Demonstration of Elastin Gene Expression in Human Skin Fibroblast Cultures and Reduced Tropoelastin Production by Cells from a Patient with Atrophoderma

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Abstract

Atrophoderma is a rare dermal disorder characterized by a patchy distribution of areas apparently devoid of elastic fibers. Skin fibroblast cultures were established from the normal and affected dermis of a patient with this disorder. Human tropoelastin was identified in culture medium by use of electroblotting and anti-elastin antisera. An enzyme-linked immunosorbent assay was used to establish that significantly less elastin accumulated in the media of cultured cells from lesional fibroblasts over a 3-d period. Since elastin biosynthesis in most tissues is under pretranslational control, molecular hybridization to a nick-translated genomic elastin probe was performed; however, elastin messenger RNA levels were equivalent in both cell strains. Both strains produced less elastin than did normal skin fibroblasts. Extracellular proteolysis of elastin was evaluated as a possible mechanism. Elastase activity was increased and porcine tropoelastin was degraded four times faster, on a per-cell basis, in lesional fibroblast cultures than in cells derived from an unaffected site. The two cell strains exhibited no significant differences in collagen production or collagenase activity. These results are the first demonstration of elastin production by cultured human skin fibroblasts, and they suggest that the primary defect in atrophoderma may be a result of enhanced degradation of newly synthesized elastin precursors.

Introduction

Elastin, a major fibrillar protein component of various connective tissues, is critical for the normal physiological function of organs such as skin, lungs, and the blood vessels (for reviews on elastin, see references 1-3). In normal human skin, the elastic fibers comprise only ~2% of the total protein in dermis (4), but it has been suggested that a number of cutaneous disorders involve defects in the structure or metabolism of elastin (5). The production of soluble elastin, or tropoelastin, the precursor of insoluble elastic fibers, has previously been demonstrated in smooth muscle cell cultures established from vascular connective tissues (6, 7). However, the cell type responsible for elastin production in the dermis has not yet been established.

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In this study, we have demonstrated that fibroblast cultures established from normal human skin synthesize tropoelastin, as detected by immunoblotting, enzyme-linked immunoabsorbant assay (ELISA),¹ and detection of elastin mRNA. We have also examined the rates of collagen and elastin production, and the activities of collagenase and elastase-like protease in cell cultures from a patient with atrophoderma; in this clinical condition, the elastic fibers are affected, as determined by histopathologic examination (8). The results indicated that in the fibroblasts from the affected area of skin, the elastin production was significantly reduced and that this reduction could be partially explained on the basis of increased degradation of the newly synthesized tropoelastin.

Methods

Clinical. Skin fibroblast cultures were established from 10 healthy adult controls; the skin specimens were obtained in connection with plastic surgery, after informed consent was obtained. Fibroblast cultures were also established from both affected and unaffected skin of a patient with atrophoderma. The patient was a 15-yr-old female with a history of gradual onset of skin lesions since 1979. Cells have been available from only the original biopsies of the affected site on the trunk and an adjacent, uninvolved area. The affected areas of skin were hyperpigmented, slightly depressed, and homogeneous in appearance. A palpable shelf bordering the normal skin could be easily demonstrated. Cutaneous blood vessels were more apparent in the skin lesions than in the adjacent normal skin. There was no evidence of sclerosis or telangiectasia. The involved areas of skin have grown progressively larger, and new areas of involvement have recently appeared. There was no history of injections in the affected areas, and review of other organ systems and family history gave negative results.

Fibroblast cultures. Human skin fibroblast cultures, derived from the arms or trunk, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing Hepes buffer, pH 7.6, 200 U/ml of penicillin, 200 µg/ml of streptomycin, and 10 or 20% fetal calf serum (FCS). Confluent primary cultures were trypsinized and subcultured in 75-cm² flasks (Falcon Plastics, Div. Becton, Dickinson & Co., Oxnard, CA) at 37°C. Confluent cells were studied in passages 3-11. Skin fibroblasts from two normal skin biopsies, 20 fetal wk and 17 yr being the donor age, were used for comparison in Northern hybridization studies.

