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Significant Decrease in Tropoelastin Gene Expression in Fibroblasts from a Japanese Costello Syndrome Patient with Impaired Elastogenesis and Enhanced Proliferation

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Costello syndrome is a connective tissue disorder associated with sparse, thin, and fragmented elastic fibers in tissues. In this study we demonstrated a significant decrease in the expression of tropoelastin mRNA in fibroblasts derived from a Japanese Costello syndrome patient with impaired elastogenesis and enhanced proliferation. In contrast, there were no changes in expression of the Harvey ras (HRAS), fibrillin-1, fibulin-5, microfibril-associated glycoprotein-1 (MAGP-1), lysyl oxidase (LOX), or 67-kDa non-integrin elastin-binding protein (EBP) gene. The proliferative activity of the Costello fibroblasts was about 4-fold higher than that of the normal and pathological control ones. However, no mutations were detected in the coding region of HRAS mRNA. Transduction of the bovine tropoelastin (bTE) gene with the lentiviral vector restored the elastic fiber formation and decreased the growth rate in the Costello fibroblasts. These results strongly suggest that the defect of human tropoelastin (hTE) gene expression should induce the impaired elastogenesis and enhanced proliferation of Costello fibroblasts, and that a primary cause other than the HRAS gene mutation should contribute to the pathogenesis in the present Costello case.

Key words: Costello syndrome, elastogenesis, lentiviral vector, proliferation, tropoelastin.

Abbreviations: bTE, bovine tropoelastin; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CS, chondroitin sulfate; DEPC, ethanol/diethylpyridylchloride; DS, dermatan sulfate; EBP, elastin-binding protein; FCS, fetal calf serum; HRAS, Harvey ras; HRP, horseradish peroxidase; hTE, human tropoelastin; Lenti-*bTE*, lentiviral vector encoding bTE cDNA; Lenti-*EGFP*, lentiviral vector encoding enhanced green fluorescent protein cDNA; LOX, lysyl oxidase; MAGP-1, microfibril-associated glycoprotein-1; PVDF, polyvinylidene difluoride; RT, reverse transcription; RT-PCR, reverse transcriptase–polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; TE, tropoelastin; VSVG, vesicular stomatitis virus.

Costello syndrome was first described by Dr. J.M. Costello (*1*, *2*). Over 100 cases have been reported, and the natural history and clinical features have been established (MIM 218040), although the genetic background remains unknown. Polyhydramnios is frequent during pregnancy (*3*, *4*). The newborn is often of excessive size and exhibits severe feeding problems during the first year with growth retardation. The clinical manifestations are coarse facial features, redundant skin with deep palmar and plantar creases, papillomata, laxity of small joints, tight Achilles tendons, cardiac malformations and developmental delay (*2*, *5*). About 60% of the patients also develop various cardiac abnormalities. Malignant tumors, including rhabdomyosarcomas, neuroblastomas, and bladder carcinomas, may occur. Recently, germ line mutations in the Harvey ras (HRAS) gene have been identified in some patients with Costello syndrome (*6*), suggesting that up-regulation of the HRAS gene may contribute to the enhanced cell proliferation in the patients. However, the pathogenic mechanism of Costello syndrome is not necessarily explained by the HRAS gene mutations, although defective elastic fiber formation is commonly seen in this syndrome.

The elastic fibers in connective tissues and blood vessel walls are made of polymeric tropoelastin, and are laid on a scaffold of microfibrils consisting of glycoproteins (*e.g.*, fibrillins and microfibril-associated glycoproteins) (*7*, *8*). Several functional proteins, including elastin-binding protein (EBP), as a tropoelastin chaperone, and lysyl oxidase (LOX) involved in cross-linking have also been demonstrated to contribute to elastic fiber formation (*9*–*12*). Impaired elastogenesis has been reported in various human diseases, including Marfan syndrome (*13*), William syndrome (*14*), and Costello syndrome (*2*, *5*), accompanied by connective tissue abnormalities and cardiovascular manifestations. Hinek et al. have demonstrated that an enzymatically inactive, alternatively spliced variant of the lysosomal β-galactosidase (β-Gal; EC 3.2.1.23) (*15*) gene (*GLB1*) product functions as an EBP that facilitates the intracellular transport of tropoelastin and its

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extracellular assembly into elastic fibers (*16*, *17*). They also reported that proteoglycans with β-galactosugar–bearing moieties, such as chondroitin sulfate (CS) and dermatan sulfate (DS), could interfere with the elastic fiber formation by interacting with the lectin-like domain of EBP and inducing its shedding from the cell surface, and suggested that the accumulation of CS and DS might cause defective elastic fiber assembly in fibroblasts in Costello syndrome via the EBP shedding mechanism (*18*, *19*). Other studies involving fibroblasts from Costello syndrome patients revealed impaired secretion of tropoelastin (TE), the monomeric precursor of insoluble elastin (*20*, *21*).

