doublings (8, 46). As shown in Fig. 2, no elastin production was detected in cultures of first-passage 140-d-old ligament cells maintained in culture for 3 mo, even though the culture period included the developmental "window" when cells in intact tissue differentiate and begin elastin synthesis. As a positive control, elastin production by differentiated cells maintained under identical culture conditions continued throughout the culture period. The failure of young cells to differentiate spontaneously in culture suggests that a developmental clock is not, in itself, influencing the transition to elastin production.

Control of Differentiation by Extracellular Matrix

Widespread evidence indicates that phenotypic differentiation of many connective tissue cells is strongly influenced or perhaps completely dependent upon cellular or tissue inter-

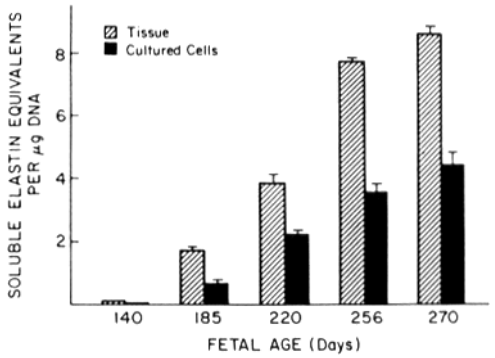

FIGURE 1 Changes in the level of elastin production in bovine ligamentum nuchae fibroblasts or tissue minces during development. Cell cultures and tissue minces were incubated overnight in DME medium containing 10% fetal calf serum, 100 μg/ml BAPN, and 100 μg/ml D-penicillamine. Elastin levels in the medium and in an acetic acid extract of tissue minces or in the cell layer of first-passage cells were determined by RIA. Values are expressed as α-elastin equivalents per microgram of DNA for the medium and acid extract combined and represent the mean and standard deviation of duplicate determinations.

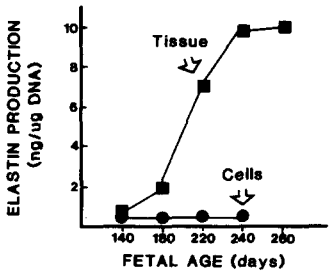

FIGURE 2 To determine if ligament cells are intrinsically programmed to begin elastin production, undifferentiated first-passage cells from a 140-d-old fetus were maintained in culture for 100 d (subcultured twice). The cells did not initiate elastin synthesis (circles) during the period when elastin production normally occurs in the tissue (squares).

actions. The results of experiments designed to determine the involvement of ECM materials in regulating elastogenic differentiation of ligament cells are shown in Fig. 3. To simulate the extracellular matrix of intact tissue, whole ligaments from fetal calves of various gestational ages were repeatedly freeze-thawed to kill endogenous cells and sliced thinly using a freezing microtome. The tissue slices were then used as a substratum upon which FCL cells were grown. In this way it was possible to expose cells from one gestational age to ECM produced by the same cell type at a different stage of development, and thus test the effects on differentiation of these extracellular matrices that are compositionally and structurally identical to the developing in situ matrix. Ligament cells plated on ligament slices formed a confluent monolayer within 3 d and did not penetrate the matrix substratum (data not shown). The morphology of cells grown on ECM was similar to that of cells on plastic with the noticeable exception that cellular cytoskeletal filaments were organized in a plane parallel to the ECM surface in the basal regions of cells grown on the biological substratum.

The results in Fig. 3 demonstrate that ECM from fetal ligaments of 270 d of gestation had a marked effect on the differentiation state of young, undifferentiated cells. After growing on the older matrix for 3 d, cells from 140 d of gestation synthesized elastin at levels equivalent to old cells from fully differentiated tissue. This degree of stimulation was characteristic for cells from all fetal ages, including cells as young as 95 d of gestation. In 13 such experiments, elastin production by induced cells ranged between 3- and 17-ng of soluble elastin equivalents per μg of DNA with a mean value of 10 ng and a standard deviation of ± 3. Variation in elastin production could be attributed to a decreased response to matrix as a cell population aged in culture (late-passage cells did not respond as well as those in early passage) and to variation inherent in the elastin RIA.