Collagen assays. For assay of total protein and collagen synthesis, three confluent monolayer cultures of fibroblasts were labeled with [³H]proline (30 µCi/flask) for 20 h in the presence of 20% FCS

1. Abbreviations used in this paper: DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; SAPNA, succinyl-L-(alanine)-₃-paranitroanilide; SSC, 0.15 M NaCl in 15 mM sodium citrate, pH 6.8.

(dialyzed) and 25 µg/ml ascorbic acid (9). At the end of the labeling period, the medium was removed, cooled to 4°C, and protease inhibitors were added to give the following final concentrations: 20 mM Na₂ EDTA, 10 mM *N*-ethylmaleimide, and 1 mM phenylmethylsulfonylfluoride. The cell layer was removed with a rubber policeman at 4°C into a solution containing 20 mM NaCl, 0.01 M Tris-HCl (pH 7.5), and the same protease inhibitors; the cells were then sonicated at 50 kHz for 30 s. To quantitate the synthesis of [³H]hydroxyproline and the total ³H-radioactivity in cultures labeled with [³H]proline, aliquots of medium and homogenized cell fractions were dialyzed against running tap water, hydrolyzed in 6 M HCl in sealed tubes at 120°C for 24 h, and assayed for [³H]hydroxyproline and total ³H-radioactivity by use of a specific radiochemical method (10).

For determination of distinct collagen types, part of the medium after labeling with [³H]proline was dialyzed against 0.5 N acetic acid at 4°C for 24 h and pepsinized for 6 h at 22°C. The collagenous proteins were precipitated by the addition of 1.8 M NaCl, and the precipitates were recovered by centrifugation at 15,000 g for 30 min at 4°C. The precipitates were electrophoresed on 6% polyacrylamide gels (11), and the ³H-peptides were visualized by fluorography (12). The bands in the fluorograms were quantitated by scanning with an integrating densitometer (Gelman ADC-18; Gelman Sciences, Inc., Ann Arbor, MI).

Elastin assays. The antiserum used in this study was created by immunization of rabbits with a purified preparation of pig α -elastin. The details of preparation of this antiserum and its affinity and cross-reactivity with human antigens are detailed elsewhere (13). Qualitative identification of human elastin and its degradation products was obtained by the Western blotting procedure (14). Confluent monolayers of cultured skin fibroblasts were washed extensively with phosphate-buffered saline (PBS) and incubated in serum-free medium for 12 h to reduce the amount of adsorbed serum albumin. Medium was removed and replaced by DMEM containing aprotinin (10 µg/ml). After a 24-h incubation, culture medium was harvested, chilled, and mixed with a cocktail of protease inhibitors (final concentration, 10 mM Na iodacetate, 1 mg/ml ϵ -aminocaproic acid, 2 mM phenylmethylsulfonylfluoride, 1 mg/ml penicillamine, 50 µg/ml β -aminopropionitrile, 25 mM EDTA). Medium was clarified by centrifugation and secreted proteins were collected by precipitation with TCA (final concentrations, 10%) at 4°C. The pellets were washed with ethanol and ether and dissolved in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gel electrophoresis and electrophoretic blotting were carried out according to standard procedures (11, 15). All electrophoresis buffers contained 0.5 M urea. Electrophoresis was performed at 60 V in a commercially available apparatus (Bio-Rad Laboratories, Richmond, CA). Human elastin antigens bound to nitrocellulose were detected by incubation of the filters in a solution containing a 1:1,000 dilution of rabbit anti- α -elastin in PBS containing 1% bovine serum albumin. Bound antibody was detected with a 1:1,000 dilution of goat anti-rabbit-immunoglobulin-horseradish peroxidase conjugate (Miles Laboratories Inc., Research Products Div., Elkhart, IN). Peroxidase activity was detected and localized with a solution (25 ml) of diaminobenzidine (1 mg/ml in 0.03% hydrogen peroxide). The incubation was continued for 5 min and the reaction was stopped by the replacement of substrate solution with ammonia in water.