In this study, we demonstrated impaired elastogenesis and a specific decrease in human tropoelastin (hTE) mRNA in fibroblasts derived from a Japanese Costello syndrome patient, which might not be directly caused by the *HRAS* mutations. We also observed the reversal of defective elastic fiber assembly and enhanced proliferation of Costello fibroblasts on transduction with a recombinant lentiviral vector encoding bovine tropoelastin (bTE) cDNA.

MATERIALS AND METHODS

Patient—The patient with Costello syndrome was a 12.8-year-old girl previously diagnosed as having idiopathic hypertrophic cardiomyopathy. She was born to healthy nonconsanguineous Japanese parents at 38 weeks of pregnancy. Polyhydramnios was recognized during the pregnancy. Her birth weight was 3,946 g (+2.9 SD), and her birth height 48.8 cm (–0.0 SD). She developed failure to thrive and curly hair after birth. At the age of 4 months, she was diagnosed as having idiopathic hypertrophic cardiomyopathy, clinically recognized as heart murmur. Although her facial appearance was noted to be mucopolysaccharidosis-like, there was nothing remarkable in her early childhood. At the age of 12.8 years, she was referred to us because of a prominent short stature. Her height and weight were 128.0 cm (–4.3 SD) and 40.7 kg (–0.6 SD), respectively. The patient showed characteristic facial findings, a coarse appearance, downslanting fissures, epicanthal folds, and a flat nasal bridge. The patient also had some skeletal characteristics, a short neck, an increased anteroposterior diameter-chest, and elbow limitation. The patient had deep creases in her hands and lentigo was noticeable in her skin. Mild mental retardation was recognized, but magnetic resonance images of the brain were normal. She was endocrinologically evaluated as being normal and exhibited a normal karyotype on chromosomal analysis. Screening for mucopolysaccharidosis, including urinary analyses and some lysosomal enzyme assays, was all negative. Her clinical features led to the diagnosis of Costello syndrome.

Fibroblasts and Cell Culture—Primary human skin fibroblasts derived from patients and control subjects; F642, Costello syndrome (unpublished case); F622, galactosialidosis with a splicing mutation in the lysosomal protective protein/cathepsin A gene (IVS7+3a/IVS7+3a) (unpublished case); F643, type I sialidosis with mutations in the NEU1 gene (unpublished case); and F592, a normal subject, were cultured in Ham's F-10 medium supplemented with fetal bovine serum (10%) and antibiotics at 37°C under 5% CO_2. The research was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and was approved by the ethical committee of the institution in which the work was performed.

Materials—Ham's F-10 and fetal calf serum (FCS) were from Sigma (St. Louis, MO). Polyclonal antiserum (PR396) against bTE was from Elastin Products Company, Inc. (Owensville, MO). Deoxy NTPs were from Life Technologies (Grand Island, NY). Molony leukemia virus reverse transcriptase and Taq DNA polymerase were from Promega Corp. (Madison, WI). The oligo(dT)12-18 primer, TRIZOL reagent and RNaseOUT were from Invitrogen Corp. (Carlsbad, CA). Hygromycin B was from Wako Pure Chemicals (Osaka, Japan). Other reagents and enzymes for molecular biology were from Nippon Gene (Osaka, Japan) and TAKARA (Tokyo, Japan).

Construction of a Lentiviral Vector Containing bTE cDNA, and Gene Transfer—Plasmid vector pCLneo-CMVbtEln encoding bTE cDNA (*22*) was kindly provided by Drs. S. Karnik and D.Y. Li. Recombinant lentiviruses, a lentiviral vector encoding bTE cDNA (Lenti-bTE), and one encoding enhanced green fluorescent protein cDNA (Lenti-EGFP), encoding bTE and EGFP cDNAs, respectively, were prepared using the pLenti6/V5 plasmid containing a blasticidin-resistant gene (*23*), the 293FT cell line and a Directional TOPO Cloning kit (Invitrogen), and transduction of Costello syndrome fibroblasts with Lenti-bTE and Lenti-EGFP was performed according to the protocol recommended by the manufacturer. Briefly, the bTE and EGFP cDNAs (Invitrogen), respectively, were amplified by PCR, and then directionally cloned into pLenti6/V5 by adding four bases necessary for overhanging. The resultant lentivector plasmid containing the blasticidin-resistant gene was cotransfected into the 293FT cell line with the optimized packaging plasmids (pLP1, pLP2, and pLP/VSVG) that allow expression in trans of proteins required to produce a replication-incompetent lentiviral progeny with the envelope G glycoprotein from vesicular stomatitis virus (VSVG). After 3 days the conditioned media derived from the transfected 293FT cells were harvested, filtered through a 0.45 μm filter (Sartorius, Göttingen, Germany), and then stored at –80°C as lentiviral stock. For gene transfer to cultured fibroblasts, the lentiviral stock diluted with the culture medium was added to Costello syndrome fibroblasts (F642) plated on a 60-mm dish at subconfluency, and then cultured in the presence of blasticidin (final concentration: 4 μg/ml). The blasticidin-resistant cells were seeded on a well chamber slide (8-well chamber slides. Nunc, Pittsburgh, PA), and then examined by cytochemical analysis for elastic fiber formation and immunoblotting for TE as described below.

