Retrovirally Mediated Overexpression of Versican V3 Reverses Impaired Elastogenesis and Heightened Proliferation Exhibited by Fibroblasts from Costello Syndrome and Hurler Disease Patients

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The phenotypic resemblance of patients with Costello syndrome and Hurler disease has been linked to impaired formation of elastic fibers that coincides with elevated cellular proliferation. Impaired elastogenesis in these diseases associates with respective abnormal accumulation of chondroitin sulfate and dermatan sulfate proteoglycans that induce cell surface shedding of elastin-binding protein (EBP) normally required for intracellular chaperoning of tropoelastin and its assembly into elastic fibers. A variant of the chondroitin sulfate proteoglycan versican, V3, which lacks chondroitin sulfate, has recently been shown to stimulate elastic fiber assembly and decrease proliferation when expressed by retroviral transduction in arterial smooth muscle cells. However, the mechanism(s) by which V3 influences this phenotype is not known. We now demonstrate that transduction of skin fibroblasts from Costello syndrome and Hurler disease patients with cDNA to versican V3 completely reverses impaired elastogenesis and restores normal proliferation of these cells. This phenotypic reversal is accompanied by loss of chondroitin sulfate from the cell surface and increased levels of EBP. Versican V3 transduction of skin fibroblasts from GM₁-gangliosidosis patients, which lack EBP, failed to restore impaired elastogenesis. These results suggest that induction of elastic fiber production by gene transfer of versican V3 in skin fibroblasts is mediated by rescue of the tropoelastin chaperone, EBP. (*Am J Pathol* 2004, 164:119–131)

Hurler disease belongs to a group of inherited metabolic storage diseases and is because of a primary deficiency in lysosomal α -L-iduronidase,^{1–4} and consequent intracellular and pericellular accumulation of dermatan sulfate and heparan sulfate glycosaminoglycans and glycolyp-

ids.^{5–8} Another striking feature that contributes to the phenotype of this disease (skeletal deformations, multiple lesions of connective tissue, hypertension, cardiac valve insufficiency, diffuse cardiomyopathy, without rhythm disturbances, and severe coronary artery stenosis) is poor development of elastic fibers.^{9–14} Sparse, thin, and fragmented elastic fibers have also been described in tissues of patients with another unique connective tissue disorder called Costello syndrome.^{15–17} The natural history of this clinical entity (Online Mendelian Inheritance (OMIM, <http://www.ncbi.nlm.nih.gov/Omim> no. 218040), caused by a still unknown genetic error, is characterized by two distinct phases. The first phase is often marked by polyhydramnios and increased birth weight and followed by the second phase that includes: failure to thrive, severe short stature, mental retardation, and a distinctive appearance with craniofacial and dermatological findings resembling those observed in lysosomal storage disorders. Other manifestations of Costello syndrome include soft skin with excess wrinkling over the dorsum of the hands and deep creases on the palms and soles, curly hair, hyperextensibility of digits, generalized hyperpigmentation, pigmented nevi, vascular birthmarks, acanthosis nigricans, papillomata, and other tumors that develop at later ages.^{18–28} Various cardiac abnormalities occur in ~60% of Costello syndrome patients.^{29–32} They differ, however, from those in Hurler disease patients.³² One-third of patients have a congenital heart defect (usually pulmonary stenosis), rhythm disturbance (usually atrial tachycardia), or cardiac hypertrophy (often as classic hypertrophic cardiomyopathy, although biventricular and concentric hypertrophy have been observed).³² In contrast to Hurler disease, Costello syndrome patients do not demonstrate occlusion of main coronary arteries, however, intimal thickenings may occur in their small intramyocardial branches.

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Despite the fact that both Hurler disease and Costello syndrome patients are characterized by impaired formation of elastic fibers, their cells did not reveal any haplo-insufficiency of the elastin gene and demonstrated normal expression of elastin mRNA.^{16,33} However, recent studies with fibroblasts from these patients^{34,35} revealed impaired secretion of tropoelastin, the 70-kd monomer precursor of insoluble elastin^{36,37} and increased shedding of the 67-kd elastin-binding protein (EBP) that normally acts as a recycling molecular chaperone protecting highly hydrophobic tropoelastin molecules from intracellular self-aggregation and premature degradation.^{38–45} Docking of EBP at the cell surface also facilitates tropoelastin assembly into new elastic fibers.^{38,39} The EBP, identified as an enzymatically inactive spliced variant of β -galactosidase (S-Gal),^{40,41} also has a galactose domain capable of interacting with free β -galactosugars or β -galactosugar-bearing moieties, such as chondroitin sulfate or dermatan sulfate.^{38–45} This interaction causes conformational changes of EBP, such that it loses its affinity for elastin and dissociates from the cell surface, disrupting elastic fiber assembly.^{38,39,46} Because Hurler disease and Costello syndrome skin fibroblasts demonstrate cell surface accumulation of dermatan sulfate and chondroitin sulfate, respectively,^{34,35} it is postulated that defective elastic fiber assembly exhibited by these cells is because of increased galactosugar-induced shedding of EBP.

