Buschke-Ollendorff Syndrome Associated with Elevated Elastin Production by Affected Skin Fibroblasts in Culture

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Buschke-Ollendorff syndrome (BOS; McKusick 16670) is an autosomal dominant connective-tissue disorder characterized by uneven osseous formation in bone (osteopoikilosis) and fibrous skin papules (dermatofibrosis lenticularis disseminata). We describe two patients in whom BOS occurred in an autosomal dominant inheritance pattern. The connective tissue of the skin lesions showed both collagen and elastin abnormalities by electron microscopy. Cultured fibroblasts from both patients produced 2–8 times more tropoelastin than normal skin fibroblasts in the presence of 10% calf serum. Involved skin fibroblasts of one patient produced up to eight times normal levels, whereas apparently uninvolved skin was also elevated more than threefold. In a second patient, whose involvement was nearly complete, elastin production was high in involved areas and less so in completely involved skin. Transforming growth factor-β1 (TGFβ1), a powerful stimulus for elastin production, brought about similar relative increases in normal and BOS strains. Basic fibroblast growth factor, an antagonist of TGFβ1-stimulated elastin production, was able to reduce elastin production in basal and TGFβ1 stimulated BOS strains. Elastin mRNA levels were elevated in all patient strains, suggesting that Buschke-Ollendorff syndrome may result, at least in part, from abnormal regulation of extracellular matrix metabolism that leads to increased steady-state levels of elastin mRNA and elastin accumulation in the dermis. *J Invest Dermatol 99:129–137, 1992*

Buschke and Ollendorff first made the association between an asymptomatic spotting of bones (osteopoikilosis) and connective-tissue nevi (dermatofibrosis lenticularis disseminata) in 1928 [1], although both had been described previously [2,3]. Over 130 cases and numerous pedigrees have been reported, establishing the BOS as an autosomal dominant disease with high penetrance, variable expression, and an incidence of 1 in 20,000 [4–13]. The basic defect is unknown, although structural abnormalities of elastic fibers, the microfibrillar component, and the collagen fibrils have been reported [11,14,15]. Using estimates of desmosine content and digital morphometric analysis of stained biopsies, Uitto et al demonstrated elevated elastin accumulation in involved skin relative to uninvolved skin and to skin of controls [16]. More recently, preliminary data on increased elastin gene expression has been reported [17].

Other dermatologic manifestations reported in association with BOS suggest that there is also an effect on collagenous tissues. Findings include Dupuytren's contractures and Peyronie's Disease, oral fibromas, keloids, histiocytomas, knuckle pads, fibrokeratomas and keratoderma palmoplantare striae, anetoderma, morphea, and scleroderma [11]. Skeletal anomalies including exostoses, enchondromas, osteofibromas, osteopetrosis, melorheostosis, arthritis, and short stature have also been reported [11].

In the present study, we sought to determine whether altered elastin accumulation was a stable marker of the disease phenotype in the skin fibroblasts of two patients with BOS by correlating ultrastructural and biochemical observations. We have also evaluated one possible site of abnormal regulation, the response to the cytokines TGFβ1 and bFGF.

CASE HISTORIES

Case 1 A 58-year-old white man was evaluated in November 1985.

At age 28, after routine X-ray for evaluation of stiff joints, patient

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Abbreviations:
bfGF: basic fibroblast growth factor
BOS: Buschke-Ollendorff syndrome
EDS: Ehlers Danlos syndromes
ELISA: enzyme-linked immunosorbent assay
FCS: fetal calf serum
MOPS: 3-N-morpholinopropanesulfonic acid
NBCS: newborn calf serum
SLE: systemic lupus erythematosus
SSC: 0.15M NaCl in 15 mM sodium citrate, pH 6.8
SSPE: 0.18M NaCl, 10 mM $NaPO_4$, pH 7.7, 1 mM EDTA
TGFβ1: transforming growth factor-β1

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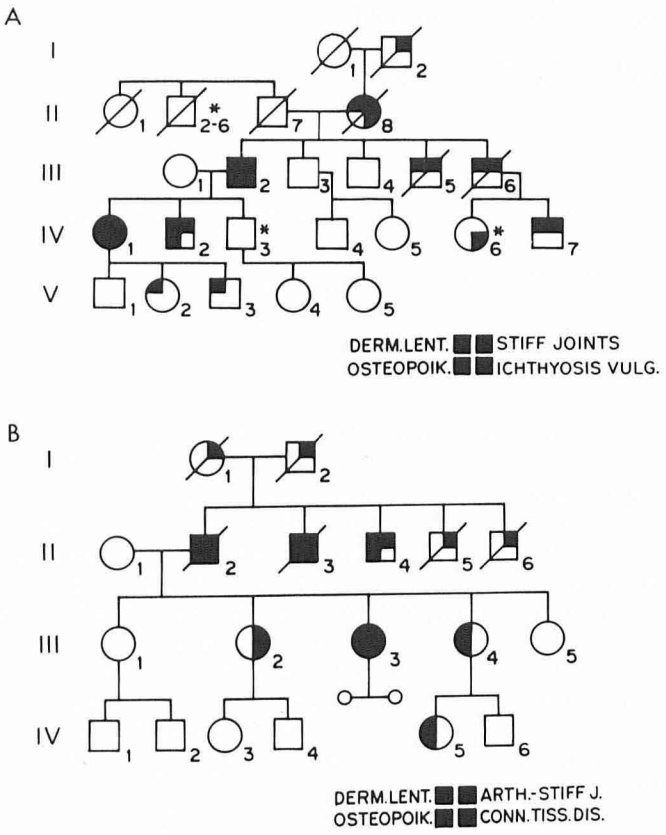