Assay for Elastic Fiber Formation—Elastic fiber formation on the surface of skin fibroblasts was assayed as immunofluorescence with anti-bTE serum. The fibroblasts (1×10^4 cells) were plated on 8-well chamber slides, and then cultured for 10 days to confluency. The cells were fixed with 2% paraformaldehyde/PBS at room temperature and then with 20% methanol/PBS overnight at 4°C. After blocking with 2.5% goat serum and 1% bovine serum albumin (BSA) for 2 h at room temperature, the cells were immunostained by a two-step incubation method; first with a 1:250 dilution of rabbit anti-bTE serum, and then with a 1:1,000 dilution of goat anti-rabbit IgG-F(ab′)$_2$ fragment conjugated with rhodamine (Biosource International,

Table 1. **PCR primers and conditions.**

Target	Forward	Reverse	Annealing temp. (°C)	Cycle number	Product (bp)
hTE part	GGAATTGGAGGCATCGCAGG-CGTTGGG	GAGCCACGCCGACACCAGG	61	30	591
hTE full	ATTTCTCCCCGAGATGGCGGG-TCTGACG	TCATTTTCTCTTCCGGCCACAAGC	59	30	2242
Fibrillin1	CACCCTATGCCAAGTTGATC	CTGCACTTAAAGCTGCCAATG	50	27	501
LOX	GATATTCCTGGGAATGGCAC	GCCAGGACTCAATCCCTGTG	57	27	567
Fibulin-5	GCCTGCCGAGGAGACATGAT	TGCACACTCGTCCACATCCA	50	27	243
MAGP-1	TGCAGGCTTGCTGGCTCAGG	CTAGCAGCTCCCACAGCTCCTGG	50	27	517
EBP	CCATCCAGACATTACCTGGC	TTGATGGGCCCAGAGGGACA	60	35	568
bTE	GGAATTGGAGGCATTCCCA-CATTTGGG	GAGCCACGCCGACTCCAGG	60	30	259
bTE/hTE	GGAGTGAAGCCTGGGAAAGTCC	GGCAGCTTGGGTGCCTTGATGGG	60	35	245/239
HRAS	GCAGGAGACCCTGTAGGAGG	CCAGGATGTCCAACAGGCACG	58	30	217
hβ-Actin	GACAACGGCTCCGGCATGTG	CCTTCTGCATCCTGTCGGCA	50	27	916

Camarillo, CA). After inclusion, elastic fiber formation was observed under a confocal laser scanning fluorescence microscope (LSM5Pascal Ver 2.8; Zeiss, Oberkochen, Germany).

RNA Isolation and Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis—Fibroblasts (approx. 5×10^5 on 60-mm plastic dishes) were rinsed with cold PBS, and then homogenized manually in 1 ml of TRIZOL reagent (Invitrogen) on ice. RNA was isolated according to the manufacturer's protocol. Briefly, 0.2 ml of $CHCl_3$ was added to the homogenate, followed by centrifugation at 20,600 × *g* for 15 min. An equal volume of 2-propanol was added to the resultant supernatant to precipitate RNA. After centrifugation, the pellet was rinsed with 75% ethanol/diethylpyridylchloride (DEPC)–treated water, followed by drying. The pellet was dissolved in an appropriate volume of DEPC-treated water as the total RNA fraction. For reverse transcription (RT), 2 μg RNA from each sample was transcribed at 37°C for 1 h in the presence of 200 U of Molony leukemia virus reverse transcriptase (Promega), oligo$(dT)_{12–18}$ primer, 0.5 mM dNTPs and 50U of RNase-OUT. The PCRs for tropoelastin, fibrillin-1, MAGP-1, LOX, fibulin-5, EBP, HRAS and β-actin were performed within the linear range of amplification using the selective primer sets and conditions, as summarized in Table 1. The PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Immunoblotting for the Expressed bTE—Human fibroblasts (F642 and F592) and the fibroblastic cell lines transduced with Lenti-*bTE* and Lenti-*EGFP* were harvested, sonicated in distilled water containing 0.1 mM leupeptin, and then centrifuged at 10,000 × *g* for 15 min at 4°C. Aliquots of the resultant supernatants were treated with 25 mM mercaptoethanol, and then subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% acrylamide gel and electroblotting onto polyvinylidene difluoride (PVDF) membranes. Proteins were visualized by immunostaining, using a 1:500 dilution of rabbit anti-bTE serum, a biotinylated anti-rabbit IgG (Vector, Burlingame, CA), horseradish peroxidase (HRP)–conjugated egg white avidin (ICN Pharmaceuticals Inc., Aurora, OH), and a chemiluminescent substrate (Western Lightning Chemiluminescence Reagent Plus; PerkinElmer Life Sciences, Boston, MA). Prestained SDS-PAGE standards (Bio-Rad, Hercules, CA) and a biotinylated protein ladder (Cell Signaling Technology Inc., Beverly, MA) were used as molecular mass standard proteins.