Matrix-induced changes in differentiation were specific for elastin production in that no significant stimulation of total protein synthesis was observed in the cells grown on ligament as compared to the control (Table I).

To determine whether ligament matrix from earlier fetal ages could stimulate elastin production in young cells, FCL-140 cells were grown on ECM from a 140-d ligament. Fig. 3 shows that the level of elastin synthesis by both young and old cells grown on the young matrix was not significantly different from cells maintained on tissue culture plastic. ECM from ligament tissue younger than 180 d (before overt differentiation of ligament cells in situ) had no stimulatory effect on differentiation as measured by elastin synthesis.

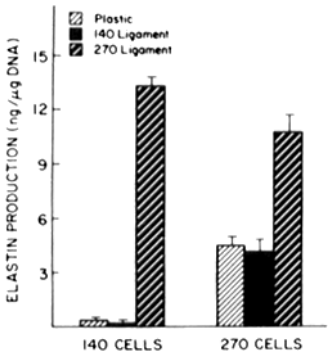

FIGURE 3 Comparison of elastin production by ligament cells grown on tissue culture plastic or on ligament ECM. Ligamentum nuchae from fetal calves of 140 and 270 d of gestation were repeatedly freeze-thawed to kill endogenous cells and sliced to thicknesses of 80 μM with a freezing microtome. Freshly trypsinized first-passage ligament cells were seeded directly onto the ligament ECM or onto tissue culture plastic and incubated with DME medium containing 10% fetal calf serum, 100 μg/ml BAPN, and 100 μg/ml D-penicillamine. Elastin levels (medium + cell layer extract) were determined by radioimmunoassay. Values are the mean plus standard deviation of triplicate determinations of duplicate plates.

TABLE I

Total Protein Synthesis by FCL-130 Cells Grown on Plastic or on Inducing ECM

	Substratum	
	Plastic	Matrix*
[^{3}H]Leucine incorporation (*cpm/μg DNA*)	2.97×10^7	2.84×10^7
Pool size factor‡	23.2	25.1

* ECM from 270-d of gestation ligamentum nuchae.
‡ [^{3}H]Leucine cpm/total free leucine (pmol).

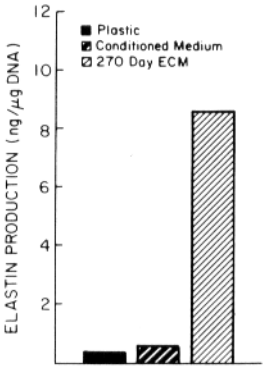

FIGURE 4 Effects of conditioned medium on elastin production. First-passage, undifferentiated ligament cells were grown on tissue culture plastic, on tissue culture plastic in medium conditioned by ECM, or in direct contact with ECM from inducing ligament. After 3 d, elastin levels were determined by RIA. Only cells in direct contact with ligament ECM expressed the elastin phenotype.

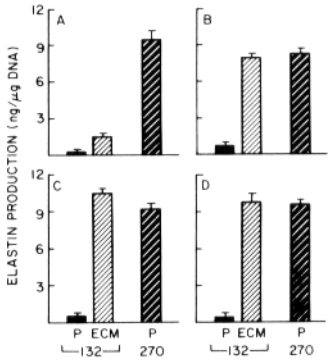

FIGURE 5 The effects of BrdU on induction of elastin synthesis by ECM. Subconfluent, first passage-FCL-132 (132) and FCL-270 (270) cells were grown for 48 h in culture medium containing 30 μM BrdU, removed from the dishes using trypsin, and reseeded onto tissue culture plastic (*P*) or onto FCL-270 ligament matrix (*ECM*) in the presence of 30 μM BrdU. Elastin production was determined by RIA after 72 h (*A*) and the cells were refed for 72 h with culture medium containing 150 mM thymidine (*B*) or were removed by trypsinization and replated onto culture dishes or matrix in the absence of BrdU (*C*). *D* shows elastin production by cells grown on plastic or on inducing matrix for 48 h before exposure to BrdU. Values are the mean plus standard deviation of triplicate determinations of duplicate plates. The level of substitution of BrdU into cellular DNA as determined by [^{3}H]-BrdU incorporation was the same whether BrdU was added before (4.58×10^6 cpm per 10^6 cells) or after (4.70×10^6 cpm per 10^6 cells) matrix induction.