Figure 1. Pedigrees of Buschke-Ollendorf Syndrome families. *Squares,* male; *circles,* female; *dots,* miscarriage; *diagonal lines,* deceased. The clinical features are denoted by the filling of the four quadrants shown in the right corner of the drawings. Osteopoikilosis is based on radiographic confirmation. *Asterisks* in *A* denote hyperextensive joints. *A:* Pedigree of case 1 family. The proband (III 2) had BOS with stiff joints and ichthyosis vulgaris. His mother (II 8), two brothers (III 5, III 6), a daughter (IV 1), a son (IV 2), and a nephew (IV 7) were similarly affected with dermatofibrosis lenticularis. X-rays were only available for the proband and his children, so the diagnosis of the osteopoikilosis *(lower left quadrant)* was unknown in the others with skin findings. All adults with BOS had stiff joints *(upper right corner).* Ichthyosis vulgaris *(lower right quadrant)* was present in the proband, in his daughter (IV 1), and a niece (IV 6) who did not have BOS. Several uncles (II 2–6), a son (IV 3), and a niece (IV 6) had joint hypermobility *(asterisk)* not associated with BOS. *B:* Pedigree of case 2 family. The proband (III 3) had BOS *(left quadrants),* mixed connective tissue disease *(lower right quadrant),* and arthritis *(upper right quadrant).* Her father (II 2), two of his brothers (II 3 and II 4), one of her sisters (III 4), and a niece (IV 5) were also affected with BOS. Connective tissue diseases were present in the father (II 2; glomerulonephritis due to SLE), one of his brothers (II 3; rheumatoid arthritis), and the proband (III 3; mixed connective tissue disease). The proband's sister (III 2) also had rheumatoid arthritis and a positive antinuclear antibody. Additionally, there was a very strong family history of degenerative arthritis in the proband's paternal grandmother, grandfather, uncles, and sister *(upper right quadrant).*

1 was diagnosed as having osteopoikilosis. He and several family members (Fig 1*A*) had noticed small, yellowish papules and nodules on the skin since childhood, accompanied by prominent joint stiffness, especially affecting the ankles and shoulders. Other family members, however, had loose joints and some had ichthyosis vulgaris, not associated with BOS. The patient had a past history of Behcet's disease, migraine headaches, hypertension, and hoarseness.

Physical examination revealed a middle-aged, hoarse man with thousands of 1-2 mm yellow papules prominent on volar wrists, inner arms, and legs (Fig 2*A*) sparing face and neck. There were firm 1-2 cm fibrous nodules on the trunk, a 1 × 3 cm firm, brown, connective tissue nevus on the buttocks, and a 10 × 10 cm elliptical nevus spilus on the left flank. The shins were xerotic, consistent with ichthyosis vulgaris. He had injected sclerae and conjunctivae. Firm 4-5 mm flesh colored papules were present on the tongue and laryngoscopy showed leukoplakia. Joint mobility was limited in the ankles with no plantar flexion possible past 95°, and near lack of inversion and eversion. Shoulders could not be abducted beyond

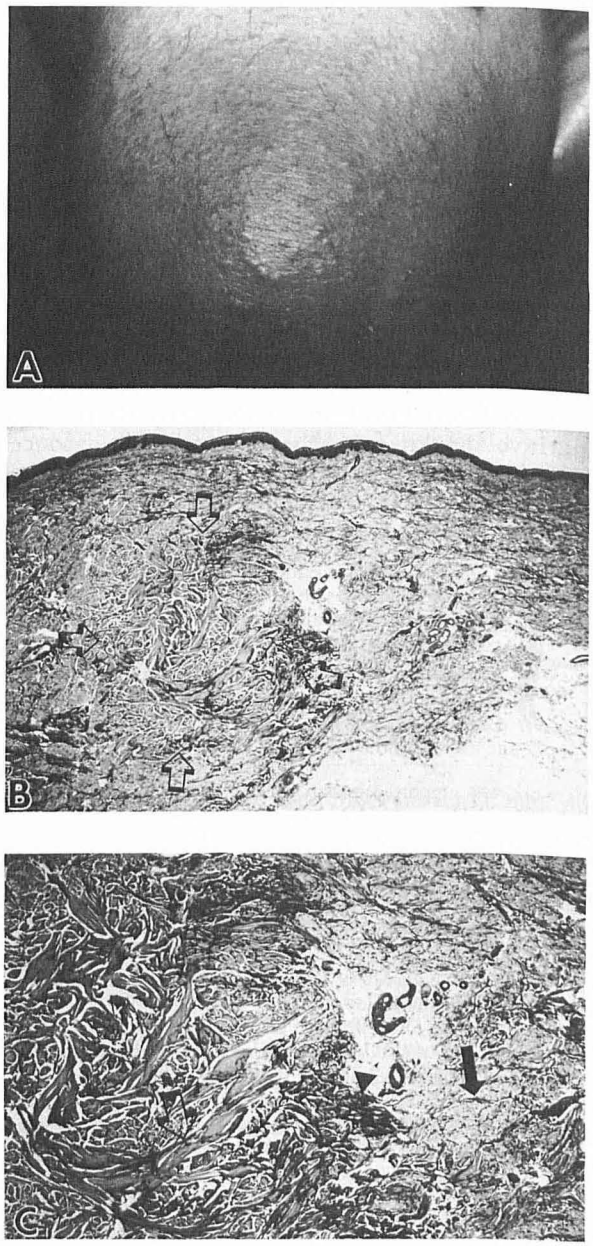

Figure 2. Gross appearance and histology of BOS lesions. *A:* Photograph showing gross appearance of the skin over the knee from case 1 (biopsy site) with 1–2 mm yellowish papules also seen over the forearms, volar wrists, and thighs, consistent with dermatofibrosis lenticularis disseminata. *B:* Biopsy of a dermal nodule from knee of patient 1 examined by light microscopy and Verhoeff-van Gieson stain (magnification ×15) shows a subtle dermal nodule *(arrows)* of thickened collagen and elastic fibers. *C:* High-power view (magnification ×30) of the nodule and surrounding normal skin showing thickened collagen fibers *(opened arrow)* and increased elastic fibers *(arrowhead).* Adjacent normal dermis is present in the other half of the photomicrograph (*solid arrow,* collagen fiber of normal thickness).

90°, and internal and external rotation were moderately restricted. Both wrists lacked full flexion and extension, and all fingers could not be fully extended by active and passive manipulation.

Laboratory evaluation included complete blood count with 10,300 white cells, 49.9 hematocrit, and 307,000 platelets. Electrolytes, thyroid function, prothrombin time, partial prothrombin time, and multichannel chemistry analysis were normal (except a serum glutamic pyruvic transaminase of 73).

Case 2 A 45-year-old female previously diagnosed and reported to have BOS [13] was evaluated. Beginning at age 3, in 1945, she experienced severe pain in the feet and was diagnosed with osteopoikilosis by X-ray. At age 15 the patient developed arthritis. In 1981 she developed a facial rash that had histology consistent with systemic lupus erythematosus. Anti-nuclear antibody and rheumatoid factor were detected in 1982. In 1983 she developed pericarditis and in April 1987 she had a muscle biopsy consistent with polymyositis. In December 1987, she complained of several months of dry eyes, mouth, and vagina and of severe proximal muscle weakness. She was taking 25 mg of prednisone alternating with 30 mg every other day and 30 mg per week of methotrexate. She was on 14 units NPH insulin for steroid-induced diabetes.