Hoechst Staining for Evaluating Proliferative Activity—Fibroblasts were seeded on 8-well chamber slides (5×10^3 cells/well). After culturing for 1, 4, 8 and 12 days, respectively, the cells were fixed with 2% paraformaldehyde/PBS for 1 h at room temperature, and then stained with Hoechst 33258 (Sigma) at a final concentration of 0.1 μg/ml for 1 h at room temperature. After washing with PBS and inclusion, elastic fiber formation was observed under a fluorescence microscope (Axiophot 2, Zeiss) with a CCD camera (AxioCam, Zeiss). The number of stained nuclei in a field was determined under magnification (×200), and then the means of the stained nuclei numbers determined in 6–10 fields were calculated to evaluate cell proliferation.

XTT Assay for Cell Proliferation—Cell proliferation was colorimetrically estimated by the XTT method with fibroblasts according to the manufacturer's protocol. The cells were seeded onto 96-well plates, and then cultured for 4 and 12 days. XTT reagent (Cell proliferation kit II, Roche) was added to each well, followed by incubation for 4 hrs at 37°C. Conversion of the XTT reagent to its formazan derivative by the succinate tetrazolium reductase in the living cells was measured as the absorbance at 492 nm measured with a microtiter reader (Fluoroskan Ascent. Thermo Electron Inc., Vantaa, Finland). The absorbance at 750 nm, as a reference, was subtracted from each value.

Protein Determination—Protein determination was performed with a *DC* protein assay kit (Bio-Rad Laboratories, Richmond, CA) with BSA as the standard.

RESULTS

Impaired Elastic Fiber Formation in Skin Fibroblasts Derived from a Japanese Costello Syndrome Patient—As shown in Fig. 1, immunofluorescence analysis with anti-bTE antibodies that cross-react with the human counterpart revealed that extracellular elastic fiber formation

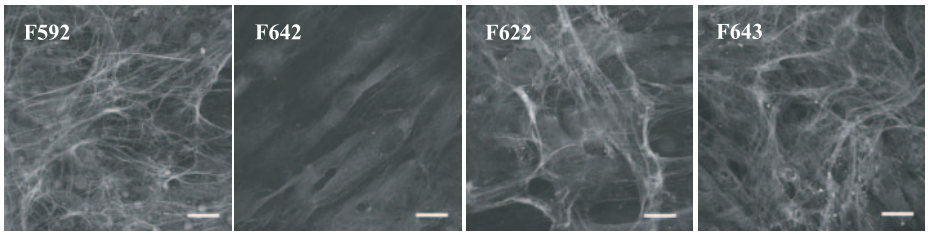

Fig. 1. **Immunofluorescence analysis for elastic fiber formation in fibroblasts with rabbit anti-bTE antibodies and rhodamine-conjugated anti-rabbit IgG antibodies (red color).** Normal control (F592); Costello syndrome (F642); juvenile/adult galactosialidosis (F622); sialidosis (F643). Bar, 20 μm.

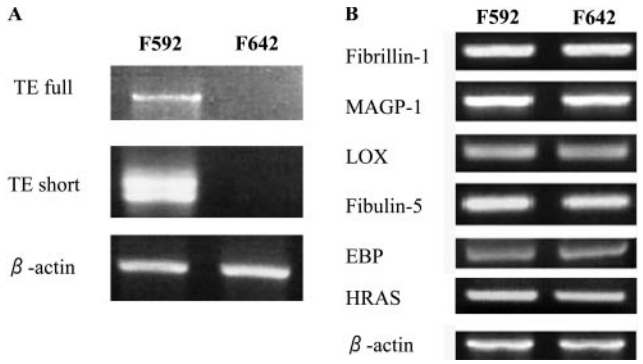

Fig. 2. **Gene expression analysis for tropoelastin and proteins involved in elastogenesis in fibroblasts by RT-PCR.** (A) RT-PCR for human tropoelastin mRNA with two different sets of primers, one for the full length open reading frame (hTE full) and the other for the partial sequence encoding the central portion of human tropoelastin (hTE part), as described under "MATERIALS AND METHODS." β-Actin was used as a control. (B) RT-PCR for proteins involved in elastic fiber formation using the specific primer sets and conditions given in Table 1, including fibrillin-1, MAGP-1, LOX, fibulin-5, EBP and HRAS. Costello syndrome (F642) and normal subject (F592).

occurred in the fibroblasts from the normal subject (F592) as well as in the patients affected by galactosialidosis (lysosomal protective protein/cathepsin A deficiency) (F622) and sialidosis (lysosomal neuraminidase-1 deficiency) (F643) as pathological controls. In contrast, elastic fiber formation was hardly observed in the fibroblasts derived from the present Costello syndrome case (F642). Control F592 cells reached confluence 6 days after seeding under the present culture conditions, and elastogenesis in F592 cells was observed 4 days after confluence. In contrast, Costello F642 cells reached confluence 4 days after seeding, but elastogenesis was not detected even 6 days after confluence.