Direct Cell-Matrix Contact Is Necessary for Differentiation

The above studies demonstrate that a differentiation-promoting activity of the developing ligament is associated with the extracellular matrix. To determine whether direct cell-matrix contact is required for ligament cell differentiation, or if diffusible factors emanating from the ligament substratum are promoting new elastin synthesis, we exposed undifferentiated cells to varying concentrations of medium conditioned by viable or dead ligament. The results, shown in Fig. 4, indicate that only those young cells in direct contact with inducing matrix initiate elastin synthesis; conditioned medium had no effect on elastin production or total protein synthesis of undifferentiated cells.

BrdU Blocks Induction of the Elastin Phenotype

In many models of differentiation, incorporation of BrdU into DNA prevents the appearance of the differentiated phenotype. Incorporation of BrdU into DNA of undifferentiated ligament cells blocked the induction of elastin synthesis when BrdU-treated cells were subsequently exposed to inducing ECM (Fig. 5). The inhibitory effects of BrdU could be reversed, however, with exogenous thymidine or by cell multiplication in the absence of BrdU. When undifferentiated cells were grown on inducing matrix for 48 h before the addition of BrdU, induction of elastin synthesis occurred unperturbed (Figs. 5 and 6).

Cell Proliferation Is a Prerequisite for Matrix-induced Differentiation

Because cell proliferation precedes cell differentiation in many developing systems, we examined whether DNA synthesis and cell replication are required for matrix activation of the elastin phenotype in undifferentiated cells. When young cells were seeded on inducing matrix at high density so that cell division was largely inhibited, elastogenic differentiation did not occur (Fig. 7). When these same cells were removed from the matrix with trypsin and were reseeded on plastic or on inducing ligament at lower densities that accommodate cell division, elastin production began in those cells in contact with inducing matrix after 18–24 h, a period that correlates with the time required for young ligament cells to traverse the cell cycle and divide (~22 h). Cells grown on plastic did not differentiate, even though cell growth and division (as measured by thymidine incorporation) was similar to cells grown on matrix.

Reversibility of the Differentiated State and Phenotypic Stability

To answer the question of whether young induced cells require continuous contact with the matrix substratum to maintain their newly acquired differentiated phenotype, FCL-120 cells were induced to differentiate by contact for 2 d with