Osteopoikilosis was present in the patient's father, two of his brothers, one of her sisters, and a niece by the affected sister (Fig 1*B*). Her father had died with glomerulonephritis secondary to systemic lupus erythematosus. One of her father's affected brothers had rheumatoid arthritis. One of her sisters had BOS and one had rheumatoid arthritis with a positive antinuclear antibody. Many family members had degenerative arthritis.

On physical examination this was a moderately obese female with generalized alopecia. The parotid glands were slightly enlarged and tender. There was an intention tremor and 4/5 muscle strength in neck flexion and in the deltoids, triceps, biceps, iliopsoas, and quadriceps muscles. There was limited joint mobility, especially of the neck, shoulders, hips, and lower back. On skin exam there were multiple, 1-2 mm, yellow, pearly papules over the volar wrists, arms, and legs. The lesions were confluent in many areas, leaving no clinically uninvolved skin.

Pertinent laboratory data included a normal complete blood count, chemistries, and creatine phosphokinase, anti-nuclear antibody, and C3 and C4 components of complement. Glucose, cholesterol, and triglycerides were slightly elevated. The patient was felt to have an overlap connective tissue disease with Sjogren's syndrome, polymyositis and lupus. In August 1988 (age 46) the proband noted increasing numbers of yellow papules such that they became confluent in most areas, and she complained of increased pain in the knees and shoulders. Skin biopsies were taken from involved and most-involved skin of the arms. There was such extensive involvement that it was not possible to find totally uninvolved skin.

MATERIALS AND METHODS

Skin Biopsies The patients signed consent forms for all studies. Punch biopsies of skin were obtained with lidocaine anesthesia from most involved, involved, or uninvolved skin on the leg. The biopsies were split for explant culture and electron microscopy. A nodule with surrounding normal skin was excised for routine hematoxylin and eosin and elastin staining (Verhoeff-van Gieson).

Light and Electron Microscopy The skin was fixed in half-strength Karnovsky's fixative at 4°C for at least 24 h [18]. The tissue was stained en bloc with 1% OsO_4, dehydrated through graded alcohols, infiltrated with propylene oxide, and embedded in epon [19]. Sections 1 μ thick were stained according to Richardson et al [20] and examined by light microscopy using a Zeiss photomicroscope. Thin sections were collected onto copper grids, stained with 1% phosphotungstic acid, 1% uranyl acetate, and lead citrate [21], and viewed with a Phillips 420 scanning transmission electron microscope.

Fibroblast Cultures Biopsies were explanted under cover slips in Dulbecco's modified Eagle's medium containing 10% bovine calf serum (Gibco Laboratories, Grand Island, NY), and 1× antibiotics (penicillin-streptomycin-fungizone; 100×, Gibco). Normal skin fibroblasts (GM 4390, age 23), used as controls, were obtained from the National Institute of General Medical Sciences (Coriell Institute, Camden, NJ), Fibroblasts were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Hyclone, Logan, UT) in an atmosphere containing 5% CO_2-humidified air until confluent and passed 1:3 using 0.025% trypsin-0.02% EDTA (Sigma, St. Louis, MO). Cells were used prior to signs of cellular senescence, between passages 4 and 8, during which time elastin production was stable. For collection of culture supernatants for elastin analysis, the serum was switched to newborn calf serum (Hyclone, Logan, UT) at confluence to reduce assay background, and tropoelastin accumulation was assayed in the medium after 2–3 d.

Cytokine Treatment Recombinant human TGFβ1 was provided by Genentech, (South San Francisco, CA); recombinant human bFGF was provided by Synergen (Boulder, CO). Affected and normal human skin fibroblasts in passages 7 and 8 were grown to pre-confluency in 35-mm dishes. Fresh medium containing 10% NBCS, antibiotics, and the desired amount of TGFβ1 (from 0 to 7.5 ng/ml) was added to duplicate dishes. In another set of experiments, cells were subcultivated to culture-chamber slides (Lab-Tek, Nunc, Naperville, IL) and exposed for 48 h to TGFβ1 (0, 0.1, 1.0, and 10.0 ng/ml) alone or combined with 100-ng/ml of bFGF. Medium and cells for elastin and DNA quantitation were harvested after 48 h and stored at −20°C for a few days, until assays were performed.

DNA Assay Cell monolayers were washed twice with cold PBS and then treated with 1.0 ml (0.5 ml in the case of chamber slides) of 10 mM EDTA, pH 12.3. Cell number was evaluated by a fluorimetric procedure using Hoechst 33258 (Boehringer-Mannheim, Indianapolis, IN) as previously described [22].

Elastin ELISA Assay A standard enzyme-linked immunoabsorbent assay (ELISA) for elastin was used as previously described [23]. To make the assay specific for human elastin epitopes, rabbit antiserum to pig α-elastin was used at a 1:3000 dilution on plates coated with 40 ng of human α-elastin. The test was made specific for tropoelastin by using 85 pg to 11 ng of porcine tropoelastin as the competing antigen in a standard curve. Results from sister cultures at three dilutions of culture medium ($n = 6$) were expressed as human elastin molecular equivalents produced per cell per hour after correction for the background present in serum-containing medium.

Elastin mRNA Studies To assess the status of elastin gene transcript levels in this disease, hybridization studies were performed with poly[A]$^+$-mRNA isolated from the BOS strains and compared to elastin mRNA levels in the normal strain that produced $30.8 \pm 1.6 \times 10^3$ molecular equivalents of elastin per cell · h. Specificity was confirmed by Northern hybridization (Fig 8*A*).

RNA Isolation Messenger RNA was extracted from cells by preparing cell lysates in an adaptation of the SDS-proteinase K method described by Pihlajaniemi and Myers [24]. Yields were equivalent for all strains. The concentration of NaCl in the cell lysates was increased to 100 mM, and the samples were heat-denatured at 70°C before passing through a poly[U]-sepharose column (Sigma, St. Louis, MO) five times. The column (1 g of dry material) was washed with NETS buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% SDS) until the OD_{260} was zero, and then eluted with 3 ml of elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% SDS, 90% formamide). Peak fractions containing poly [A]$^+$-mRNA were pooled, ethanol-precipitated, and resuspended in sterile water at 150 μg/ml.