Significant Reduction of Tropoelastin mRNA in Costello Fibroblasts—We next examined the differential expression of mRNAs encoding proteins that are involved in elastogenesis, including hTE, HRAS, fibrillin-1, MAGP-1, LOX, fibulin-5 and EBP, by RT-PCR using the selective primer sets listed in Table 1. We isolated RNA from normal control (F592) and Costello (F642) cells (5×10^5 cells) 10 days after seeding (above 4 days after the confluence of F592 cells). As shown in Fig. 2A, a significant decrease in the transcriptional expression of hTE mRNA was demonstrated in the fibroblasts from a Japanese patient with Costello syndrome (F642), as compared to in a normal subject (F592), with two different primer sets, one for the full length open reading frame (hTE full) and the other for the partial sequence encoding the central portion of hTE (hTE part). Tropoelastin mRNA was expressed in the normal fibroblasts (F592) 3 days after seeding (at 50% confluency) (data not shown). On the other hand, such a change in mRNA expression was hardly observed for HRAS or the other examined proteins involved in elastogenesis (Fig. 2B). We also examined the base sequence of the open reading frame (ORF) in HRAS cDNA derived from the Costello fibroblasts, but could not detect any mutations in the ORF (data not shown).

Enhanced Proliferation of Skin Fibroblasts in Costello Syndrome—Next we examined the proliferative features of the fibroblasts in Costello syndrome. Under the present culture conditions, the control fibroblasts (F592) reached confluence 6 days after seeding whereas the Costello fibroblasts (F642) reached confluence 4 days after seeding. Figure 3A shows the nuclear staining pattern with Hoechst33258. Costello fibroblasts (F642) exhibited a remarkable and time-dependent increase in the number of nuclei in a definite optical field, *i.e.*, enhanced cell proliferation, compared to in a normal control. The inset shows the features of the overlapping nuclei, indicating that the Costello fibroblasts could grow in layers. As shown in Fig. 3B, quantitative evaluation was performed by determining the nuclear numbers in several optical fields and then averaging them. The nuclear number in the Costello fibroblasts was about 4-fold greater than those in the normal (F592) and pathological controls (F622 and F643) at 12 days after seeding. These results indicated that the Costello fibroblasts could continue to grow by escaping the contact inhibition.

Restoration of Elastic Fiber Formation in Costello Fibroblasts on Transduction with a Lentiviral Vector Encoding the Normal Tropoelastin Gene—A recombinant Lenti-bTE was constructed for normal gene transfer into the Costello fibroblasts. Transduced Costello fibroblastic cell lines were further selected in the presence of blasticidin. Costello fibroblastic cells transduced with the recombinant Lenti-EGFP were also isolated as a control. Figure 4A shows that impaired elastogenesis in the parent Costello fibroblasts (F642) was partly reversed in the cell lines transduced with Lenti-bTE (F642/Lenti-bTE), but not with Lenti-EGFP (F642/Lenti-EGFP), although the latter cell line expressed a significant level of fluorescent EGFP.

Significant bTE gene expression in the Costello fibroblasts transduced with Lenti-*bTE* (F642/Lenti-*bTE*) was confirmed by immunoblotting with anti-bTE antibodies (Fig. 4B) and RT-PCR (Fig. 4, C and D) with different primer sets. The intensities of the bands due to the human and bovine TE mRNAs observed on RT-PCR with the common primer sets (Fig. 4D) seemed to correlate with the degree of restoration elastic fiber formation (Fig. 4A).

Inhibitory Effect of Transduction with Lenti-bTE on the Proliferation of Costello Fibroblasts—The proliferation of