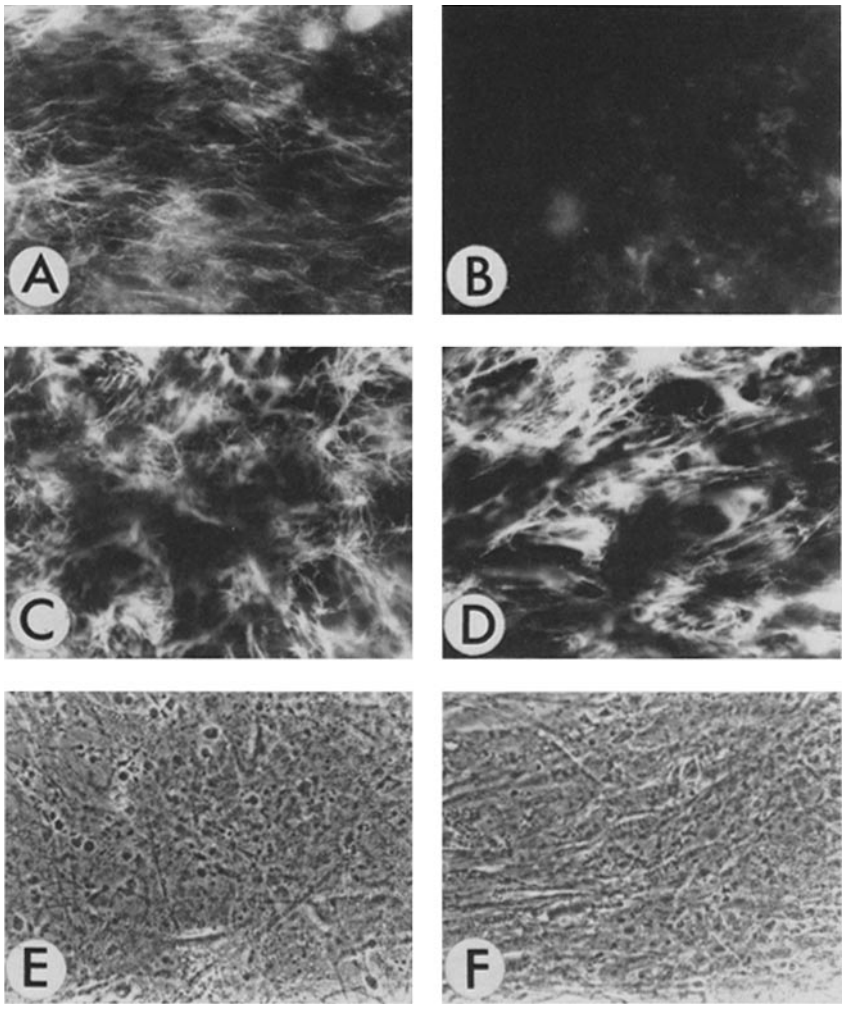

FIGURE 6 FCL-132 cells were grown on inducing matrix for 48 h before and after exposure to 30 μM BrdU. After an additional 48 h, the cells were removed from the matrix by trypsinization and reseeded on culture slides without matrix. Elastin and fibronectin synthesized by the cells were visualized after 4 d by immunofluorescence microscopy. BrdU had no effect on elastogenic differentiation when added after cells had contacted inducing matrix for 48 h (*A*) but blocked the induction of elastin synthesis if incorporated into cellular DNA before matrix exposure (*B*). Fibronectin synthesis was not affected by BrdU (*C–D*), nor did BrdU alter cell morphology (*E–F*, phase contrast). *A* also demonstrates that elastin production continues after the ligament cell is removed from the inducing matrix. × 200.

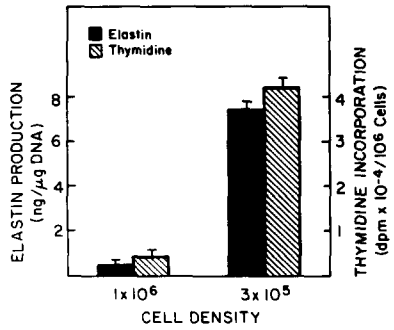

FIGURE 7 Effects of cell density and DNA synthesis on matrix-induced differentiation. First passage FCL-120 cells were plated at 1×10^6 cells onto inducing ligament ECM in 35-mm^2 culture dishes. After 6 h, [^{3}H]thymidine was added to the growth medium and after an additional 3 h the incorporation of [^{3}H]thymidine into DNA was determined as described in Materials and Methods. Elastin levels were quantified by RIA after 72 h and the cells were trypsinized from the matrix and replated onto fresh ECM or onto plastic at 3×10^5 cells per dish. [^{3}H]Thymidine incorporation into DNA and elastin production were then determined as above.

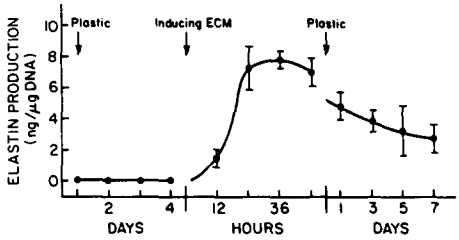

FIGURE 8 Stability of elastin phenotype in ECM-induced cells. First passage FCL-120 cells were grown on tissue culture plastic for 4 d and then stimulated to differentiate by plating onto inducing ECM. After 48 h, the cells were removed from the ECM substratum and replated on tissue culture plastic. Elastin levels were determined by radioimmunoassay at the times indicated.

FCL-270 matrix and passed from the matrix on to tissue culture plastic. Fig. 8 shows that exposure to ECM stimulated elastin production from the basal rate of barely detectable levels to ~8 ng elastin per μg of DNA. Passage of the cells back to tissue culture plastic resulted in a progressive decrease in elastin production to ~50% of the maximal level (4 ng of elastin per μg of DNA). However, even the lowest level observed was still many times higher than elastin production before matrix-induced differentiation. In agreement with the known instability of the elastin phenotype in culture, elastin production by the induced cells decreased rapidly with subsequent subculture.