Enzyme-linked immunoassay. Elastin production in the cell cultures was quantitated by an ELISA which used anti-porcine- α -elastin antiserum (13). Production was defined as the net result of synthesis minus degradation, as measured by accumulation of tropoelastin over 3 d in culture medium.

Each well of flat-bottomed polystyrene microtiter plates (Flow Laboratories, Inc., McLean, VA) was coated with 20 ng of human α -elastin in 200 µl of 20 mM carbonate buffer, pH 9.6 containing 0.02% Na₂N₃ at 4°C overnight (16). Human α -elastin was prepared from human aorta as previously described (13). Pig aorta tropoelastin isolated from copper deficient animals (kindly provided by Dr. L. B. Sandberg, University of Utah) was used as a competitive inhibitor in

the construction of a standard binding curve; 110 µl of a stock solution of 2.0 µg/ml in PBS-Tween (0.025% Tween 20) was serially diluted by two in round-bottomed microtiter wells (Linbro, Flow Laboratories, Inc.). Rabbit antiserum to pig α -elastin (110 µl) was added to yield a 1:4,000 dilution in PBS-Tween and binding was continued at 4°C for >12 h. Competition between bound and unbound antigen was carried out for 30 min. After washing was done, horseradish peroxidase conjugated to goat anti-rabbit IgG (Kirkegaard and Perry, Gaithersburg, MD) was used at a 1:1,000 dilution. *O*-phenylenediamine was used as the enzyme substrate and the reaction was measured with a microspectrophotometer (Flow Laboratories, Inc.) at 492 nm.

To evaluate human fibroblast elastin production, cell culture media were lyophilized and reconstituted to one-fifth volume in PBS-Tween before serial dilution. Production data were estimated by fitting of unknown values to a standard curve constructed from a four-parameter, nonlinear regression analysis performed on a microcomputer (Apple Computer Inc., Cupertino, CA).

Detection of elastin mRNA by northern hybridization. Poly[A]⁺-RNA (0.5 µg) was isolated from control fibroblasts (2×10^8 cells) by standard procedures (17, 18). Sheep nuchal ligament RNA (poly[A]⁻) was used as a standard because of the species homology, the relative overabundance of ribosomal RNA in this preparation, and the high proportion of mRNA sequences coding for elastin. Samples were denatured and size fractionated on a 1% agarose gel containing 2.2 M formaldehyde (18). RNA was transferred to a nylon-based membrane filter (Zeta-bind; AMF Cuno Div., AMF Inc., Meriden, CT) by electrophoretic transfer in 0.025 M phosphate buffer, pH 6.5, at 12.5 V for 9 h and 40 V for 7 h. The filter was rinsed in 0.1 $\times$ 0.15 M NaCl in 15 mM sodium citrate, pH 6.8 (SSC), 0.5% SDS at 65°C for 1 h and then subjected to RNA-DNA hybridization. Prehybridization was performed in a solution of 50% formamide, 0.1% SDS, 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 50 mM Na phosphate, pH 6.7, and 250 µg/ml of denatured, sonicated herring sperm DNA at 42°C overnight. Hybridization was performed in the same solution as that used for prehybridization plus 250 ng of nick-translated (19) elastin probe. The probe used was a subcloned fragment of a sheep genomic clone, pSEI-1.3 (pSS1), which represents a portion of the 3' untranslated region of the sheep elastin gene (20, 21). This probe has been shown to react specifically with unique single-copy fragments of human DNA (LuValle, P. A., R. Leach, and J. M. Davidson, unpublished observations). Specific activity of the probe was $\sim 2 \times 10^7$ cpm/µg. Hybridization was allowed to take place for 48 h at 44°C, after which filters were washed twice with 2 $\times$ SSC at room temperature, and then washed more stringently with 0.5 $\times$ SSC at 55°C. Filters were exposed to X-ray film (X-Omat AR; Eastman Kodak Co., Rochester, NY) at -70°C in cassettes equipped with intensifying screens.

