Biochemical Analysis of Elastic Fiber Formation with a Frameshift-Mutated Tropoelastin (fmTE) at the C-Terminus of Tropoelastin

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(Received January 6, 2006; Accepted February 23, 2006)

Elastic fibers play an important role in characteristic elastic properties of tissues such as skin, lungs, ligaments, and blood vessels. Many of the disorders related to elastic fibers are due to errors in assembly, yet the basic mechanisms of this process remain unclear. Cutis laxa, which is caused by an elastin gene mutation, includes a rare and heterogeneous group of the disorders characterized by lax, inelastic skin. To investigate whether elastic fiber assembly insufficiency is a possible factor in cutis laxa, we compared normal tropoelastin (nTE) and frameshift-mutated tropoelastin (fmTE) from a patient with autosomal dominant cutis laxa using an *in vitro* elastic fiber assembly model, in which purified recombinant tropoelastin was added to the culture medium. Assembly was evaluated by immunofluorescence staining, the quantitative analysis of cross-linked amino acids, and semi-quantitative analysis of matrix-associated tropoelastin. Immunofluorescence microscopy indicated an approximate 50% decrease in the deposition of fmTE in the extracelluar matrix, in contrast to that of nTE. The amount of cross-linked amino acids unique to mature insoluble elastin in the fmTE assembly was also decreased by approximately 20%. In addition, we clarified that the molecular interaction between fmTE and the amino-terminal domain of fibrillin-1 or full-length fibulin-5 was significantly decreased. Our results suggest that the defect of fmTE fiber assembly is due, at least in part, to decreased molecular interactions between tropoelastin and the microfibrillar components fibulin-5 and fibrillin.

Key words —— tropoelastin, molecular interaction, cross-linked amino acid, elastic fiber formation

INTRODUCTION

Elastin is an insoluble extracellular matrix (ECM) protein and is the core protein of the elastic fibers that impart elasticity to elastic tissues such as skin, lungs, ligaments, and arterial walls. Elastin is secreted from cells as a soluble protein of approximately 70 kDa referred to as tropoelastin. Tropoelastins are cross-linked in the extracellular space by one or more members of the lysyl oxidase gene family to form an elastin polymer, which is the functional form of the mature protein. Fibrillin containing microfibrils are thought to play an important role in the assembly process by serving as a scaffold for aligning cross-linking domains within tropoelastin molecules. A direct molecular interaction between tropoelastin and microfibril proteins has been demonstrated.[1–3] Domain-mapping studies have localized the microfibril-binding domain to the C-terminus of tropoelastin.[4–6] However, the mechanisms of elastic fiber assembly are still not fully understood.

It has been reported that genomic disorders in the elastin gene are closely related to the pathogenesis of diseases of the arteries or skin such as supravalvular aortic stenosis (SVAS) or cutis laxa.[7] Cutis laxa is an acquired or inherited condition characterized by redundant, pendulous, and inelastic skin.[8] A recent study suggests the possibility that elastin gene mutations in a patient with cutis laxa resulted in severe disease in pulmonary or aorta.[9] Previously, the elastin gene mutations in patients with autosomal dominant cutis laxa have been reported to be frameshift mutations of the elastin gene.[10–12] In these frameshift mutated elastin gene, it is believed that a frameshift mutation in exon 32 of the elastin gene replaces the 37 amino acids at

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the C-terminus of elastin with a novel sequence of 62 amino acids. In the cutis laxa patient with the frameshift mutation, abnormal branching and fragmentation in the amorphous elastin component has also been demonstrated by electron microscopy of skin sections. Immunocytochemistry indicated elastin deposition in elastic fibers with a reduced number of microfibrils.[11]

We have recently developed an *in vitro* model of elastic fiber assembly, which enables the quantitative comparison of various tropoelastin molecules.[13] In the present study, we investigated the elastic fiber formation of the frameshift-mutated tropoelastin (fmTE) using an *in vitro* model of elastic fiber assembly and biochemical analysis for the deposition of tropoelastin, the cross-linked amino acids, and the molecular interactions between tropoelastin and scaffold proteins such as fibulin-5 or fibrillin-1 (FBN-1).