These results suggest that, in addition to inducing a stable phenotypic change in young cells, ECM may also play an important role in maintaining phenotypic stability in cells after differentiation has occurred. We tested this possibility by growing first-passage FCL-270 cells that synthesize elastin on inducing ligament matrix (270 d), noninducing matrix (140 d), and on plastic. As shown in Fig. 3, elastin production by FCL-270 cells grown on older ligament matrix is almost twice that of the same cells grown on tissue culture plastic in the absence of matrix and approximates that of cells in the intact tissue. However, extracellular matrix from younger ligaments produced no enhancement of elastin synthesis in the differentiated cells (Fig. 3).

Effects of ECM on Insoluble Elastin Production

An important consequence of growing ligament cells on ECM was an alteration in the distribution of tropoelastin between the medium and the matrix cell layer. Ligament cells grown on plastic release 80–90% of newly synthesized tropoelastin into the culture medium. When grown on ligament matrix from a 270-d fetus, however, this ratio reverses with >70% of the newly synthesized elastin remaining associated with the cell layer (Fig. 9). The demonstration of [^{14}C]desmosine in the matrix (Table II) after a 3-d incubation with medium containing [^{14}C]lysine, the precursor amino acid for elastin cross-links (12, 31), provides unequivocal evidence for cross-linking of tropoelastin chains in FCL cells associated with ligament substratum (24). Addition of BAPN prevented desmosine formation but altered only slightly the distribution of elastin between the medium and cell layer (Fig. 9). However, the total amount of soluble elastin extractable from the cell layer increased by 60% with the addition of BAPN, suggesting that the ligament ECM provides a favorable environment for deposition of tropoelastin (perhaps to preexisting elastin fibers in the ligament matrix), even in the absence of cross-linking. These results provide two important pieces of information. First, that ECM promotes fiber organization and facilitates cross-link formation, and second, that these two processes are independent; i.e., cross-linking is not a prerequisite for association of tropoelastin with ECM.

Cell-Matrix Model: Artifact or Altered Gene Expression?

Throughout this study, tropoelastin levels were determined

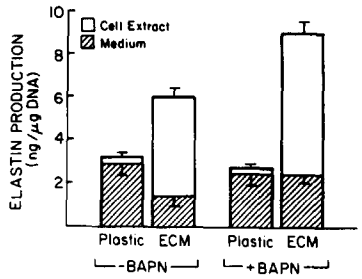

FIGURE 9 Distribution of tropoelastin between the medium and cell layer in first-passage ligament cells grown on tissue culture plastic or on ligament ECM. FCL-270 cells, plated on tissue culture plastic or on FCL-270 matrix, were grown for 4 d in the presence or absence of BAPN. Elastin levels in cell culture medium and in an acid extract of the matrix cell layer were determined by radioimmunoassay. Values are mean plus standard deviation of triplicate values of duplicate plates.

TABLE II

Formation of [^{14}C]Desmosine by FCL-270 Cells Grown on Tissue Culture Plastic or on Ligament ECM during a 7-d Incubation with [^{14}C]Lysine in the Presence or Absence of BAPN

	FCL-270 cells grown on	
	Plastic	ECM‡
	[^{14}C]Desmosine* dpm/10^6 cells	
−BAPN	0	1,364
+BAPN§	0	25

* Values represent total ^{14}C radioactivity in the collected desmosine peak from amino acid analysis.
‡ 4.2×10^7 FCL-270 cells on ECM from a 270-d-old bovine fetus.
§ 100 μg/ml.