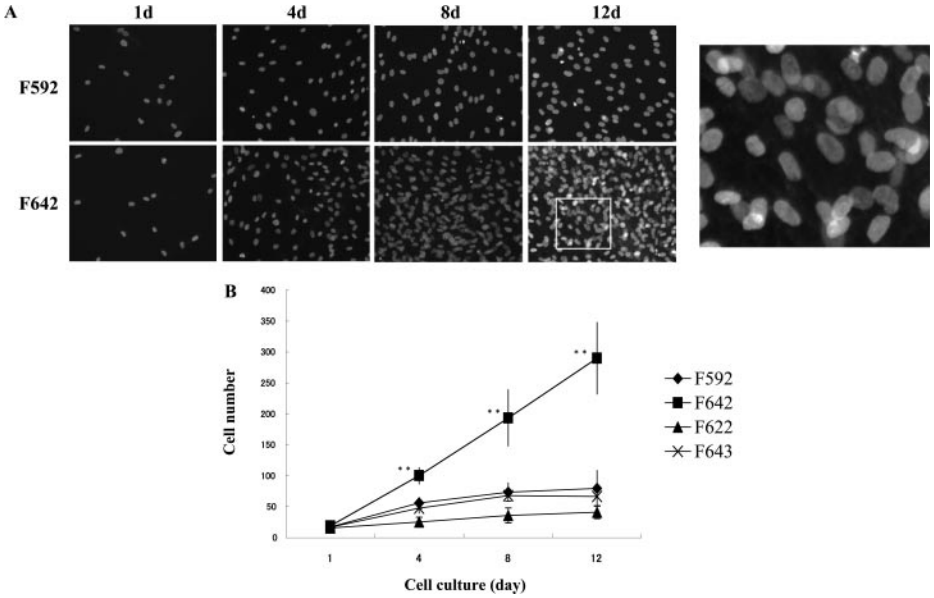

Fig. 3. **Proliferation assaying by nuclear staining with Hoechst dye.** Normal subject (F592); Costello syndrome (F642); juvenile/adult galactosialidosis (F622); sialidosis (F643). (A) Nuclear staining patterns in fibroblasts. The cells were fixed at 1, 4, 8 and 12 days after seeding, respectively, and then stained with Hoechst 33258 and examined under a fluorescence microscope. Magnification, ×200. Inset, an enlarged image. (B) Time-dependent changes in the nuclear number determined in a field under magnification (×200) were analyzed. The means of the nuclear numbers determined in 6–10 fields are plotted. $^{**}P < 0.01$ (*versus* F592).