MATERIALS AND METHODS

Cell Culture —— Retinal pigment epithelia cells (ARPE-19 cells; ATCC, Manassas, VA, U.S.A.) derived from human retinal pigment epithelial and human aortic smooth muscle cells (HASMCs; KURABO, Kyutaro-machi, Osaka, Japan) were grown as described previously.[13]

Antibodies —— The antibodies used for immunofluorescence and Western blot analyses included human tropoelastin specific monoclonal antibody-MAB2503 (Chemicon International, Inc., Temecula, CA, U.S.A.) and anti His-G antibody (Invitrogen Japan K. K., Chuo-ku, Tokyo, Japan); in addition, an antibody against human FBN-1[14] was used. The secondary antibodies included Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, U.S.A.), and horseradish peroxidase conjugated anti-mouse IgG and anti-rabbit IgG (ICN/Cappel, Costa Mesa, CA, U.S.A.).

Purification of Recombinant Tropoelastin —— Full-length human tropoelastin cDNA was cloned into the *Mlu* I-*Xba* I site of the pCIneo vector (Promega, Madison, WI, U.S.A.) (FL). It should be noted that the human template lacked exon 22. Frameshift-mutated elastin cDNA in elastin gene exon 32, which was extended to a 72-base pair sequence at the C-terminus of elastin, was constructed by RT-PCR amplification of total RNA isolated from HASMCs. The sense primer began with a *Pst* I restriction site and a mutation sequence as follows: 5′-AACTGCAGCCGCTAAAGCAGCTAAATAC-GGTGCTGCTGGCCTTGGAGGTGTCC**TG**GGG-GTGCCGGGCAG-3′. The antisense primer contained an *Xba* I restriction site as follows: 5′-CATGCTCTAGATTACAAAGGGTTTAC-3′. The PCR products were also cleaved with *Pst* I and *Xba* I and inserted into the similarly cleaved FL vector. To generate normal tropoelastin (nTE) and fmTE cDNA, PCR was performed using the following primer sequences: forward primer for the cDNA of nTE and fmTE, respectively: 5′-GGAGGGGTCCC-TGGGGCCATTCC-3′, reverse primer for nTE: 5′-TCA TTTTCTCTTCCGGCCACAAG-3′; and reverse primer for fmTE: 5′-CATGCTCTAGATTA-CAAAGGGTTTAC-3′. The PCR products of nTE and fmTE were verified by sequence analysis. These amplifications were inserted into a bacterial expression pTrcHis-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.). The bacteria were treated with isopropyl-β-D-thiogalactoside (IPTG) to induce the expression of recombinant tropoelastin and were then purified as described previously.[13] Purified nTE and fmTE were verified by Western blot analysis.

Immunofluorescence, Semi-Quantitative ELISA, and Determination of Cross-Linked Amino Acids into the Cell Matrix —— The methods used for the immunofluorescence study, ELISA, and determination of the desmosine content have been described previously.[13] For the immunofluorescence assay, ARPE-19 cells were plated at a density of 5×10^4 cells on eight-well LabTek chamber slides (Nunc no. 177445, Fisher Scientific, Pittsburgh, PA, U.S.A.) in dulbecco's modified eagle medium (DMEM) supplemented with L-glutamine and penicillin/streptomycin and 10% cosmic calf serum (CCS). The cells were washed with phosphate buffer saline (PBS) and fixed with 2% paraformaldehyde in PBS. After several washes with PBS, nonspecific immunoreactivity was blocked with Block Ase (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) for 5 min at room temperature. The cell layers were then incubated with primary antibody diluted at 1 : 100 (MAB2503) and 1 : 400 (FBN-1) for 30 min at room temperature, followed by several washes with PBS, and the secondary antibody was used in conjugation with Alexa fluor® 488 and 546 diluted with Block Ase. The cell layers were then washed with PBS and mounted in glycerol containing sodium phosphate and citric acid (Sigma, St. Louis, MO, U.S.A.). Fibrils were visualized with a KEYENCE microscope with epifluoresence using

20 × phase/fluorescence objectives.

For semi-quantitative ELISA, The ARPE-19 cells were plated at a density of 3×10^4 cells on 96-well micro-titer plates (FALCON) in DMEM supplemented with L-glutamine and penicillin/streptomycin and 10% CCS. The cells were then washed with PBS and fixed. Blocking agent and primary antibody diluted at 1 : 1000 were added as described above. The cell layers were then washed several times with PBS, incubated with horseradish-peroxidase linked secondary antibody, and washed again. The elastic fibers recognized by the antibodies were quantified with a colorimetric assay using the 3,3′,5,5′-tetramethylbenzidine (TMB)-substrate reagent (Fermentas Inc., Hanover, MD, U.S.A.) for 10 min at room temperature. The individual wells in the culture plate were read at a wavelength of 450 nm.