RNA Hybridization Studies After boiling in water for 5 min or after treating with 6.5% formaldehyde solution at 65°C for 10 min,

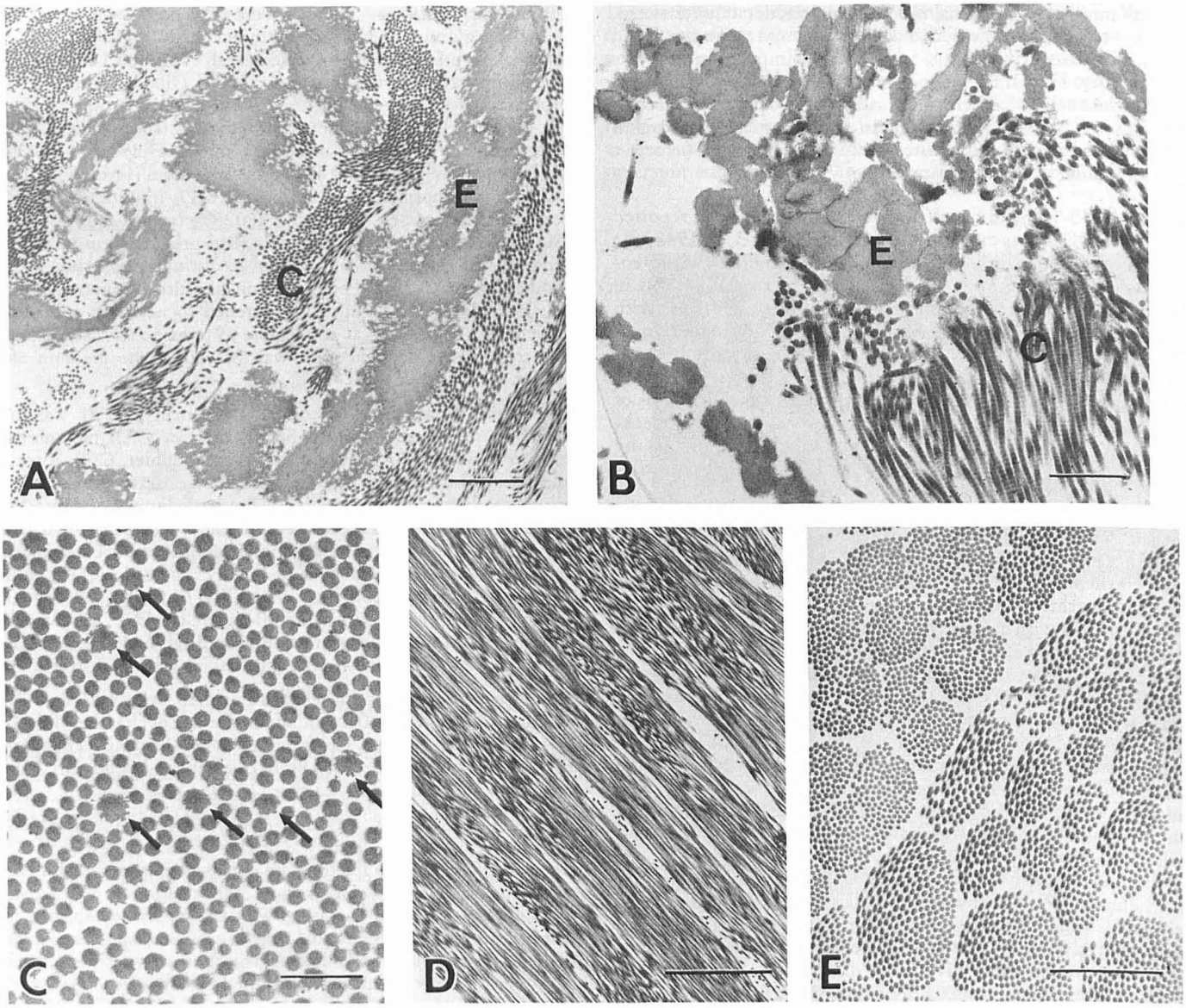

Figure 3. Transmission electron micrographs of skin from a patient with BOS (case 1). *A:* The elastic fibers (E) were abundant relative to the amount of dermal collagen (C). In some areas the elastic fibers had frayed edges but fairly normal proportions of microfibrils and amorphous elastin. *Bar,* 2 μ. *B:* In other areas, the elastic fibers appeared more matted and tangled, and they lacked the regular deposition of microfibrils within the elastin matrix. *Bar,* 1 μ. *C–E:* Transmission electron micrographs of collagen from involved (C,D) and uninvolved (E) skin are shown. Composite collagen fibrils (*C, arrows; bar,* 0.5 μ) were present among normal fibrils in involved skin. Large collagen fibers appeared to be made up of parallel cords of smaller fibers (*D; bar,* 5 μ). A similar cordlike organization of collagen fibers was present in the papillary dermis of uninvolved skin (*E: bar,* 2 μ). Magnification: (*A*) ×5400, (*B*) ×11,400, (*C*) ×24,000, (*D*) ×3300, (*E*) ×7400.

mRNA was applied to Zeta Bind nylon membrane filters (AMF Cuno, Meriden, Connecticut) in a vacuum manifold with circular orifices (Schleicher and Schüell, Keene, NH) and baked at 80°C in vacuo for 2 h. Northern blots were run in 1% agarose gels containing 6.5% formaldehyde in MOPS buffer [25] and then capillary-blotted onto membranes using 6 × SSC. Filters were baked in vacuo at 80°C. The presence of elastin mRNA on filters was detected with a 1.1-kb DNA restriction enzyme fragment isolated from a human elastin cDNA clone CHE-4 [26], by Bam HI and HindIII digestion, agarose gel electrophoresis, and electroelution. This fragment includes about half of the coding region of the elastin mRNA molecule, including exons 18 through 36, as well as 26a but lacking the 3′-untranslated region. DNA was subsequently labeled with [^{32}P]-dCTP by random priming [27,28] according to manufacturer's instructions (Pharmacia LKB Biotechnology, Piscataway, NJ). The clone D5, a cDNA for cytochrome C oxidase subunit II, was used as a constitutive probe* to normalize for mRNA recovery. Prehybridization (4–12 h) and hybridization (18–20 h) were carried out at 42°C in 1.5 × SSPE (0.18 M NaCl, 10 mM $NaPO_4$, pH 7.7, 1 mM EDTA) [25], 10% nonfat dry milk, 2% SDS, 10% dextran sulphate, 50% formamide, and 420 μg/ml of salmon sperm DNA. Final stringency washes were in 0.1 × SSC, 0.1% SDS, 65°C. Blots were exposed to pre-flashed film (Kodak X-Omat AR, Kodak, Rochester, NY) at −70°C.