Assay of elastin mRNA by cytoplasmic dot hybridization. The relative abundance of elastin mRNA in affected and normal skin fibroblasts from the patient with atrophoderma was measured by cytoplasmic dot hybridization (22). Confluent monolayers of cultured skin fibroblasts (three T-25 flasks each) were scraped into PBS and centrifuged, and the cell pellet was lysed with detergent containing buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.6, 1.5 mM MgCl₂, 0.5% Nonidet P-40). Nuclei were fractionated from cytoplasm by low speed centrifugation, and supernatants containing total cytoplasmic RNA were treated with SDS as described previously (23). RNA was isolated by extraction with phenol/chloroform/isoamyl alcohol (24:24:1), and the aqueous phase was precipitated with 3 M sodium acetate at -20°C to remove contaminating DNA. RNA concentrations were estimated by optical density at 260 nm.

Dot hybridization was performed as previously described (23) in a manifold by use of nitrocellulose paper. It was found that binding of RNA was more quantitative if the nitrocellulose was first pretreated with 20 $\times$ SSC. Hybridization conditions were as described above.

Assay of collagenase and elastin-like protease activities. For assay of collagenase and elastase activities, cells were plated into 75-cm² flasks. The medium was removed and the cells were rinsed twice with serum-free DMEM. 7 ml of fresh DMEM was then added, and the

cultures were incubated for 6 h. The medium was collected and assayed for collagenase and elastase activities.

For collagenase assay, the enzyme preparations were activated by a brief trypsin proteolysis (10 $\mu\text{g}/\text{ml}$ for 10 min at 25°C) (24), trypsin was inactivated by soybean trypsin inhibitor, and collagenase activity was assayed by incubation of samples with ^3H -labeled type I collagen, as described previously (25). The enzyme activity was expressed as degradation of ^3H -labeled collagen in counts per minute per milligram DNA $\times$ hours. The cleavage products after collagenase assay were also studied by means of 6% polyacrylamide gel electrophoresis.

Elastase-like neutral protease activity was assayed by incubation of 50- μl aliquots of the medium with a synthetic substrate, succinyl-L-(alanyl) $_3$ -paranitroanilide (SAPNA) in 50 mM Tris-HCl, pH 7.8, for various periods up to 24 h (26). The reaction was monitored by the change in absorbance at 410 nm. The enzyme activity was expressed as hydrolysis of SAPNA in nanomoles per milligram DNA $\times$ hours.

Degradation of ^{125}I -labeled tropoelastin in fibroblast cultures. The relative elastin-degrading capacity of the fibroblast cultures was evaluated by use of a highly purified tropoelastin as substrate. Porcine tropoelastin that was prepared from copper-deficient pig aortas by standard procedures and further purified by reverse-phase high-performance liquid chromatography (20) was kindly provided by L. B. Sandberg. The substrate was radioactively labeled by use of Iodogen (ref. 27) (Pierce Chemical Co., Rockford, IL). Specific activity of the substrate was $\sim 1 \times 10^7$ cpm/ μg . To examine the degradation of tropoelastin, 1×10^6 cpm of substrate in 2.5 ml of DMEM containing 10% FCS was added to confluent fibroblast cultures in 35-mm dishes. Substrate was also incubated with DMEM-FCS without cells at 37°C; 100- μl aliquots of medium were harvested at different intervals and precipitated with TCA (10%). Degradation was expressed as counts per minute of TCA-soluble radioactivity released per microgram of cultured cell DNA. The pattern of elastin degradation products was also visualized by SDS-PAGE of the TCA-precipitable material, by use of autoradiography of the dried gels.