For the assay carried out to determine the cross-linking amino acids, ARPE-19 cells were plated at a density of 1×10^6 cells on 60-mm dishes (FALCON, Tokyo, Japan) in DMEM supplemented with L-glutamine and penicillin/streptomycin and 10% CCS. The cells were washed three times with PBS and were pooled by scraping into 1 ml of distilled water. The sample was centrifuged for 5 min, and the supernatants were removed. The pellets were then hydrolyzed overnight at 110°C using constant boiling of 6 N HCl. Desmosine levels in the hydrolysates were determined by radioimmunoassay,[15] and total protein concentrations were determined by amino acid analyses. The values were normalized to the total protein per dish.

In all assays, 48 hr after seeding the ARPE-19 cells, the cells were treated with various concentrations of nTE or fmTE for 8 days, respectively.

Production of Recombinant Fibulin-5 and the First Residue of the Proline-Rich Domain of FBN-1 and Ends with the Last Residue Predicted for the Second 8-Cysteine domain (PET) —— Fibulin-5 cDNA was constructed by RT-PCR. RT-PCR and performed with 1 μg of total RNA extracted from human aortic smooth muscle cells using the following primer sequence: forward primer 5′-GCCACCATGCCAGGAATAAAAAGG-3′ and reverse primer 5′-GAATGGGTACTGCGACACATATATCC-3′. The PCR products were separated by electrophoresis on 2% agarose gels and were visualized by ethidium bromide staining. Previously reported PET cDNA was kindly provided by Dr. Robert P. Mecham (Washington University, St. Louis, U.S.A.). Fibulin-5 and PET cDNA were inserted into the pcDNA 3.1/V5-His-TOPO© expression vector (Invitrogen, Carlsbad, CA, U.S.A.). CHO-K1 cells (ATCC, CCL-61) were transfected with the Fibulin-5 and PET in the vector, respectively. After 24-hr incubation, the culture media were replaced with CHO-SFM-II (Invitrogen, Carlsbad, CA, U.S.A.), and the cells were incubated for 24 to 72 hr At the end of the incubation period, the culture media were collected and concentrated using Amicon Ultra-15 (Millipore, Bedford, MA, U.S.A.). The products were used in the experiments as conditioned medium. The conditioned medium was electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and subjected to Western blotting analysis.

Solid-Phase Binding Assay —— Micro-titer plates (FALCON) were coated at 4°C overnight with 10 μg/ml of each recombinant tropoelastin or bovine serum albumin in bicarbonate buffer (14 mM sodium bicarbonate, 6 mM sodium carbonate, pH 9.5), respectively. The plates were washed several times with PBS containing 5% Tween 20 and were then incubated with 5%(w/v) non-fat milk/PBS at room temperature for 60 min in order to block the non-specific binding site. After blocking, a concentrated fibulin-5 or PET conditioned medium was diluted with 0.5% bovine serum albumin (BSA)/PBS (dilution buffer) and was then added to the wells. After incubation at 37°C for 60 min and several washes with dilution buffer, the plates was incubated with anti-V5 epitope antibody diluted 1 : 5000 in dilution buffer at 37°C for 60 min. After several washes with dilution buffer again, the plates was incubated with the anti-mouse IgG antibody diluted 1 : 5000 in dilution buffer at 37°C for 60 min. The amount of bound protein was quantified using a colorimetric assay carried with TMB-substrate reagent for 30 min at room temperature. The plates were read at a wavelength of 450 nm.