Versican is a large chondroitin sulfate containing proteoglycan that interacts with hyaluronan and associates with the surface of a variety of cells.⁴⁷ There are four spliced mRNA variants of versican with the smallest isoform (V3) lacking chondroitin sulfate chains.^{48–50} Recent studies show that retroviral transduction of V3 cDNA into rat vascular smooth muscle cells induces elastic fiber assembly *in vitro* and *in vivo* when these transduced cells are seeded into injured arteries.^{51,52}

To determine whether V3 gene transfer could reverse the abnormal phenotype of skin fibroblasts from Hurler disease and Costello syndrome patients and, if so, influence the association of EBP with the cells, we transduced these cells with retroviral vectors containing V3 cDNA and evaluated elastic fiber assembly by immunohistochemical and biochemical methods. Our results demonstrate that the overexpression of V3 in these cells reverses the impaired elastic fiber assembly phenotype and restores the normal proliferative rate of these cells. Furthermore, we present evidence that the crucial mechanism responsible for the V3 effect on elastogenesis depends on rescue of the tropoelastin chaperone, EBP.

Materials and Methods

Materials

All chemical grade reagents, chondroitin sulfate A, and monoclonal antibody to chondroitin sulfate A were from Sigma (St. Louis, MO). α -Minimum essential medium, fetal calf serum, and other cell culture products were obtained from Gibco Life Technologies (Burlington, Can-

ada). Polyclonal antibodies to tropoelastin, microfibril-associated glycoprotein (MAGP), and fibrillin 1 were purchased from Elastin Products Co., Inc. (Owensville, MI). Polyclonal antibody to human versican (LF 99) was a generous gift from Dr. Larry W. Fisher of The Craniofacial and Skeletal Disease Branch of the National Institutes of Health (Bethesda, MD). Fluorescein-conjugated antibodies, goat anti-rabbit (GAR-FITC) and goat anti-mouse (GAM-FITC) were purchased from Sigma. Horseradish peroxidase-conjugated goat anti-rabbit antibody (GAR-HRP) used for Western blotting was from Bio-Rad (Hercules, CA). The chemiluminescence detection kit and radiolabeled reagents, [³H]-valine, [³H]-serine, [³⁵S]-methionine, and [³H]-thymidine were purchased from Amersham Canada Ltd. (Oakville, Canada).

Fibroblast Cultures

With parental consent and Institutional Ethics Committee approval, skin biopsies of two Hurler disease patients [9-month-old female (patient 8180) and 21-month-old male (patient 8339)], two Costello syndrome patients [9-month-old male (patient 12,195) and 24-month-old female (patient 12,368)], and one 18-month-old female patient (patient 4032) diagnosed with the infantile phenotype of GM₁-gangliosidosis bearing nonsense mutation of β -Gal gene, therefore deficient in EBP,⁵³ were used as the source of fibroblasts. Fibroblasts derived from one normal 36-month-old female (patient 4184) were also used for comparison. All donors of fibroblasts were clinically diagnosed at The Hospital for Sick Children in Toronto. Clinical diagnosis of Hurler disease, and GM₁ gangliosidosis patients were confirmed by genetic tests. The diagnosis of Costello patients was based on the presence of a cardinal clinical features previously ascribed to this phenotype^{23–25} (coarse facies and thick lips, mental retardation, postnatal growth retardation, sparse and curly hair, deep palmar and plantar creases, loose skin of the hands and feet, hypertrophic cardiomyopathy and arrhythmia, and papillomata) and by our previously described³⁵ immunohistochemical evaluation of their cultured skin fibroblasts demonstrating impaired elastogenesis and higher than normal deposition of chondroitin sulfate.

All skin fibroblasts collected from patients with these rare genetic diseases were originally isolated by collagenase digestion of the biopsies and then stored as multiple samples of passage two in the cell repository of The Hospital for Sick Children in Toronto. All mentioned fibroblasts were previously used in our published studies on Hurler disease³⁴ Costello syndrome,³⁵ and GM₁ gangliosidosis.⁵³ In the present study fibroblasts taken from the repository were maintained in α -minimum essential medium supplemented with 20 mmol/L HEPES, 1% antibiotics/anti-mycotics, and 10% fetal bovine serum and passaged two to four times by trypsinization before their retroviral transduction. Both control and transduced cells were further passaged two times and then used in immunohistochemical, biochemical, and cell proliferation test. We have previously established that both Costello syn-

drome and Hurler disease fibroblasts maintain their phenotype (lack of elastic fiber assembly and high levels of CS- or DS-proteoglycans) up to passages 10 to 12.

Retroviral Vector Construction and Infection

The rat V3 cDNA, rVe,⁵¹ had an upstream sequence of 18Ts that we believed to be an artifact of its cloning into the vector in the reverse direction. To remove this sequence, the V3 sequence was prepared as follows, using standard molecular biology protocols.^{54,55} The rVe cDNA in pBluescript sk(−) was linearized with Nar I, at position −188 of the 5′-untranslated region. The overhanging ends were filled in with Klenow fragment and *Bam*HI linkers were attached. After digestion with *Bam*HI, which cut both the linker and a sequence in the multicloning site at the other end of the versican sequence, the V3 sequence was inserted into the *Bam*HI site of the retroviral vector LXS_N (courtesy of Dr. A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA). The retroviral vector containing the V3 gene (LV), as well as the empty control vector (LX), were used to transduce cultured fibroblasts derived from patients with Costello syndrome and Hurler disease as previously described.^{56–58} The EBP-deficient fibroblasts from the GM₁-gangliosidosis patient bearing a nonsense mutation of β -galactosidase gene were similarly transduced for the comparison. Effectiveness of the transduction procedure was confirmed by a Northern blot with the V3 cDNA probe.