Other assays. DNA was quantitated either by the fluorometric assay according to Hinegardner (28) or by diphenylamine method as described by Burton (29).

Results

Demonstration of elastin production by cultured human skin fibroblasts

In preliminary studies, normal human foreskin fibroblasts were incubated in serum-free medium, and the newly synthesized proteins were examined on immunoblots by use of anti-pig- α -elastin antiserum. A distinct band comigrating with intact, purified porcine tropoelastin standard was seen (Fig. 1, lane B). Several bands of slightly lower molecular weight, also detected in the standard, reflect protease contamination. This standard had not been subjected to high-performance liquid chromatography purification.

Quantitation of the elastin production was obtained by an ELISA assay specific for human tropoelastin. A typical standard curve generated in this inhibition assay is shown in Fig. 2. Although anti-porcine- α -elastin antisera were used to generate this curve, the test was made more specific for human elastin by coating of the ELISA plates with human α -elastin. Moreover, the test was made specific for elastin precursors by use of (porcine) tropoelastin as the competing antigen. In this way, the ELISA was made selective for epitopes shared by both human α -elastin and soluble elastin precursors.

Reduced tropoelastin production in fibroblast cultures from a patient with atrophoderma

Qualitative changes. Media from equal numbers of fibroblasts grown from the lesional and unaffected skin of a patient with

Figure 1. Qualitative identification of human tropoelastin in fibroblast culture media by Western blotting. Lane A, reaction products of porcine tropoelastin (100 ng) with anti- α -elastin antiserum. Faint bands above and below the principal (M_r 70,000) band are polymerized and degraded forms of the molecule, respectively. Lane B, reaction product from the culture medium of human foreskin fibroblasts. Several-fold less medium was analyzed from these normal cells. Lane C, reaction products from the culture medium of lesional atrophoderma fibroblasts. Lane D, reaction with a comparable volume of culture medium from an equivalent number of fibroblasts derived from uninvolved skin of the atrophoderma patient.

atrophoderma were qualitatively analyzed for elastin production by immunoblotting. Cultures were analyzed between the sixth and eleventh passages. Polypeptides cross-reacting with anti-elastin antibody were detected in both types of fibroblast cultures. Qualitative comparison of the patterns indicated that more degradation had taken place in both of the proband's fibroblast cultures than in a normal foreskin fibroblast control (Fig. 1). Much less medium had been loaded in the normal control (foreskin fibroblast) lane. The total amount of immunologically detectable material appeared to be decreased in the cell cultures derived from the lesional skin relative to uninvolved skin.

Quantitative changes. Precise quantitation of the elastin production was obtained by ELISA. A significant reduction in the amount of elastin produced (i.e., net accumulation during a 3-d period) was noted in the fibroblast cultures from affected skin as compared with cells from the unaffected skin in the

Figure 2. Typical competitive binding curve for the determination by ELISA of elastin in human fibroblast culture media. Ordinate: absorbance at 492 nm. Abscissa: natural logarithm of the dose of human α -elastin. The assay has a useful sensitivity of about 5 ng.

same patient (Fig. 3). However, the elastin production in both affected and unaffected skin fibroblast cultures from the patient with atrophoderma was markedly reduced in comparison to control fibroblasts. Assays of culture media from 10 other skin fibroblast lines, including an age- and sex-matched control, indicated that normal cells produced elastin in the range of 30 to 120 ng tropoelastin/ μ g DNA in 3 d ($2.4\text{--}9.6 \times 10^4$ molecules/cell $\cdot$ h), which is about 10 times greater than production by either of the cell strains from this patient.