Immunoprecipitation —— The amount of nTE or fmTE was adjusted to 0–1000 ng/ml in PBS, and fibulin-5-conditioned medium was added to each of the solutions. The mixtures were incubated for 30 min at 4°C with immobilized Protein A-Sepharose (PIERCE Rockford, IL, U.S.A.), followed by incubation overnight at 4°C with 2 μl of anti V5-epitope monoclonal antibody. The immunoprecipitates were collected by centrifugation, were then washed with PBS containing 0.5% Tween 20. The immunoprecipitates were eluted by boiling the samples in 1 × SDS-PAGE buffer containing 100 mM dithiothreitol (DTT). The samples were

electrophoresed on 10% SDS-PAGE gels and subjected to Western blot assay using anti V5-epitope or His-G monoclonal antibody. Detection was performed using an ECL Kit (Amersham, Buckinghamshire, U.K.). Each membrane was exposed to X-ray film (Kodak, Rochester, NY, U.S.A.) and analyzed by Kodak-1D Image Analysis Software (Kodak).

Statistical Analysis —— Data were statistically analyzed using analysis of variance (ANOVA). The results were considered statistically significant when the p-value was < 0.05 (* or # $p < 0.05$; *** or ### $p < 0.001$). All data are shown as the mean value ± standard error of the mean (SEM).

RESULTS

Immunofluorescence Analysis and Semi-Quantitative Assay of ARPE-19 Cells Treated with Recombinant nTE and fmTE

The schematic diagram of nTE and fmTE, in which 37 amino acids were replaced at the C-terminus of elastin, are illustrated in Fig. 1. The replacement of amino acids in fmTE was determined by DNA sequence analysis and Western blot analysis (data not shown).

Double immunofluorescence staining showed less of an fmTE network than an nTE network, whereas the network of FBN-1 remained unchanged (Fig. 2A). To quantify the amount of recombinant tropoelastin associated with the ECM, the tropoelastin was semi-quantified with a colorimetric assay using human tropoelastin specific antibody. We confirmed that nTE and fmTE have no effect on the cell number (data not shown). The absorbance detected with FBN-1 antibody in all wells remained constant under these culture conditions (data not shown). The fmTE associated with the ECM showed low absorbance under all concentrations tested (Fig. 2B). This observation was consistent with the results obtained by immunofluorescence assay.

The Determination of Cross-Linked Amino Acids in Elastic Fibers

The cross-linked amino acids, desmosine and isodesmosine are found only in elastin and are routinely used as specific markers of fiber maturation and elastin quantitation. When the cell cultures were assayed by radioimmunoassay for desmosine, very low levels of desmosine were found in the untreated ARPE-19 cell matrix. Addition of nTE resulted in a marked increased in desmosine after 8 days in culture. Cells treated with fmTE also increased desmosine concentration but significantly less than with the addition of nTE (Fig. 3).

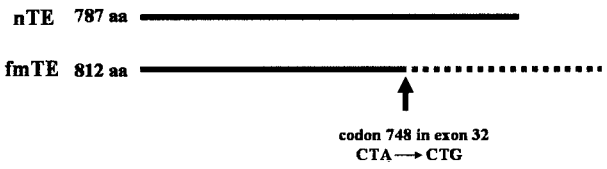

Fig. 1. Schematic Depiction of Normal Tropoelastin and Frameshift Mutated Tropoelastin Used in this Study

Deletion of an adenine in codon 748 in exon 32 (arrow) cause a frameshift mutation that replaced the 37 amino acids of tropoelastin with a novel sequence of 62 amino acids.[11] The mutated sequence is indicated by a broken line. aa: amino acid.

Molecular Interaction between Each Recombinant Tropoelastin and PET or Fibulin-5

To examine the molecular interactions between each type of recombinant tropoelastin and the microfibril components, we performed solid-phase and solution-phase binding assays. Fibulin-5 and PET from the conditioned medium were detected as proteins of approximately 66 and 42 kDa, respectively, by Western blot assay (data not shown). There was a 70% increase in nTE binding to fibulin-5 compared to fmTE binding (solid bars). The interaction of each recombinant tropoelastin was much less to PET, however, the binding of nTE to PET was also increased 30% above fmTE binding. Addition of 30 mM Ca^{2+} to the incubation media increased the binding to both nTE and fmTE to fibulin-5; however, the differences remained the same (Fig. 4). On the other hand, we confirmed that there was no difference in the amount of coated-nTE and -fmTE (data not shown).