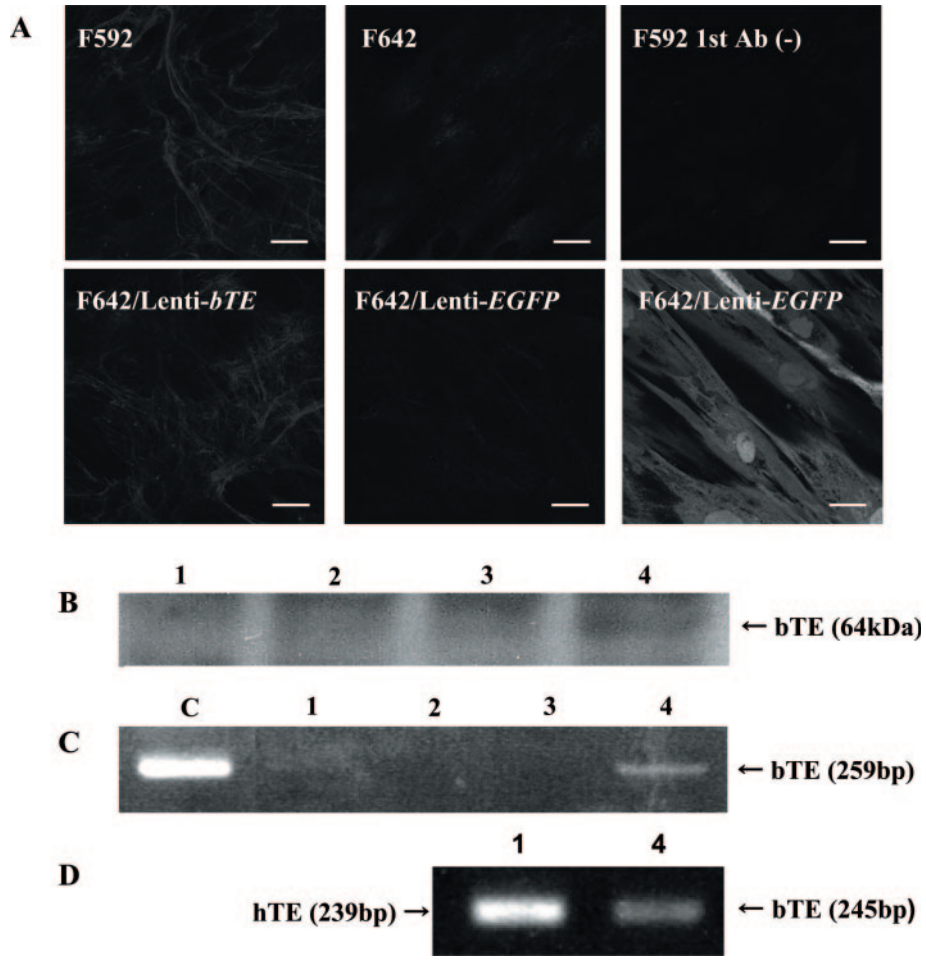

Fig. 4. **Restoration of elastic fiber formation in Costello fibroblasts on transduction with the recombinant Lenti-*bTE* virus.** (A) Immunofluorescence with a rabbit anti-bovine tropoelastin and rhodamine-conjugated anti-rabbit IgG antibody. Fluorescence of EGFP (right be on panel). F592, normal human fibroblasts; F642, Costello fibroblasts; F642/Lenti-*EGFP*, Costello fibroblasts transduced with Lenti-*EGFP*; F642/Lenti-*bTE*, Costello fibroblasts transduced with Lenti-*bTE*; F592 1st Ab (–), F592 untreated with the primary antibody. (B) Immunoblotting with anti-bTE antibodies for the cell lysate, and (C) RT-PCR for the exogenous bTE using specific primers. Lane 1, normal fibroblasts (F592); lane 2, Costello fibroblasts (F642); lane 3, Costello fibroblasts transduced with Lenti-*EGFP*; lane 4, Costello fibroblasts transduced with Lenti-*bTE*; lane C, control prasmid pCLneo-CMVbtEln.

skin fibroblasts was also analyzed by the XTT method, as shown in Fig. 5. In this assay, the fibroblasts were seeded at a cell density (1×10^3 cells/cm^2). The Costello (F642) and other control cells (F592, F642/Lenti-EGFP and F642/Lenti-bTE) reached confluence 6 and 8 days after seeding, respectively. The elevated OD_{492nm} value based on the mitochondrial dehydrogenase activity and cell number in the Costello fibroblasts at 12 days after seeding was

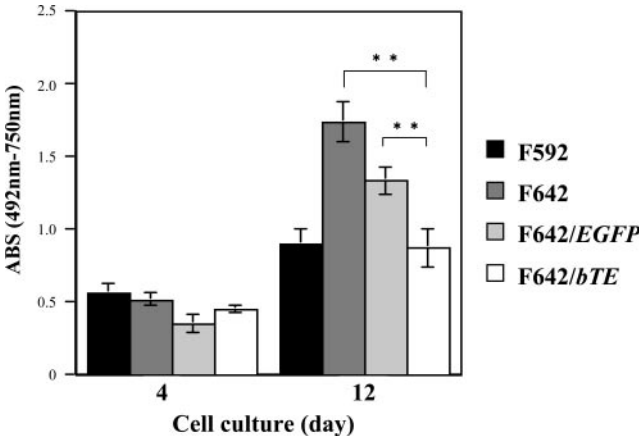

Fig. 5. **Normalization of proliferative activity of Costello fibroblasts transduced with the Lenti-*bTE* virus.** The time-dependent proliferative activity of fibroblasts was estimated by the XTT method. F592, normal fibroblasts; F642, Costello fibroblasts; F642/*EGFP*, Costello fibroblasts transduced with Lenti-*EGFP*; F642/Lenti-*bTE*, Costello fibroblasts transduced with Lent-*bTE*. $^{**}P < 0.01$ (versus F642/Lenti-*bTE*).

significantly decreased in the Costello cell line transduced with Lenti-bTE, although the OD_{492nm} value was slightly decreased in that transduced with Lenti-EGFP as a control, probably due to the cytotoxic effect of lentiviral infection or overexpression of EGFP on cell proliferation. The results indicate that the enhanced proliferation of Costello fibroblasts could be normalized by means of bTE gene transfer with Lenti-bTE.

DISCUSSION

Costello syndrome is characterized by loose skin, mental retardation, a coarse face, skeletal deformity, cardiomyopathy, and a predisposition to numerous malignancies in association with impaired elastogenesis and enhanced cell proliferation (*1*–*5*). The genetic origin of Costello syndrome has not been established, but recently germ line mutations of the HRAS gene were identified in Japanese and Italian Costello syndrome patients (*6*), suggesting that the tumorigenesis might be caused by up-regulation of the mutated HRAS gene in the somatic cells from these Costello patients.

In this study we demonstrated a remarkable defect of hTE gene expression in skin fibroblasts derived from the present Japanese patient with Costello syndrome. Immunocytochemical analysis with anti-bTE antibodies revealed impaired formation of elastic fibers in the Costello fibroblasts. RT-PCR analysis indicated a specific decrease in the expression of hTE mRNA in the Costello fibroblasts, while that of HRAS and the other major proteins involved in elastogenesis, including fibrillin-1, MAGP-1, LOX, fibulin-5 and EBP, did not change as compared to in a normal subject. In addition, the decrease in hTE mRNA was revealed to be significant because the hTE cDNA was hardly amplified even with two different primer sets. These results strongly suggest a defect in the hTE gene expression might be the primary cause of the impaired elastogenesis in the present Costello case, although we could not specify the important factors here. Hatamochi et al. have also reported decreased hTE gene expression in cultured dermal fibroblasts derived from a 2-year-old Japanese male patient with Costello syndrome, associated with a coarse face, curly hair, and loose skin of the dorsal aspects of the hands and feet with dark pigmentation (*24*). They showed that the Costello fibroblasts expressed 19% of the hTE mRNA detected in normal fibroblasts on Northern blot analysis. These findings suggest that some patients with Costello syndrome might have a defect in hTE gene expression. Although cutis laxa is one of the typical manifestations of Costello syndrome, a reduction in the hTE mRNA level has also been reported for several fibroblast strains of generalized "cutis laxa" (*25*, *26*). Accordingly, it is possible that a reduction in hTE gene expression might be involved in the pathogeneses of some Costello syndrome cases. At present, the reason why the hTE mRNA level was severely decreased in our case is unknown. Mutation analysis of the hTE gene itself or its regulatory element as well as biochemical analysis of the signal pathway leading to induction of hTE gene expression, including specific growth factors and transcription factors, are necessary to determine the reason.