Immunostaining

Skin fibroblasts from all patients, and additionally normal fibroblasts incubated in the presence of 200 μ g/ml of exogenous chondroitin sulfate A (preparation from bovine trachea containing 70% of chondroitin 4-sulfate and 30% chondroitin 6-sulfate, C-8529; Sigma), to alter elastic fiber assembly were fixed in cold 100% methanol at −20°C for 30 minutes and immunostained with each of the following antibodies: 10 μ g/ml of polyclonal antibody raised to the elastin-binding domain of the alternatively spliced variant of β -galactosidase (anti-S-Gal) that recognizes the EBP⁴⁰, 10 μ g/ml of monoclonal antibody to chondroitin sulfate (C-8035, Sigma), 10 μ g/ml of antibody recognizing the isoforms of versican (LF 99), 5 μ g/ml of antibody (DAKO, Denmark) recognizing ki-67 antigen present in proliferating cells,⁵⁹ and with 20 μ g/ml of polyclonal antibody to tropoelastin.⁵⁹ Parallel cultures scheduled for immunohistochemical assessment of microfibrillar components of the elastic fibers were fixed in 0.5% paraformaldehyde for 15 minutes, blocked in phosphate-buffered saline (PBS) containing 0.1 mol/L ammonium chloride, and then treated with 20 μ g/ml of polyclonal antibody to human fibrillin 1. Replicates were additionally pretreated for 10 minutes with PBS containing 50 mmol/L dithiothreitol, alkylated with 100 mmol/L iodoacetamide for 15 minutes, washed in PBS, and then immunostained with a polyclonal antibody to MAGP at the same concentration.⁶⁰ All cultures were incubated with appropriate fluorescein-conjugated secondary antibodies (GAR-FITC

or GAM-FITC) for an additional hour. Nuclei were counterstained with propidium iodide (Sigma, Tokyo, Japan).

Morphometric analysis was performed using an Olympus AH-3 microscope attached to a CCD camera (Optonix, Staffordshire, UK) and a computerized video analysis system (Image-Pro Plus software 3.0 for Macintosh; Media Cybernetics, Silver Spring, MD) as previously described.^{34,35} Fifty low-power fields ($\times 20$) from three separate cultures derived from each analyzed group were analyzed, and the area occupied by the particular immunodetectable component was quantified. The abundance of each component was then expressed as a percentage area of the entire analyzed field.

Tropoelastin and Insoluble Elastin Assays

Fibroblasts from all experimental groups were plated in quadruplicates either subconfluently (2×10^4 cells per 30-mm dish) or densely (1×10^5 cells per 30-mm dish) to reach immediate confluency. Twenty-four hours after cell seeding, [³H]-valine (20 μ Ci) was added to each dish in fresh media. Cultures were then incubated for 2 or 6 days, and soluble and insoluble elastin were assessed separately from each culture.^{34,35} Parallel cultures were also incubated in the presence of 10 to 4 mol/L of β -amino-propionitrile (BAPN, Sigma), a lysyl oxidase-inhibitor, to prevent deposition of insoluble elastin.⁶¹ At the end of each experiment, the conditioned media were collected and immunoprecipitated with a polyclonal antibody to tropoelastin. The soluble proteins in the intracellular compartments were extracted with 0.1 mol/L of acetic acid, and intracellular tropoelastin was immunoprecipitated from these extracts and quantitatively assessed by scintillation counting. The remaining cultures containing cell remnants and deposited insoluble extracellular matrix were scraped and boiled in 0.5 ml of 0.1 N NaOH for 45 minutes to solubilize all matrix components except elastin. The resulting pellets containing the insoluble elastin were then solubilized by boiling in 200 μ l of 5.7 N HCl for 1 hour and the aliquots were mixed with scintillation fluid and counted.

Northern Blots

Total RNA was isolated from 15-day cultures using Trizol solution following the manufacturer's instructions. Purified samples were fractionated on 0.8% formaldehyde-agarose gels, alkali-denatured in 50 mmol/L NaOH and 10 mmol/L NaCl, neutralized, transferred to nylon blotting membranes (Zeta Probe; Bio-Rad, Richmond, CA) and crosslinked by UV-irradiation. Blots were hybridized with [³²P]-dCTP random-labeled cDNA probe to full-length rat versican V3 splice variant (clone V5a2).^{52,54} In addition, the membranes were probed with a [³²P]-dCTP random-labeled 0.9-kb human tropoelastin cDNA recombinant H-11 probe for 16 hours, followed by two washes with 2 $\times$ standard saline citrate/0.1% sodium dodecyl sulfate at 55°C for 30 minutes each and one wash with 0.2 $\times$ standard saline citrate/0.1% sodium dodecyl sulfate at 65°C for 1 hour. For quantitation of mRNA levels, autoradio-

grams were normalized to the amount of 28S ribosomal RNA as revealed by ethidium bromide staining.