Autoradiographs were scanned with a Bio-Rad 6520 video densitometer, and the output was recorded and integrated using MS-

* Matrisian L (personal communication).

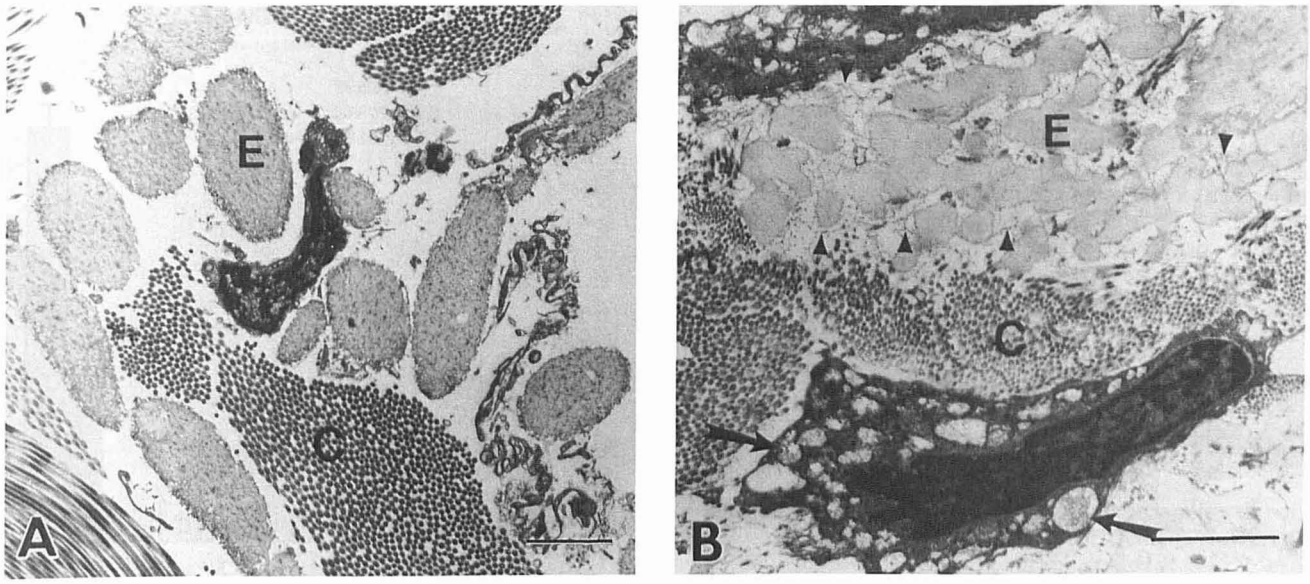

Figure 4. Transmission electron micrographs of involved *(A)* and most-involved *(B)* skin from case 2. Lesions were nearly confluent in this patient. In the involved skin *(A)*, the elastic fibers (E) were abundant relative to normal appearing collagen (C). The elastic fibers were frayed around the edges and had holes within the elastic matrix. *Bar*, 2 μ. In most-involved dermis *(B)*, there were dense networks of elastin matrix (E) with proteoglycan-like matrix in between *(arrowheads)*. Many of the adjacent collagen fibrils (C) were abnormal. Cells retained a filamentous material within the cisternae of the rough endoplasmic reticulum *(arrows)*. *Bar*, 2 μ. Magnification: (*A*) ×5400, (*B*) ×8200.

DOS based software (Bio-Rad, Richmond, CA). Data were compared among dots showing a linear response to several dilutions.

Analysis of Elastin Gene Polymorphism by Southern Blotting Genomic DNA from fibroblasts was prepared using a standard SDS-proteinase K extraction procedure [29]. Ten micrograms of DNA per lane was digested with Eco RI, Msp I, Pst I, Taq 1, and Hinf II (Bethesda Research Laboratories, Gaithersburg, MD) at 4 units/μg DNA in the manufacturer's recommended digestion buffers. The DNA was electrophoresed on 0.8% agarose gels and transferred to Zeta Bind membranes for standard Southern hybridization [30] as per the manufacturer's conditions.

DNA from a sheep elastin genomic probe (pSE1-1.3) [31] and the human cDNA probe (CHE-4 [26]) were labeled to 1×10^9 cpm/μg using a random primer oligo-labeling kit (Boehringer Mannheim Biochemicals) with [^{32}P]-dCTP (Amersham) [27,28]. Hybridization was at 42°C for 24 h in 50% formamide, 5 × SSC, 1 × Denhardt's, 100 μg/ml herring sperm DNA, and 1% SDS. Washes were done at room temperature in 2 × SSC, 0.5% SDS, and at 60°C in 2 × SSC, 0.1% SDS.

RESULTS

Histology and Electron Microscopy A biopsy taken from a nodule and surrounding normal skin (case 1, Fig 2*A*) showed compact and large collagen bundles in the center of the nodule compared to normal surrounding areas of the dermis (Fig 2*B*). Verhoeff-van Gieson stain for elastic fibers showed increased numbers of thickened elastin fibers in the lesion (Fig 2*C*).

Biopsies from yellow papules and so-called normal skin were examined by transmission electron microscopy. In case 1, the involved dermis had clumps of irregularly shaped elastic fibers with frayed edges (Fig 3*A*). Other areas of the lesion exhibited a disproportionately high content of amorphous elastin relative to microfibrils (Fig 3*B*). Composite collagen fibrils (Fig 3*C*) were present in collagen fibers adjacent to abnormal elastic fibers. Large collagen fibers containing fibrils with normal cross-sections appeared to have been assembled from smaller cordlike fibers (Fig 3*D*). This unusual organization of cords of collagen fibers was also present in the papillary zones of uninvolved skin (Fig 3*E*).