by RIA using antibodies to insoluble elastin. Because antielastin antibodies recognize peptide fragments from mature elastin, the RIA cannot differentiate between newly synthesized tropoelastin and peptide fragments from degraded, insoluble elastin (27). Because ligament slices that induce elastin production in young cells (i.e., from tissue older than 180 d) contain substantial amounts of insoluble elastin in the extracellular matrix, it was important to show that elastin levels measured by RIA in the induction experiments were not merely an artifact of degradation of elastin in the ligament substratum by elastase produced by the ligament cells. Although most known elastases are inactive in culture medium that contains fetal calf serum, we investigated the possibilities that (*a*) ligament fibroblasts secrete a unique elastin-degrading protease that is not inactivated by serum inhibitors or (*b*) ligament fibroblasts exhibit an elastolytic enzyme bound to the plasma membrane or secreted beneath the cell at the site of cell matrix contact, inaccessible to serum inhibitors. No elastin-degrading activity could be detected using either soluble substrates for elastase (data not shown) or ^{14}C-labeled elastin (Table III) in conditioned serum-free or serum-containing cell culture medium, in detergent or neutral salt extracts of elastin-producing FCL cells, or associated with purified plasma membranes. These results suggest that FCL cells do not degrade elastin in the ligament substratum and that the cell-matrix model is appropriate to evaluate matrix induction of elastin synthesis.

Further evidence that ECM is stimulating elastin gene expression in young cells is given in Table IV. Immunoprecipitation of tropoelastin from cultures of young cells grown on inducing ECM shows an almost twofold increase in immunoprecipitable counts from the medium and a nearly fivefold increase in counts from an acid extract of the cell layer compared with cells grown on plastic. Although this increase in elastin production is not as great as that observed by RIA (due to inherent difficulties associated with immunoprecipitation using low titer antibodies and problems involved in concentrating and preparing samples), the results support the conclusion that young cells initiate tropoelastin synthesis after contacting inducing ECM. This point is shown conclusively in Fig. 6: elastin is clearly evident by immunofluorescence microscopy in cultures of young cells after matrix induction but no elastin is present when matrix-induced differentiation is blocked by BrdU. The results in Fig. 6 also demonstrate the stability of the induced phenotype in that induced cells continue to synthesize elastin when removed from the matrix substratum.

TABLE III

Assay for Elastase Activity in Ligamentum Nuchae Fibroblast Conditioned Medium, Cell Extracts, and Plasma Membranes

^{14}C-radioactivity released from ^{14}C-labeled insoluble elastin* by			
Human leukocyte elastase	cpm released	Ligament cells‡	cpm released
ng	*Standard curve*		
0	206 ± 21	medium–0% serum	206 ± 21
10	923 ± 46	medium–10% serum	267 ± 18
20	1,623 ± 84	cell extract–salt	278 + 16
30	2,348 ± 30	cell extract–detergent	234 ± 11
40	2,798 ± 45	plasma membranes§	211 ± 14
50	3,033 ± 143		

* 50 μl of radiolabeled elastin suspension (300 cpm/μg) prepared according to Bielefeld et al. (2) and 50 μl of the sample to be assayed were thoroughly mixed in 150 μl of reaction buffer (50 μl polyoxyethylene lauryl ether/100 ml PBS) and incubated overnight at 37°C. Insoluble elastin was pelleted by centrifugation at 10,000 *g* for 5 min in a microfuge and solubilized peptides in the supernatant were determined by scintillation spectrometry for ^{14}C radioactivity. The results are the mean ± SD of triplicate measurements.

‡ Conditioned medium from 245-d-old ligament cells or neutral salt and detergent extracts of the cell layer were concentrated 10-fold under positive pressure using an Amicon PM-10 membrane before analysis.

§ Plasma membranes from ~7 × 10^7 cells.

TABLE IV

Immunoprecipitation of [^{14}C]Valine-Labeled Tropoelastin from FCL-117 Cells Grown on Plastic or on Inducing ECM

[^{14}C]Valine counts bound to elastin antibody		
	FCL-117 cells grown on	
	Plastic	ECM*
	dpm/10^6 cells	
Culture medium	511	882
Cell layer extract	329	1573
Total dpm bound	840	2455

* From 270-d-old gestation ligamentum nuchae.

DISCUSSION

Ligamentum Nuchae as a Model for Elastogenic Differentiation

The bovine ligamentum nuchae was chosen as an experimental model to study elastogenic differentiation partly due to the elegant studies of Greenlee et al. (13) and Cleary and colleagues (5, 7, 45) that describe morphological and biochemical changes that occur in the ligament during development. With this important background information, it was possible to predict, with surprising accuracy, when developmental transitions were occurring in the tissue.