Isolation of EBP

Results of our previous studies demonstrated that initial EBP synthesis did not differ between Hurler and Costello fibroblasts when compared to normal fibroblasts.^{34,35} Furthermore, these studies showed that EBP was quickly shed from the surface of Hurler and Costello fibroblasts (30 to 45 minutes) because of its interaction with dermatan sulfate or chondroitin sulfate. To assess the influence of V3 transduction on the processing of EBP, fibroblasts from each experimental group were pulsed-labeled with 15 μ Ci/ml [¹⁴C]-serine in serine-free medium for 1 hour. The cultures were then rinsed well and chased in fresh Medium 199 for 45 minutes. An additional experimental group consisted of normal fibroblasts that were pulsed and chased in the presence or absence of 200 μ g/ml of exogenous chondroitin sulfate A. At the end of the chase period, the media were removed; the cell layers were washed, scraped, and suspended in 1 ml of 0.1 mol/L bicarbonate buffer, pH 8. Extraction was performed with 0.1 mol/L lactose, 0.1 mol/L dithiothreitol, and 0.25% octyl- β -glucoside in the presence of proteinase inhibitors in the following final concentrations: 2 mmol/L benzamide, 2 mmol/L epsilon amino capronic acid (EACA), 2 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L ethylenediaminetetraacetic acid, and 1 mg/ml trasylol. Extraction took place throughout 3 hours at 4°C with constant stirring and the remaining insoluble material was removed by centrifugation. The supernatants were dialyzed exhaustively (12,000 to 14,000 MW cutoff) at 4°C against 0.1 mol/L sodium bicarbonate, pH 8, containing proteinase inhibitors. The extracts were run over an insoluble elastin affinity columns for 1 hour at 4°C.^{34,35,41} The unbound material was removed by washing the insoluble elastin affinity slurries with 0.1 mol/L sodium bicarbonate buffer, pH 8, until the absorption A_{280} of the eluant returned to background level. The elastin slurries were then pelleted; suspended in 62.5 mmol/L Tris-HCl buffer, pH 6.8, containing 2% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, and 0.001% bromophenol blue with dithiothreitol; and boiled for 5 minutes. The elastin-bound proteins were resolved by 7.5 to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. The identity of EBP was additionally confirmed by immunoblotting with an affinity-purified anti S-Gal antibody that recognizes the 67-kd EBP,⁴⁰ followed by GAR-HRP-conjugated secondary antibody and amplification with the enhanced chemiluminescence detection system.

Assessment of Fibroblast Proliferation

Fibroblasts from all experimental groups were seeded in quadruplicates either subconfluently (2×10^4 cells/30-mm dish) or densely (1×10^5 cells per 30-mm dish) in α -minimal essential medium containing 10% fetal bovine serum to reach immediate confluency. The medium was

Figure 1. A: Representative Northern blots illustrating expression of V3 versican mRNA in Costello syndrome, Hurler disease, and GM1-gangliosidosis fibroblasts cells retrovirally transduced with V3 cDNA (LV) or with empty vector (LX). **B:** Northern blots probed with 0.9 kb of human tropoelastin cDNA demonstrate that all tested fibroblasts, either nontransduced (NT) or LX- and LV-transduced, express comparable levels of tropoelastin mRNA.

changed at 24 hours after seeding, and parallel cultures were maintained for the next 2 days in the presence of [³H]-thymidine (2 μ Ci/well). All cultures were then washed in PBS and treated with cold trichloroacetic acid twice for 10 minutes at 4°C. Then 0.5 ml of 0.3 N NaOH was added to all dishes for 30 minutes and 200- μ l aliquots of each extract were mixed with scintillation fluid and counted.⁶² Additionally, expression of Ki-67 proliferative antigen and growth curves of quadruplicate cultures of fibroblasts from all experimental groups were performed as previously described.⁶³ In all above-mentioned studies, means and standard deviations were calculated, and statistical analyses were performed by analysis of variance.

Results

Northern blot analysis confirmed that, in contrast to fibroblasts transduced with the empty vector (LX), fibroblasts transduced with V3 cDNA (LV) displayed strong mRNA expression of this short variant of versican (Figure 1A). Probing these same blots for tropoelastin mRNA revealed no differences in expression of this ECM gene among the different experimental groups (Figure 1B). Immunostain-

ing of 48-hour-old subconfluent cultures confirmed our previous observation that, in contrast to fibroblasts derived from normal children, fibroblasts derived from Costello syndrome patients accumulate chondroitin sulfate at the cell surface and in the pericellular space (Figure 2A). On the other hand, LV Costello fibroblasts accumulated less chondroitin sulfate, as compared to LX counterparts. Similar patterns of immunostaining were also seen with antibody recognizing CS-containing endogenous versican (Figure 2A). Furthermore, the LV Costello fibroblasts immunostained considerably more intensely for EBP than LX Costello cells and resembled the staining pattern for normal fibroblasts. A similar change in the amount and distribution of EBP was observed in LV Hurler fibroblasts (Figure 2B). Neither LX nor LV fibroblasts derived from the GM₁-gangliosidosis patient, bearing a nonsense mutation of β -galactosidase gene and lacking EBP, immunostained for EBP. The increase in EBP associated with the cell surface of LV Costello and LV Hurler fibroblasts was additionally confirmed by metabolic radioactive pulse-labeling and chase experiments. More metabolically labeled 67-kd protein could be isolated from cell extracts by elastin-affinity chromatography from the LV Costello and LV Hurler fibroblasts after a 45-minute chase than from their LX counterparts (Figure 2C). This experiment additionally demonstrated that normal fibroblasts incubated in the presence of exogenous chondroitin sulfate lose their synthesized (labeled) EBP and that LX or LV GM₁-gangliosidosis fibroblasts do not produce EBP.