Elastic fibers were relatively overabundant in case 2, but the biopsies from the involved (Fig 4*A*) and the most involved skin (Fig 4*B*) showed several differences. The elastic fibers in involved skin (Fig 4*A*) were frayed around the edges, contained abundant electron-dense material and had holes within the elastic matrix. The most involved dermis (Fig 4*B*) showed disproportionately high amounts of amorphous, electron-lucent elastin (E) relative to collagen (C). There were pools of ground substance and proteoglycans surrounding the elastic fibers and pools of fibrillar material within swollen cisternae of the rough endoplasmic reticulum. Adjacent collagen fibers had fibrils with irregular cross-sectional contours.

The degree of skin involvement differed in the two cases. In case 1, biopsies could be made from apparently uninvolved and involved sites. In contrast, in case 2, the near confluent state of the lesions made it difficult to obtain completely uninvolved dermis; moreover, the most involved areas showed further degeneration of normal fibrous architecture as well as fibroblasts with swollen endoplasmic reticulum.

Elastin Production and Cytokine Response Elastin production levels in a normal skin fibroblast strain were compared with production levels in uninvolved and involved skin fibroblasts from case 1 (Fig 5, *first column*) or involved and most involved skin from case 2 (Fig 6, *first column*). Because of the extensive involvement found in case 2, fibroblasts from completely uninvolved dermis were unavailable. In the most involved area, the histopathology was so severe that few normal fibroblasts or connective tissue were present. Thus, cells taken from the most involved areas may have represented end-stage lesions. Expression of elastin in the presence of 10% NBCS was about 7–8 times higher than control levels in involved BOS cells of case 1; in addition, uninvolved fibroblasts of case 1 were also about three times as active as the control strain in accumulating soluble elastin in the culture medium. Elastin accumulation was also markedly elevated in cells from the involved areas of case 2, whereas in the most involved lesions of case 2, production was only doubled.

To determine whether increased elastin production may have been due to altered responsiveness to an elastogenic effector TGFβ1, the cytokine was added to BOS cultures. Elastin production was stimulated in all BOS skin fibroblasts as well as dermal cells from a normal donor (Fig 5). Production was stimulated more than two-

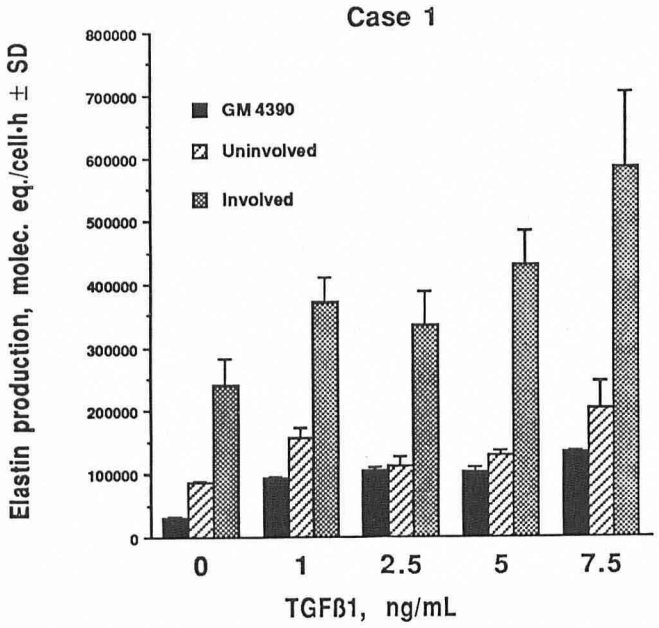

Figure 5. Elastin production in fibroblasts of a BOS patient (case 1) and effect of increasing concentrations of recombinant human TGFβ1. Data on cellular production was obtained in low-passage (p6 – p8) cultures. Ordinate: elastin production in molecular equivalents per cell · h. Normal values *(solid bars)* are represented by strain GM 4390 (age 23) also used as a reference source of elastin mRNA. *Hatched bars,* uninvolved skin: *shaded bars,* involved skin. Data was obtained in passage 6–8 cultures. TGFβ1 (0 to 7.5 ng/ml) was added immediately before confluency to cultures in 35-mm culture dishes in the presence of 10% NBCS and antibiotics. Media were harvested 48 h later. Elastin production was expressed as molecular equivalents per cell · hour, ±SD. Our unpublished studies have shown that little, if any, newly synthesized elastin remains in the cell layer, and analysis of this compartment was omitted from the present study.

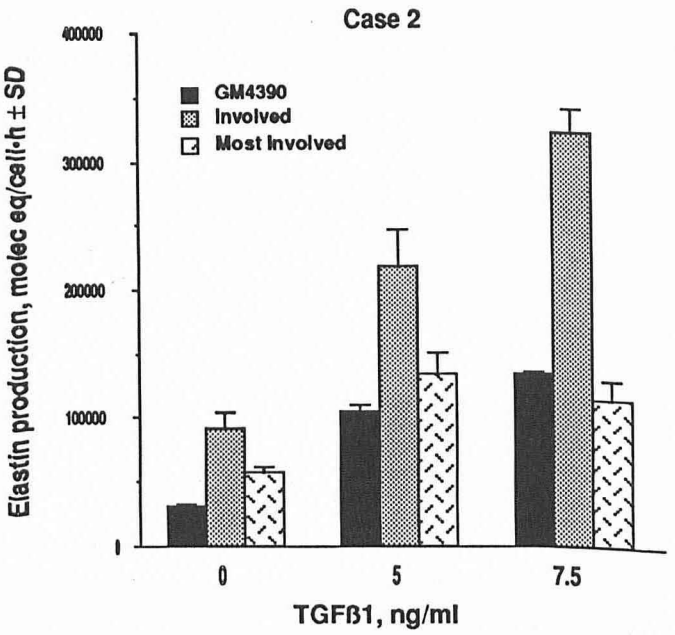

Figure 6. Effect of increasing concentration of TGFβ1 on elastin production in cultured fibroblasts from a BOS patient (case 2) and normal skin fibroblasts. Control strain was GM4390 *(solid bars). Shaded bars,* involved skin; *cross-hatched bars,* most-involved skin; TGFβ1 (0; 5.0; 7.5 ng/ml) was added immediately before confluency to 35-mm dishes. Media was harvested after 48 h. Elastin production was expressed as molecular equivalents per cell · h, ±SD.

fold in both cell strains from BOS case 1 and more than threefold in normal cells. In case 2 (Fig 6), involved lesions exhibited highest basal and TGFβ1-stimulated levels of elastin production, whereas most involved skin fibroblasts were not as responsive to TGFβ1 stimulation.