Assay of elastin mRNA levels in skin fibroblasts by cytoplasmic dot hybridization

One possible mechanism that could result in decreased production of elastin by affected skin fibroblasts is decreased biosynthesis of elastin. Because all other elastin-synthesizing systems that have been examined have been shown to be largely under transcriptional control (20), a direct comparison of the elastin mRNA levels between affected and unaffected cells was made by cytoplasmic dot hybridization by use of a genomic elastin subclone, pSS1. This probe was derived from a 1.3-kilobase fragment near the 3' terminus of the sheep elastin gene, and it has been shown to anneal to homologous sequences in sheep (18, 20), bovine (Mecham, R. P., and J. M. Davidson, unpublished observations), porcine (20, 31), and rat RNAs (32), as well as hamster and human DNAs (LuValle, P. A., R. Leach, and J. M. Davidson, unpublished observations). Shown in Fig. 4 A is the specific hybridization of the sheep elastin genomic DNA probe to a 3.5-kilobase mRNA prepared from two normal human skin fibroblast strains. These bands comigrated with authentic sheep elastin mRNA. The relative abundance of elastin RNA sequences in cytoplasmic RNAs from both affected and unaffected skin fibroblasts from the atrophoderma patient was evaluated with the heterologous DNA probe, as shown in Fig. 4 B. The results indicate that, over the range of serial dilutions applied, no significant differences in the level of radioactive signal were seen between the uninvolved and lesional fibroblasts. These results imply that elastin synthesis, on a per-cell basis, was similar in each of these cell types.

Degradation of tropoelastin in skin fibroblast cultures

Another possible reason for the reduced elastin production in the affected atrophoderma fibroblast cultures is the accelerated

degradation of newly synthesized elastin by a proteolytic mechanism. To measure the rate of tropoelastin degradation, highly purified porcine tropoelastin labeled with ^{125}I was added to the fibroblast cultures from the atrophoderma patient. Aliquots of culture media were removed at various times and the proteins were precipitated with TCA. The acid-soluble radioactivity in the supernatant increased with time in both cultures but did not increase significantly in medium incubated under the same conditions but without cells (Fig. 5). The rate of tropoelastin degradation was about four times higher in affected cell cultures than in fibroblast cultures from the unaffected skin. Examination of the TCA-precipitable, ^{125}I -labeled polypeptides by SDS-PAGE did not indicate significant differences in the pattern of the degradation products in these two fibroblast cultures (data not shown).

In further studies, the activity of elastase-like enzymes in the culture media was determined by incubation with SAPNA, a synthetic substrate for assay of elastolytic enzyme activity. The results indicated that the elastase-like enzyme activity was significantly increased in the affected fibroblast cultures (Table I). This observation suggests that the increased degradation of tropoelastin in cell cultures from the affected skin is mediated by proteases secreted into the cell culture medium.

The significance of the increased elastase activity in the culture medium was further examined by assay of collagenase activity in the same cultures. No differences in the collagenase activity between the lesional and unaffected skin fibroblast cultures were noted (Table I).

Collagen studies

Collagen production was evaluated in fibroblast cultures by biosynthetic labeling of procollagen with [^3H]proline followed by assay of nondialyzable [^3H]hydroxyproline. The amount of [^3H]hydroxyproline produced was the same in fibroblast cultures from both the lesional and unaffected skin (Table I). Also, the total incorporation of radioactivity, as an indication of the rate of total protein synthesis, was the same in both fibroblast cultures. SDS-PAGE of newly synthesized collagens, isolated by limited pepsin proteolysis and salt precipitation, revealed that type I and type III collagen were synthesized in affected skin fibroblast cultures in a $4.9 \pm 0.3/1$ ratio (mean $\pm$ SD from three determinations); the corresponding values in three parallel control cultures were $6.4 \pm 0.8/1$. Thus, the collagen production, both quantitatively and qualitatively, appeared to be normal in the atrophoderma fibroblast cultures.