Immunostaining of 7-day-old normal fibroblast cultures with an anti-elastin antibody demonstrated a dense network of elastic fibers that was absent in the nontransduced (NT) and LX fibroblasts derived from Costello syndrome and Hurler disease patients (Figure 3). Normal fibroblasts treated with chondroitin sulfate also showed an absence of elastic fiber network, confirming that these glycosaminoglycans interfere with elastic fiber assembly. Importantly, transduction of the Costello and Hurler fibroblasts with V3 completely converted these cells to an elastogenic phenotype that resembled normal fibroblasts. This was in striking contrast to GM₁-gangliosidosis-derived fibroblasts that did not produce an elastic fiber network after V3 transduction.

Morphometric analysis of immunostained cultures revealed that the lack of mature elastic fibers in cultures of NT Costello and Hurler fibroblasts, as well as in their LX counterparts, coincided with a marked accumulation of chondroitin sulfate associated with their cell surfaces (Figure 4A). However, immunostaining for other components of elastic fibers such as fibrillin 1 and MAGP revealed no differences (Figure 4, A and B). Interestingly, similar analyses of LX and LV GM₁-gangliosidosis fibroblasts further demonstrated the V3 transduction of these cells had no effect on fibrillin 1 and MAGP distribution, and did not improve their low deposition of extracellular elastin (Figure 4C). These results support the postulate that impaired elastic fiber assembly in Hurler disease and Costello syndrome fibroblasts is likely because of impaired elastin secretion and assembly and not to alterations in the microfibrillar scaffold.

Quantitative assessment of [³H]-valine incorporation into total immunoprecipitable soluble tropoelastin (media and cell layers) showed that fibroblasts from all experimental groups synthesized comparable amounts of this precursor protein (Figure 5A). Immunoprecipitation of soluble tropoelastin extracted separately from the cell layers and conditioned media revealed however, that in contrast to normal fibroblasts, the majority of the metabolically labeled tropoelastin was retained intracellularly and not secreted from the nontransduced (NT) and LX Costello, Hurler, and GM₁-gangliosidosis fibroblasts (Figure 5B). However, transduction of Costello and Hurler fibroblasts with V3 significantly reduced the levels of newly synthesized intracellular tropoelastin and increased the amount of newly synthesized elastin secreted to the media (Figure 5C). Consistently, LV Costello and Hurler fibroblasts incorporated more radioactive valine into the NaOH-insoluble elastin than their NT or LX counterparts (Figure 5D). Importantly, the compartmental distribution of newly synthesized tropoelastin in the LV GM₁-gangliosidosis fibroblasts was unaffected. We have also mentioned that the high V3 expression in LV Costello and Hurler fibroblasts and the consequent reversal of their diseased phenotype remained stable in four successive passages tested.

To determine whether reversal of elastic fiber phenotype by V3 in Hurler disease and Costello syndrome fibroblasts was accompanied by changes in their proliferative phenotype, thymidine incorporation and growth curve experiments were performed. We found that DNA synthesis as measured by thymidine incorporation was significantly reduced in high-density cultures of LV Costello and LV Hurler fibroblasts (Figure 6A). Furthermore, LV Costello and LV Hurler fibroblasts grew significantly more slowly than their NT and LX counterparts (Figure 6B) confirming that elastic fiber assembly and cell proliferation are inversely correlated. The inhibition of proliferation in cultures of LV Costello and LV Hurler fibroblasts was also confirmed by a significant decrease in the percentage of cells demonstrating the presence of Ki-67 antigen (data not shown). To further establish the inverse relationship between deposition of insoluble elastin and cellular proliferation, LV Hurler disease and LV Costello syndrome fibroblasts were grown at low density to limit deposits of insoluble elastin. Sparsely seeded NT, LX, and LV Costello and Hurler fibroblasts that deposit only traces of insoluble elastin (Figure 7A) grew at a faster rate and incorporated more [³H]-thymidine than normal fibroblasts (Figure 7B). Furthermore, inhibiting the deposition of insoluble elastin in confluent cultures by the lysyl oxidase inhibitor, BAPN eliminated the inhibitory effect of V3 on thymidine incorporation by these cells (Figure 8). Together, these results support previous observations that cell proliferation of tested fibroblasts is affected by the deposition of insoluble elastin.

Discussion

In this study, we have used retroviral mediated transfer of the gene encoding the V3 splice variant of versican to

reverse two phenotypic traits, impaired elastic fiber assembly and elevated cell proliferation, in skin fibroblasts from patients with Hurler disease and Costello syndrome.