Because bFGF is known to antagonize the effect of TGFβ1 on elastin production [32], cells were treated with bFGF at a concentration that inhibits TGFβ1 effects in normal fibroblasts and smooth muscle cells [32] (100 ng/ml) and increasing concentrations of TGFβ1 (0–10 ng/ml). Basal levels of elastin production in this strain of BOS fibroblasts (involved, case 1) were reduced by 85% with 100 ng/ml of bFGF (Fig 7). Although addition of 10 ng/ml of TGFβ1 induced a nearly sixfold rise in elastin production, bFGF treatment reduced this parameter by a factor of 12.5, to 47% of basal production level.

Analysis of Elastin mRNA Levels Messenger RNA levels were quantified in two ways (Table I): by densitometry of either Northern blots (Fig 8*A*) or dot blots (Fig 8*B*), hybridized with either CHE-4, a human elastin cDNA probe [26] or D5, a constitutively expressed, mitochondrial gene product [33] (Fig 8*C*). The raw densitometric data gave clear evidence of increased steady-state levels of elastin transcripts in BOS cells relative to a normal fibroblast strain; moreover, correction for mRNA recovery showed that the involved cells (case 1) had about a fivefold relative increase in elastin mRNA content (Table I). Although Northern hybridization showed little relative increase in elastin mRNA from uninvolved fibroblasts of case 1, about a 70% increase was measured by the more quantitative blot hybridization procedure in which efficiency of transfer and binding is uniform and high.

Analysis of Elastin Gene by Southern Blotting Compared to normal fibroblast DNA, no differences in restriction patterns were noted in BOS fibroblast DNA with any of the enzymes tested, and no restriction fragment length polymorphisms that would be useful for linkage studies were identified for the elastin gene (data not shown).

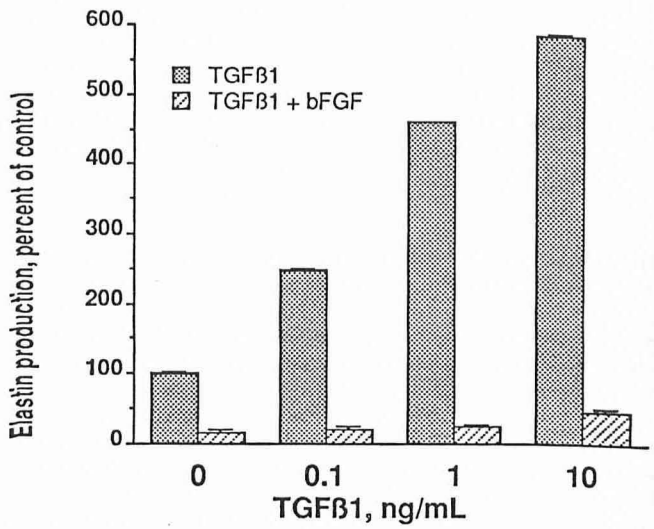

Figure 7. Basal and TGFβ1-stimulated elastin production in cultured BOS skin fibroblasts is inhibited by bFGF. Involved fibroblasts from case 1 were subcultivated in culture-chamber slides and exposed for 48 h to TGFβ1 (0–10.0 ng/ml in DMEM, antibiotics, and 10% NBCS) alone *(shaded bars)* or combined with 100 ng/ml bFGF *(hatched bars).* Elastin production is expressed as percent of control (10% serum only), ±SD.

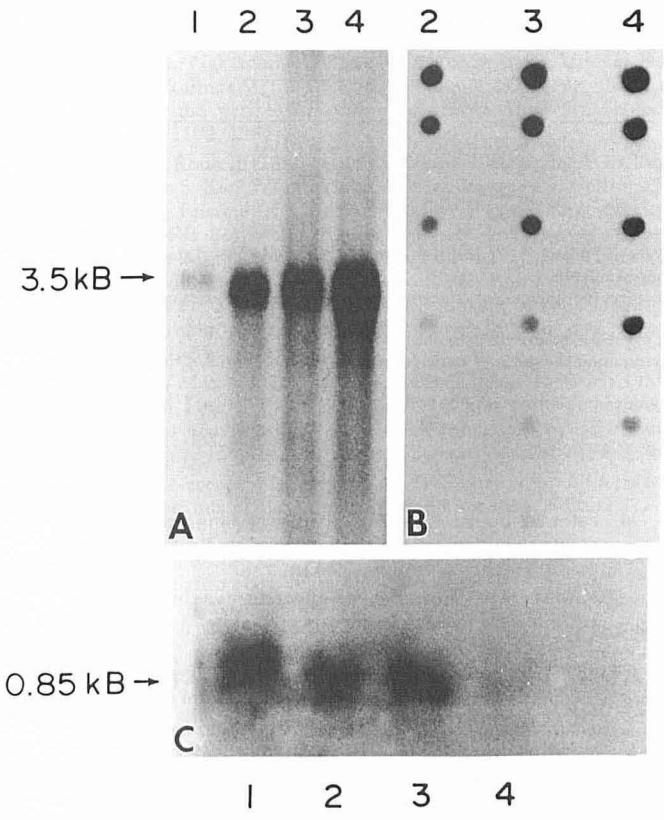

Figure 8. Detection and quantification of elastin mRNA in normal and BOS fibroblasts. *A:* Northern blot hybridization of RNA to CHE-4, a human elastin cDNA probe, labeled with [^{32}P]-dCTP as described in *Materials and Methods. Lane 1,* 10 μg total RNA from sheep nuchal ligament, used as a marker and positive control; *lane 2,* poly[A]$^+$-mRNA (0.75 μg) from GM 4390 as the normal fibroblast strain; *lane 3,* poly[A]$^+$-mRNA from uninvolved BOS fibroblasts of case 1 (0.75 μg); *lane 4,* poly[A]$^+$-mRNA from involved BOS fibroblasts of case 1 (0.75 μg). *B:* Dot hybridization of twofold dilutions of RNA samples (beginning at 250 ng) corresponding to *lanes 2–4* of *A.* Loading of RNA was normalized by rehybridization of the Northern blot with a probe for D5, a constitutively expressed mitochondrial gene, after stripping the original probe with 96% formamide at 65°C (2×). *C:* Constitutive expression is shown by the D5-generated signal of each of the samples in *A.* Responses in *lanes 2–4* were quantified by densitometry and used to correct the hybridization data of *A* and *B,* as shown in Table I.