Discussion

In this study we have demonstrated that human skin fibroblasts can synthesize tropoelastin in culture. Tropoelastin production was demonstrated by immunoblotting and could be quantitated more precisely by an ELISA assay. This obviated our recurring difficulties in obtaining quantitative immunoprecipitation of this low-abundance protein from culture medium of labeled cells. Furthermore, the results demonstrated the presence of elastin mRNA in cultured human skin fibroblasts, as detected by an elastin-specific genomic subclone, which represents a portion of the 3' untranslated region of sheep elastin that is known to have homology with an mRNA cloned human elastin cDNA sequence (33). Since the results indicate that skin fibroblasts contain elastin gene transcripts and secrete

Figure 3. Comparative levels of production of elastin by atrophoderma fibroblasts from lesional and uninvolved dermis. Affected cells (left) produce $\sim 40\%$ less elastin per cell than do unaffected cells (right). The normal range for 10 control fibroblast lines is 30–120 ng elastin per μ g DNA per 3 d, which is considerably higher than the range for either atrophoderma cell strain. Values are expressed as mean $\pm$ SE ($n = 6$).

Figure 4. (A) Specificity of the pSE1-1.3 elastin probe. Northern hybridization analysis was performed with poly[A]⁺RNA (0.5 µg) obtained from normal human skin fibroblasts (lanes A and B) and poly[A]⁻ RNA from sheep nuchal ligament (2 µg, lane C). RNA was denatured and fractionated in the presence of 2.2 M formaldehyde and electrophoretically transferred to a nylon-based membrane filter. The filter was hybridized with 250 ng (10⁷ cpm) of ³²P-pSE1-1.3 DNA for 48 h at 44°C in the presence of 50% formamide. Final stringency wash was 0.5× SSC, 56°C. The lines and arrow mark the position of the ribosomal RNA (in Svedberg units) present in lane C (not shown) and elastin mRNA in kilobases (Kb), respectively. (B) Dot hybridization analysis of elastin mRNA levels in atrophoderma fibroblasts. RNA from affected (left column) and unaffected fibroblasts (right column) was prepared as described in the text and applied as serial twofold dilutions; the upper dots represent the hybridization signal with 1.0 µg of total cellular RNA. In the range of linearity (dots 3–5 from top), no significant difference in reactivity with pSE1-1.3 DNA could be measured by scanning densitometry (not shown).

tropoelastin, this cell type is confirmed biochemically to be the source of elastic fibers in human skin in vivo.

The applicability of the techniques used in this study to the study of human diseases was demonstrated by assay of elastin production in fibroblast cultures established from a patient with atrophoderma. Atrophoderma is a rare cutaneous disorder that primarily affects young females (8). The individual lesions, which have a predilection for sites on the trunk, appear as hyperpigmented, slate-colored, depressed areas of the skin. The histopathology of the affected skin initially demonstrated a mild inflammatory cell infiltrate with a slight thickening of the collagen fibers. Elastin degradation products are known to be chemotactic to mononuclear phagocytes (30, 34). Later, the dermis appeared atrophic, and the elastic fibers were scanty and clumped (8, 35). Unlike another atrophic disorder, morphea, the dermis had not become hypopigmented or sclerotic.

Our results indicated that the production of tropoelastin

by the fibroblasts established at that time from the affected areas of skin was markedly reduced as compared with that by fibroblasts from unaffected dermis of the same individual.

Table I. Total Protein and Collagen Synthesis and Activities of Collagenase and an Elastase-like Protease in Fibroblast Cultures from a Patient with Atrophoderma*

	Total protein synthesis‡	Collagen synthesis§	Collagenase activity	Elastase activity¶
	$\frac{\text{cpm} \times 10^6}{\text{mg DNA}}$	$\frac{\text{cpm} \times 10^{-6}}{\text{mg DNA}}$	$\frac{\text{cpm} \times 10^{-6}}{\text{mg DNA} \times \text{h}}$	$\frac{\text{nmol} \times 10^{-2}}{\text{mg DNA} \times \text{h}}$
Unaffected skin	33.1±4.8	3.8±0.4	1.5±0.1	3.0±0.9
Lesion	34.4±3.1	3.9±1.2	1.8±0.2	4.9±1.2**

* Fibroblast cultures established either from the unaffected skin or a lesion of the patient with atrophoderma were incubated as described in Methods, and the parameters indicated were assayed either after 20 h incubation in medium containing 20% FCS (total protein and collagen synthesis) or after 6 h of incubation in serum-free medium (collagenase and elastase activities). The values are mean±SD of three parallel flasks, each assayed in duplicate.