Versican is an extracellular matrix proteoglycan that is synthesized as multiple splice variants.^{47–50} Three of these variants (V0, V1, and V2) contain one or both of the domains that include multiple chondroitin-sulfate chain attachments sites and thus differ in core protein length and number of chondroitin sulfate chains. A fourth variant, V3, lacks both glycosaminoglycan attachment exons and is thus predicted to be a glycoprotein that lacks chondroitin sulfate chains. All versican isoforms possess conserved globular domains at their amino and carboxy termini⁴⁷ and can interact with similar ligands via these domains. The anti-adhesive function of the chondroitin sulfate-containing isoforms of versican has been attributed to the high negative charge and hygroscopic properties of the glycosaminoglycan chains. Overexpression of V3 by rat arterial smooth muscle cell increases cell adhesion and decreases cell proliferation and migration,⁵² suggesting that V3 counteracts the anti-adhesive effects of the larger chondroitin sulfate-containing isoforms of versican.⁴⁷ This V3-dependent phenotype is also associated with the formation of elastic fibers in 2- to 3-week arterial smooth muscle cell cultures.⁵¹ This phenotype is maintained when V3-transduced arterial smooth muscle cells are seeded into balloon-injured rat carotid arteries.⁵¹ The presence of V3-expressing arterial smooth muscle cells in the injured blood vessels produces a highly structured neointima that is significantly enriched in elastic fiber lamellae.^{47,51} The mechanism for this proelastogenic effect of V3 is not known.

Although the basic genetic defect in Costello syndrome remains obscure, previous studies demonstrate that cultured fibroblasts derived from patients with this disorder accumulate β -galactosugars such as chondroitin 4-sulfate at their cell surfaces.³⁴ Interestingly, the clinical phenotype of Costello syndrome resembles Hurler disease, and skin fibroblasts from these patients accumulate dermatan sulfate as part of their altered phenotype.^{4–7} Because both of these disorders involve defective formation of elastic fibers,^{10,15,16,34,35} and treatment of normal cells with exogenous chondroitin sulfate and dermatan sulfate, but not with heparan sulfate inhibits elastogenesis *in vitro*,^{34,35} we hypothesized that accumulation of β -galactosugars associated with these cells interferes with normal assembly of elastin on the microfibrillar scaffold. This hypothesis is supported by observations that demonstrate that both Hurler disease and Costello syndrome fibroblasts synthesized tropoelastin at a normal rate but are unable to secrete tropoelastin or to

assemble extracellular insoluble elastin. Furthermore, impaired secretion of tropoelastin coincides with low amounts of EBP, the elastin receptor, in both Hurler and Costello fibroblasts, suggesting that a deficiency in this recyclable tropoelastin chaperone^{38–45} is a crucial factor in the disruption of elastic fiber formation. Pulse-chase experiments indicate however, that fibroblasts from both diseases produce equal amounts of EBP, but quickly lose it into the conditioned media.^{33,34} These observations plus studies that demonstrate that β -galactosugars can bind to the galactose domain of EBP and inhibit tropoelastin binding to EBP supports a regulatory role for chondroitin sulfate and dermatan sulfate glycosaminoglycans in elastic fiber assembly.^{38–42} The fact that addition of galactosugars to cultures of normal fibroblasts, chondroblasts, and smooth muscle cells causes depletion of EBP and disruption of elastic fiber production^{38,44–46} suggests that rapid shedding of the EBP from the Costello and Hurler cells occurs as a result of the respective accumulation of chondroitin sulfate and dermatan sulfate on the surface of these cells. This proposal is further supported by studies that demonstrate that enzymatic degradation of chondroitin sulfate and dermatan sulfate by exogenous chondroitinase ABC leads to restoration of elastic fiber assembly in cultures of skin fibroblasts derived from patients with Costello syndrome or Hurler disease.^{33,35}

Although the mechanism(s) responsible for the V3 effect on elastic fiber assembly and proliferation of arterial smooth muscle cells has not been established,^{51,52,54} the results of the present study suggest that V3 could be acting by competing for chondroitin sulfate-containing isoforms of versican associated with the cell surface. For example, all isoforms of versican can interact with hyaluronan that is associated with the cell surface via hyaluronan receptors such as CD44. It could be that V3 competes with V1 for binding sites on hyaluronan and displaces the chondroitin sulfate-containing isoform of versican from the cell surface. Our findings of reduced immunostaining for cell surface chondroitin sulfate isoforms of versican in the LV Costello syndrome fibroblasts coupled to loss of EBP and enhanced elastic fiber assembly by these cells supports a role for V3 in regulating elastic fiber assembly by preventing loss of EBP and restoring normal secretion of tropoelastin. Further support for this proposed mechanism is the finding that V3 transduction of fibroblasts derived from a GM₁-gangliosidosis patient (primary deficient in EBP) failed to restore elastogenesis in these cells.

Although our studies strongly support a role for EBP in regulating the V3 elastogenic effect, we cannot rule out

Figure 2. A: Representative photomicrographs of 48-hour-old cultures of human skin fibroblasts. Note that positive immunostaining is FITC (green) with a red nuclear counterstain. Immunostaining indicates that, in contrast to cultures of normal fibroblasts, Costello syndrome fibroblasts, either nontransduced (NT) or LX-transduced, show strong deposition of chondroitin sulfate-containing moieties (CS), including versican 1, in their extracellular matrix. The high expression of chondroitin sulfate and versican 1 coincided with greatly diminished levels of the cell-surface elastin-binding protein (EBP). In contrast, the LV fibroblasts demonstrated lower levels of immunodetectable chondroitin sulfate moieties and versican 1 and strong cell-surface expression of the EBP, with a pattern that resembled normal fibroblasts. **B:** Similarly, LV but not LX Hurler fibroblasts showed intense immunostaining for EBP. The LX or LV GM₁-gangliosidosis fibroblasts did not reveal immunodetectable EBP. **C:** Representative autoradiographs showing levels of the radioactive 67-kd EBP protein purified by elastin affinity columns from the fibroblasts metabolically pulsed with [¹⁴C]-serine for 1 hour and then chased for 45 minutes. In contrast to normal fibroblasts, which retain the majority of metabolically labeled EBP after a 45-minute chase, fibroblasts maintained in the presence of exogenous chondroitin sulfate (CS) lose a large portion of this initially synthesized protein. Moreover, LV fibroblasts from Costello syndrome and Hurler disease retain more 67-kd EBP than their LX counterparts. Both LX and LV GM₁-gangliosidosis fibroblasts do not contain any metabolically labeled EBP.