DISCUSSION

The cultured dermal fibroblasts from two patients with BOS showed increased elastin production and elastin mRNA levels consistent with a phenotype of abnormal elastogenesis. This effect could not be attributed to increased sensitivity to the cytokine, TGFβ1, and elevated elastin production was reduced by treatment of cells with another cytokine, bFGF. Although the genetic defect in BOS could be constitutive overproduction of elastin, the time of onset of elastosis, changes in bone, and the initial, focal nature of the lesions suggest that other factors influence the expression of this pleiomorphic defect.

The BOS in these patients presented a complex phenotypic background, possibly indicating the contribution of multiple (genetic) factors. In addition to the dermatofibrosis lenticularis disseminata characteristic of BOS, patient 1 and family members also had large plaque-like connective tissue nevi, and dermatofibromas. The patient had a nevus spilus, considered to be a *forme fruste* of neurofibromatosis. Von Recklinghausen's neurofibromatosis is also characterized by fibrous tumors in skin and bone [34] inherited in an autosomal dominant pattern with variable penetrance. This disorder has recently been linked to the pericentric region of 17q12–22 in humans [35] and mutations have been described [36] and mapped [37], but linkage analysis of BOS is not yet available. Hinrichs et al have inserted the *tat* region of the human T-cell retrovirus, HTLV-1, into transgenic mice to create a neurofibromatosis phenotype that was then passed through three generations [38]. A retrovirus affecting skin and/or bone fibroblasts could conceivably explain the heterogeneity of skin and joint involvement of BOS, the altered matrix regulation, and the autosomal dominance inheritance pattern.

The regulation of the connective tissue matrix is extremely complex, and the interrelationships among the genes for matrix proteins and proteglycans are not fully understood. Abnormalities of one matrix component may affect the structural organization of another [14]. Elastin and collagen are fibroblast products that can be interwoven in various proportions to determine the biomechanical properties of an extracellular matrix. Disproportionate synthesis or accumulation of elastic tissue components may act to disrupt delicate feedback regulatory systems that play a role in the formation of normal matrix.

Patients with BOS had ultrastructural abnormalities of both collagen and elastic fibers. The elastic fiber changes, however, were much more pronounced. Studies of cell strains derived from different biopsy sites in vitro revealed obvious increases in elastin production under standard culture conditions. Collagen production, although not systematically examined, was variably lower in BOS strains,† possibly contributing to the presence of composite collagen fibrils in both patients, a feature also seen in EDS and other disorders of connective tissue [14]. Changes in collagen production may have been secondary to elastin overproduction, the consistent feature of the BOS phenotype. The fibroblasts explanted from affected skin produced a five to eightfold excess of tropoelastin relative to controls, uninvolved fibroblasts (case 1) produced a two to threefold excess, and elastin mRNA levels were likewise elevated. However, mRNA levels may not have fully accounted for the increases in elastin accumulation, because the apparent increase in protein accumulation was somewhat higher than mRNA increases. Turnover of newly synthesized tropoelastin or efficiency of mRNA utilization could be playing a role in this defect. Southern blotting data of case 1 have failed to show a gross structural rearrangement of the elastin gene, but we cannot exclude the elastin gene as being one target of a structural mutation. Restriction fragment length polymorphisms, rare in the elastin gene [39], have not been informative in these families to date. A newly discovered polymorphism may be more informative [40].

Regulation of elastin production is governed by many environmental factors, including the matrix per se [41], glucocorticoids [42], and growth factors [43,44]. The pleiotropic phenotype of BOS could result from the derangement of a common control or signal mechanism such as cellular receptor or a signal-transduction pathway, one of the consequences being elevated cutaneous elastin gene expression. Other elastic tissues such as lung and aorta are not known to be involved in this syndrome. It is conceivable that expression is driven by factors from the epidermis or the cutaneous environment. Elastin production in vitro is dependent on the serum concentration [45], which implies that cytokines released at injury sites are involved in regulation of elastin production. This dominant disorder could represent derangement of a serum-responsive regulatory mechanism operant in skin, rather than a defect in the elastin gene itself. Although BOS cells appeared more sensitive to TGFβ1 in preliminary experiments, this was a variable feature. Defective regulation may be more consistent with the very generalized connective tissue defects seen in these patients. Even if the primary genetic lesion is not in the elastin gene, the present findings show altered elastogenesis to be a highly significant biochemical marker that may help to unravel the actual basis of this genetic defect.

† Duvic M (unpublished findings).

Table I. Relative Elastin mRNA and Protein Levels in Buschke-Ollendorff Fibroblasts

	Ratio[a]			Corrected Ratio[b]		
Cell Strain	Northern[d]	Dot Blot[e]	D5[f]	Northern/D5	Dot Blot/D5	Protein[c]
Case 1 involved	1.95	2.63	0.46	4.23	5.70	7.8
Case 1 uninvolved	1.30	2.22	1.32	0.99	1.68	2.8
Case 2 involved	6.64	ND	0.53	12.53	ND	3.2
GM 4390[g]	1.00	1.00	1.00	1.00	1.00	1.0

[a] Densitometric scans of Northern and dot-blot data from equivalent amounts of RNA are expressed relative to signals from control strain, GM 4390. Although A_{260} values were equal, densitometry of D5 hybridization showed substantial differences in actual concentrations of mRNA.
[b] Uncorrected ratios from the preceding columns were divided by the ratio of D5 signal to correct for differences in the amount of mRNA present in samples from each strain.
[c] Data derived from ELISA assay shown in Figs 5 and 6.
[d] Densitometric data from Northern hybridization to DNA from the elastin probe CHE-4, expressed relative to the control strain, GM 4390.
[e] Densitometric data from serial dilution of cytoplasmic mRNA hybridized to CHE-4, representing the mean of ratios from three serial dilutions of mRNA giving a linear dose-response signal.
[f] Derived from Northern blot hybridization to a constitutively expressed human gene recognized by plasmid D-5.
[g] Control strain, elastin production = $30.8 \pm 1.6 \times 10^3$ molecular equivalents/cell · h.

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ADDENDUM

Bristol Meyers Squibb also sponsored the 40th Annual Symposium on the Biology of the Skin, published in June [JID 98(suppl):1S–106S].