The biochemical changes that occur in the developing ligament suggest that connective tissue deposition by the ligament fibroblast occurs in a well-defined, temporal sequence that leads to the ultimate differentiated state that can be characterized by synthesis of elastin, the elastomeric protein that will make up >70% of the total protein in the adult ligament (10). Until the last trimester of development, the ligament fibroblast synthesizes and secretes predominantly collagen, proteoglycans, and other glycoproteins. Elastin deposition increases markedly during the last 3 mo of pregnancy, is maximal at about the time of birth, and continues for 2–5 mo postnatally before declining to a much reduced rate (5). Ligament cells removed from their in situ ECM and maintained under tissue culture conditions fail to differentiate (i.e., synthesize elastin) spontaneously, suggesting that epigenetic factors and not an intrinsic developmental program direct gene expression during differentiation.

Role of ECM in Elastin Gene Induction

Our results suggest that the ECM plays an important role in determining when elastin production begins in the ligament fibroblast. Undifferentiated ligament cells initiate elastin synthesis after contacting ECM of fetal ligaments from a more advanced stage of differentiation (fetal ages >180 d).

The ability of ECM to promote differentiation of young

cells is critically dependent upon the cell's ability to undergo mitosis in the presence of the inducing matrix. When cell division is inhibited by high cell density, inducing matrix does not alter the cellular phenotype until the growth inhibition is removed by replating the cells at a lower density. Once committed to elastin production, however, continuous contact with the matrix inducer is not required for young cells to maintain their differentiated state. Ligament cells that have been induced by ECM to differentiate continue to divide and synthesize elastin, suggesting that there is no strong antagonism between mitosis and differentiation as has been reported in some other cell types (8; discussed in reference 21).

The possibility of selective overgrowth by an elastin-producing cellular subpopulation in response to contact with ECM can be discounted by the short time course of the induction experiments. As shown in Fig. 8, elastin production in cultures of young cells in contact with inducing ECM reaches a maximal level in 24–48 h—sufficient time for only one or two rounds of cell division (the average doubling time for young cells averages between 18 and 24 h). It is highly unlikely that a small number of cells could overgrow the cultures during this period. Further evidence that matrix is inducing phenotypic alterations and is not selecting for growth of an elastin-producing cell type is provided by the observation that elastin production in cultures of induced young cells is equivalent to the level of elastin synthesis for an equal number of older, fully differentiated cells, suggesting that (*a*) the entire daughter cell population expresses the differentiated phenotype after induction and (*b*) the entire parent cell population was competent to respond to the matrix inducer.

BrdU Blocks Matrix-induced Differentiation

Further evidence that new DNA synthesis is a prerequisite for FCL fibroblast differentiation is evident from the inhibition by BrdU of the matrix-induced onset of elastin production. BrdU has been shown to inhibit a variety of differentiated functions in many cell types (19, 23, 35) under conditions where its effects on normal cell functions are minimal or undetectable (43). When log phase FCL fibroblasts are cultured with BrdU, BrdU is incorporated into cellular DNA and blocks the induction of the elastin phenotype when the cells are subsequently exposed to inducing matrix. However, sensitivity to BrdU is critically dependent upon the stage in cell development when exposure to the BrdU occurs. If undifferentiated FCL fibroblasts are exposed to BrdU after they have contacted the inducing matrix, differentiation occurs unimpeded and BrdU has no observable effect on elastin synthesis. These findings indicate that incorporation of BrdU into the FCL fibroblast genome before exposure to inducing matrix prevents expression of the differentiated state characterized by elastin production, but once the cell initiates the new developmental program by contact with inducing matrix, BrdU has no effect. The requirement for new DNA synthesis and the interruption of differentiation by BrdU provide strong evidence that matrix induction of the elastin phenotype in FCL fibroblasts is a consequence of transcription of a previously inactive elastin gene.