Normal

**Normal
+
Chondr.
Sulfate**

**Costello
LX**

**Costello
LV**

**Hurler
LX**

**Hurler
LV**

**GM1
LX**

**GM1
LV**

Figure 4. Morphometric analysis of immunostained components of extracellular matrix in 7-day-old cultures of normal and Costello syndrome fibroblasts (A), and in cultures of Hurler disease (B) and GM₁-gangliosidosis fibroblasts (C). The nontransduced (NT) and empty vector-transduced (LX) Costello and Hurler fibroblasts deposit only negligible amounts of immunodetectable extracellular elastin. Amounts of chondroitin sulfate produced by these fibroblasts significantly exceed those present in cultures of normal fibroblasts. In contrast, both V3-transduced (LV) Costello and Hurler, but not GM₁-gangliosidosis-derived fibroblasts, demonstrate significantly higher rates of immunodetectable elastin in their ECM, but lower accumulation of chondroitin sulfate. Deposition of fibrillin 1 and MAGP (marking microfibrillar scaffold) in all tested cultures was not affected by LX or LV transduction. In each analyzed group, 50 low-power fields ($\times 20$) from three separate cultures (per independent patients) were analyzed and the area occupied by the particular immunodetectable component quantified. The abundance of each component was then expressed as a percentage of the entire analyzed field (mean $\pm$ SD), and results from cultures of fibroblasts from Costello syndrome and Hurlers disease were statistically compared with those in cultures of normal skin fibroblasts (*, $P < 0.001$).

the possibility that V3 may act also as a structural component in elastic fiber assembly. Versican has been detected within elastic fibers and shown to interact with a major component of the microfibrillar scaffold, fibrillin 1.⁶⁴ In addition, versican interacts with fibulin 1 and fibulin 2,^{65,66} two proteins that also associate with elastic fiber microfibrils. Because the interaction of all versican isoforms with these proteins involves the C-type lectin in the G3 domain of versican, V3 could play a role in facilitating the interaction of these elastin-associated proteins during the formation of the elastic fibers. Our immunohistochemical evidence that V3-expressing cells form microfibrillar scaffolds that do not differ in fibrillin 1 and MAGP content

Figure 5. A: Quantitative analysis of [³H]-valine-labeled immunoprecipitable tropoelastin indicates that normal (patient 4184), Costello syndrome (patients 12,368 and 12,195), Hurler disease (patients 8180 and 8339), and GM₁-gangliosidosis (patient 4032) fibroblasts synthesize comparable amounts of total metabolically labeled tropoelastin. B: In contrast to normal fibroblasts, all nontransduced (NT) and LX fibroblasts retain the majority of their metabolically labeled tropoelastin intracellularly. Consistently, those fibroblasts demonstrate lower than normal levels of immunoprecipitable tropoelastin from conditioned media (C) and incorporate significantly less [³H]-valine into extracellular insoluble elastin than normal fibroblasts (D). In contrast, cultures of both Costello syndrome and Hurler disease fibroblasts transduced with the V3 versican gene (LV) demonstrate much lower levels of intracellular tropoelastin and significantly higher levels of insoluble elastin than their NT and LX counterparts. LV transduction of GM₁-gangliosidosis fibroblasts did not improve their ability to secrete newly synthesized tropoelastin and deposit insoluble elastin.

and distribution from vector alone cells suggests that V3 is not affecting the formation of the microfibrillar scaffold involving these proteins. However, more work is needed to determine whether different isoforms of versican play other structural roles in the formation of elastic fibers.

Our finding that overexpression of V3 by Costello and Hurler fibroblasts also influences the proliferation of these cells supports other studies that illustrate a relationship between elastic fiber assembly and cell proliferation. For example, transgenic mice with homozygous null mutation for elastin develop fibrocellular occlusive arterial disease because of subendothelial proliferation and accumulation of arterial smooth muscle cells.⁶⁷ Furthermore, arte-

Figure 3. Representative photomicrographs of 7-day-old cultures immunostained with anti-tropoelastin antibody, indicate that normal fibroblasts produced long, branching elastic fibers, whereas normal fibroblasts maintained in the presence of exogenous chondroitin sulfate do not deposit any extracellular elastin. The lack of elastic fibers is also apparent in cultures of Costello syndrome and Hurler disease fibroblasts transduced with empty retroviral vector (LX). In contrast, Costello and Hurler fibroblasts transduced with cDNA encoding V3 versican (LV) produce elastic fibers that are similar to those deposited in cultures of normal fibroblasts. The EBP-deficient fibroblasts derived from GM₁-gangliosidosis patients did not display any elastic fibers in the newly produced extracellular matrix regardless of LX or LV transduction.