We also observed enhanced proliferation of fibroblasts in the present Costello case, which is one of the phenotypic characteristics of Costello syndrome. Elevated proliferation of fibroblasts in Costello syndrome has been shown to coincide with impaired elastic fiber formation (*18*, *19*). Hinek *et al.* reported that a functional deficiency of 67-kDa EBP, an alternative splicing product of the lysosomal β-galactosidase gene, is associated with the impaired elastogenesis in Costello syndrome (*18*). EBP plays physiological roles not only as a molecular chaperone for hTE through the secretory pathways to its extracellular assembly, but also as a component of a cell surface receptor complex for soluble elastin and its degradative peptides involved in the signal transduction coupled with G proteins for cell growth and migration (*28*). They also suggested that impaired elastic fiber formation in Costello fibroblasts, which express a normal level of hTE, should be caused by the enhanced EBP shedding from the cell surface through binding between the lectin-like domain of EBP and the β-galactosugar–bearing moieties on extracellular glycoconjugates, such as glycosaminoglycans including CS and DS (*18*, *27*). The findings suggest that an EBP dysfunction should be responsible for the pathogenesis of another group of Costello syndrome cases, which is not directly associated with a defect of hTE gene expression.

Recently, Hinek *et al.* demonstrated that overexpression of a versican isoform, V3, in fibroblasts from Costello syndrome patients transduced with a retroviral vector reversed their impaired elastogenesis and elevated cell proliferation (*19*). They also proposed that the restoration of normal phenotypes on overexpression of a variant of the CS proteoglycan versican, V3, which lacks CS, might be due to loss of CS from the cell surface and inhibition of EBP shedding, and that a therapeutic effect of versican V3 gene transfer in fibroblasts in Costello syndrome could be expected on rescue by the tropoelastin chaperone, EBP.

In this study we constructed a lentiviral vector encoding bTE cDNA and examined the effect of gene transfer into fibroblasts derived from the present Costello patient, in which hTE mRNA was significantly decreased. We

demonstrated here the restoration of elastic fiber formation and normal proliferative activity of fibroblasts transduced with the lentivirus encoding the bTE cDNA. These results strongly suggested that the impaired elastogenesis and enhanced proliferation of our Costello fibroblasts must have been directly caused by a defect of hTE gene expression.

Recently, we also demonstrated that the gene product secreted from Chinese hamster ovary (CHO) cell lines stably expressing bTE cDNA is deposited in the extracellular space, which causes a significant decrease in the proliferation of the transformed CHO cells, although elastic fiber formation was hardly observed, probably due to a lack of the proteins involved in elastogenesis in the parent CHO cells (data not shown). These results suggest that a growth-inhibitory effect of bTE gene transfer could be observed for normal cultured cells. Gene knock-out mice with a homozygous null mutation for the tropoelastin gene were demonstrated to develop occlusive arterial disease because of subendothelial proliferation and accumulation of arterial smooth muscle cells (*29*), which proliferate at a higher rate than in the wild-type *in vitro* culture system. However, this elevated proliferation could be reduced by the additon of elastin to the cultures (*30*). The enhanced proliferation of fibroblasts and aortic smooth muscle cells from patients with supravalvular aortic stenosis and Williams-Beuren syndrome exhibiting a primary haplodefect of the elastin gene can be reversed by adding insoluble elastin (*31*). In addition, the enhanced proliferation of cultured Costello syndrome fibroblasts could be normalized by administration of exogenous insoluble elastin (*18*). The lentiviral vector constructed in this study might also be an effective therapeutic tool for gene therapy for some clinical forms of Costello syndrome caused by defects of hTE gene expression and production.

The URL for data presented is as follows: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for Costello syndrome [MIM 218040]). We wish to thank Drs. S. Karnik and D.Y. Li (Dept. of Medicine and Oncological Science, Univ. of Utah, Salt Lake City, UT, USA) for providing the pCLneo-CMVbtEln plasmid. This work was supported by JST, CREST, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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