Mechanism of Matrix-induced Differentiation

Although it remains an intriguing question as to whether ECM specifies or permits elastin production, several inferences about the specificity and mode of action of matrix induction can be made from our observations. Our results imply that important signals appear in the matrix at a well-specified time during development and that the inducing factor(s) remains associated with the ECM throughout the period of elastin production. It is unlikely that the developmental signal is a serum component that binds to matrix since fetal calf serum was included in all induction experiments. Fetal calf serum is routinely harvested from late gestation fetal calves that are at the stage of development when elastogenic differentiation is occurring in the ligament. If the "inducer" is a circulating serum component, it is likely to be present in the fetal calf serum utilized in our study. As we have shown, however, young ligament cells do not differentiate in serum-containing medium unless they contact an appropriate ECM. Although it is possible that a serum inducer is active only when bound to ECM, the failure of young cells to differentiate when grown on ECM from early gestation ligaments, in the presence of 10% fetal calf serum, makes this explanation doubtful.

Because induction is not associated with a diffusible substance and because direct cell-matrix interaction is necessary to initiate differentiation, it is intriguing to speculate that individual macromolecular components of the ECM are directly involved in the genetic reprogramming of young cells (31). This may occur through a number of possible mechanisms. For example, sequential alterations in the differentiation state of the ligament cell with concomitant changes in the types of matrix macromolecules produced could result in the appearance of a discrete, tissue specific inducer molecule at the appropriate time in development. Alternatively, changes in the composition of the matrix could result from alterations in the relative concentrations of individual matrix components as a consequence of variations in synthetic or degradative rates. Elastogenic induction could then ensue when the concentration of a specific matrix inducer surpasses (positive induction) (34) or falls below (release from inhibition) (41) a critical threshold level.

Recently, several examples of developmental control have been proposed that do not require the presence of a unique inducer or the exchange of informational molecules. Slavkin (37) has proposed that changing combinations of connective tissue components and the resultant rearrangement of the ECM provides structural signals that direct cell differentiation. Similarly, it is now clear that cytoskeletal organization can alter cell metabolism (12, 22) and that molecules of the ECM affect the organization of the cytoskeleton (9, 39). In this regard, Frisch and Werb (11) have observed that changes in cell shape may regulate elastin gene expression in rat aortic smooth muscle cells. An understanding of the specific mechanisms for matrix regulation of elastin gene expression will require a detailed knowledge of biochemical changes that occur in the ligament ECM during development.

Other Changes Associated with Ligament Cell Differentiation

In this report we have only addressed how the elastin phenotype is altered by ECM, but clear differences occur in the synthesis of other secreted matrix macromolecules during differentiation of FCL cells. For example, we (29) and Davidson et al. (6) have shown that collagen production decreases as a consequence of FCL fibroblast differentiation. In addition, there is evidence that FCL fibroblasts secrete a low

molecular weight (12,750) hydroxyproline-containing protein that is collagenase sensitive and is secreted to a greater extent by cells from later stages of embryonic development (36). Another important difference between differentiated and undifferentiated ligament cells is their ability to respond to elastin peptides in a chemotaxis assay. Those results, detailed in the following paper (25), suggest that differentiation may also regulate the appearance of cell surface determinants that are important in cell-matrix recognition.

In summary, our results provide compelling evidence that the extracellular matrix provides a factor(s) that directs the acquisition of the differentiated state and either specifies or permits elastin production in ligament cells committed to the elastin pathway. Because of the homogenous nature of the ligament cell population, the matrix inducer most likely originates from the ligament cell itself, and in this respect the developing ligament cell is responsible for creating the proper microenvironment for its own differentiation. Implied in this model is the suggestion that a major source of information guiding the cell through successive stages of differentiation is contained in the same molecules that are used by the cell to organize a structurally important ECM.

It should be emphasized, however, that while our findings document a differentiation pathway of elastin production in the ligament fibroblast, they are not necessarily applicable to other elastin producing cells. In more complex elastic tissues, such as blood vessels and lung, ECM components from multiple cell types may interact to play a regulatory role in directing differentiation, and other inductive factors may be involved besides ECM. Nevertheless, we believe the simplicity of the ligamentum nuchae presents an ideal model to begin to delineate the role of the cellular microenvironment in influencing a cell's genetic expression.

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