Figure 6. A: Assessment of [³H]-thymidine incorporation into 3-day-old cultures (initially densely seeded at 100,000 cells/dish to reach immediate confluency and deposition of elastin-containing ECM). Results indicated the V3 transduction of Costello and Hurler, but not GM₁-gangliosidosis fibroblasts, causes a significant decrease in their proliferation rate. **B:** Estimation of cell number and total DNA assayed in dense cultures (initially plated at 100,000 cells/dish). The NT and LX Costello and Hurler fibroblasts exhibit significantly higher growth rates. In contrast, growth curves of their LV counterparts are similar to normal fibroblasts.

rial smooth muscle cells from elastin knockout mice proliferate at a higher rate than wild-type when placed in cell culture and this elevated proliferation can be reduced by the addition of elastin to the cultures.⁶⁸ Other studies demonstrate that heightened proliferation of fibroblasts and aortic smooth muscle cells from children with supra-valvular aortic stenosis and Williams-Beuren syndrome exhibiting primary haploinsufficiency of the elastin gene can be reversed by adding insoluble elastin.⁶⁹ Additionally, the elevated proliferation of cultured Costello syndrome fibroblasts and Hurler disease fibroblasts can be normalized by administration of exogenous insoluble elastin.^{34,35} Our finding that inhibition of tropoelastin polymerization into cross-linked insoluble elastin by BAPN eliminates the inhibitory effect of V3 on the proliferation of LV Costello syndrome and LV Hurler disease cells (Figure 8) further supports a role for insoluble elastin in controlling cell proliferation.

Our present studies do not directly address the molecular mechanism by which insoluble elastin regulates cell proliferation. At present, we can only speculate that a decrease in proliferation of LV fibroblasts, detected only when they deposit insoluble elastin, likely results from several overlapping mechanisms. It may be that contact between elastin and the cell surface initiates anti-mitogenic signals.⁶⁸ However, previous results demonstrat-

Figure 7. Incorporation of [³H]-valine into NaOH-insoluble elastin (**A**) and incorporation of [³H]-thymidine (**B**) in subconfluent 3-day-old cultures of Costello syndrome and Hurler disease fibroblasts (initially sparsely seeded at 20,000 cells/dish). Both NT and LX Costello and Hurler fibroblasts kept in subconfluent cultures do not deposit significant amounts of elastin and demonstrate only slightly higher than normal proliferation rates. The proliferation of LV fibroblasts kept in subconfluent cultures does not differ from their NT and LX counterparts.

ing that rapidly proliferating fibroblasts from supra-valvular aortic stenosis and Williams-Beuren syndrome reduce their proliferation when maintained in media that were only preincubated with exogenous insoluble elastin⁶⁹ suggests that insoluble elastin may block the mitogenic signals by interfering with growth factor availability. For example, insoluble elastin can trap platelet-derived growth factor.⁷⁰ Physical contact between large particles of insoluble elastin and the cell surface may also cause aggregation of EBP and masking of adjacent growth factor receptors that normally transduce mitogenic signals. This suggestion is consistent with previous findings that demonstrate that cell-surface EBP can mask adjacent interleukin type I receptors and decrease the cellular response to this cytokine.⁷¹ Moreover, large hydrophobic particles of exogenous insoluble elastin attract soluble tropoelastin-derived peptides present in the conditioned media and cause their precipitation (co-acervation).^{72,73} Such a depletion of soluble tropoelastin and its soluble degradation products reduces the likelihood of a mitogenic response to these factors such as has been demonstrated for many cell types, including vascular smooth muscle cells, skin fibroblasts, and astrocytoma cells.^{62,74-76}

In summary, our results demonstrate that overexpression of V3 in Hurler disease and Costello syndrome-derived fibroblasts rescues the tropoelastin chaperone, EBP, reverses the impairment of elastic fiber assembly and restores the normal proliferative activity of these cells. Furthermore, our data supports previous studies that show a link between the accumulation of galactosugars such as chondroitin sulfate and the altered phenotype

A Incorporation of [³H]-Valine into Insoluble Elastin

B Incorporation of [³H]-Thymidine

Figure 8. A: Quantitative analysis of [³H]-valine-labeled insoluble elastin indicates that treatment with inhibitor of lysyl oxidase, β -amino-propionitrile (BAPN) caused radical inhibition in deposition of insoluble elastin in densely plated (100,000 cells/dish), 7-day-old cultures of normal fibroblasts and V3-transduced (LV) Costello fibroblasts. Treatment with BAPN further deteriorates low elastin deposition in cultures of NT and LX Costello fibroblasts. **B:** Assessment of [³H]-thymidine incorporation in parallel 7-day-old cultures demonstrates that V3-transduction of (LV) did not reduce heightened proliferation of Costello fibroblasts cultured in the presence of BAPN, which prevents the efficient deposition of insoluble elastin. Interestingly, inhibition of insoluble elastin deposition in cultures of normal fibroblasts also caused an increase in their proliferation rate.

of fibroblasts taken from patients with these two diseases. Because Costello syndrome and Hurler disease remain incurable, data presented in this report may encourage development of therapeutic strategies aimed at using this technology to enhance elastin deposition and reverse major phenotypic features in these two genetic disorders.

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