‡ Expressed as incorporation of [³H]proline into nondialyzable fraction in cells plus medium.

§ Expressed as synthesis of nondialyzable [³H]hydroxyproline in cell plus medium fraction.

|| Expressed as degradation of [³H]proline-labeled type I collagen.

¶ Expressed as hydrolysis of SAPNA, as monitored at 410 nm.

** Statistically different from cultures established from unaffected skin; *P* < 0.05.

Figure 5. Degradation of tropoelastin by fibroblast cultures. The extent of release of TCA-soluble radioactivity from radioiodinated porcine tropoelastin as a function of incubation time is shown. Line A (open circles), radioactivity released by affected atrophoderma fibroblasts. Line B (closed triangles), release by a comparable number of unaffected atrophoderma fibroblasts. Line C (closed squares), radioactivity released by incubation of culture medium with tropoelastin substrate.

Both types of fibroblasts produced significantly less elastin than did normal controls. Among controls, production did not depend significantly on the biopsy site (Sephel, G., and J. M. Davidson, unpublished observations). Since the elastin mRNA levels were similar in both cell strains, the relatively reduced elastin production in lesional fibroblasts might have been due to decreased mRNA utilization or, alternatively, to post-translational events, such as increased degradation of the newly synthesized protein. Cell-free translation of elastin mRNA, which is less sensitive and more subjective because of the optimization necessary (21, 36, 37), was not used for quantitation. In support of the latter possibility were two observations. First, the rate of degradation of ^{125}I -labeled tropoelastin, when added to the cell culture medium, was markedly accelerated in the fibroblast cultures from the patient's affected skin relative to the unaffected fibroblast cultures. Second, elastase-like enzyme activity, assayed by use of a synthetic, low molecular-weight substrate, was significantly increased in the lesional cell cultures. The importance of the latter observation was emphasized by the fact that the activity of collagenase, another protease secreted into fibroblast culture medium, was not different between the uninvolved and lesional skin cell cultures. No significant changes were observed between the lesional and uninvolved cell cultures in the rate of total protein production or in the production of collagen. Thus, it is conceivable that the decreased elastin production by the fibroblast cultures is due to increased degradation of the newly synthesized tropoelastin. It should be noted that the elastin production in the fibroblast cultures established from an apparently unaffected area of the skin of the same patient was significantly lower than that in normal, age- and sex-matched control fibroblast cultures. We regret that we have been unable to continue evaluation of the molecular basis for this reduction at this time, since the cells from the original biopsy acquired an altered morphology after ~25 population doublings, i.e., the large cytoplasts typical of senescent cells (38) at higher population doubling levels. Since cellular senescence per se appears to reduce elastin production (Sephel, G., and J. M. Davidson, unpublished observations), these higher-passage cultures were eliminated from the study. It is possible that other changes in the elastin metabolism are contributing to the development of the skin lesions in this patient with actively progressing disease.

The demonstration of elastin gene expression in human skin fibroblast cultures provides us with a convenient source to study elastin abnormalities in a variety of acquired and heritable disorders of connective tissues. In many of these conditions, including morphea, cutis laxa, and Marfan syndrome, the elastic fibers have been shown to be diminished or abnormal by histopathologic or ultrastructural examinations (5), but the biochemical basis of such changes has not been elucidated. The demonstration of elastin production in these cultures also allows us to examine the factors responsible for the normal control of elastin gene expression in this cell system (21, 37), which in turn provides us with important information on the maintenance of the normal elastic fiber network in the skin and other connective tissues.

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