The molecular interactions between each type of recombinant tropoelastin and fibulin-5 in the solution phase were determined by immunoprecipitation assay using anti V5-epitope antibody. Co-immunoprecipitated nTE and fmTE with fibulin-5 were determined by Western blot analysis with anti His-G antibody, and then the area of each band was measured using Kodak-1D Image Analysis Software in order to quantitatively compare the samples. The band of tropoelastin co-precipitating with fibulin-5 was normalized to the amount of fibulin-5 in each precipitate. There was a linear response in binding of nTE to fibulin-5 over the range of concentrations

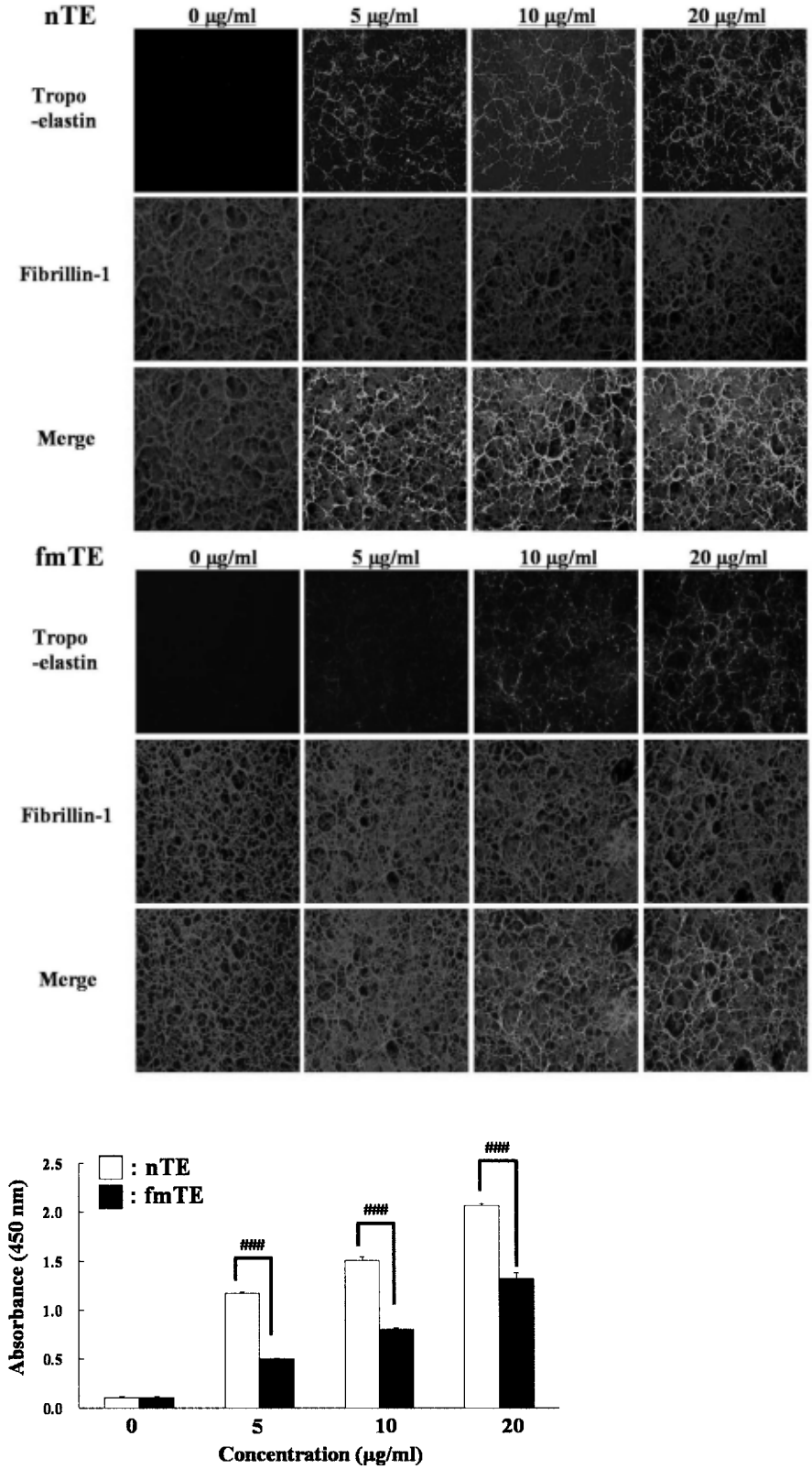

Fig. 2. Immunofluorescence of Tropoelastin and FBN-1 in ARPE-19 Cells

(A) ARPE-19 cells were incubated with various concentrations of nTE (upper panel) or fmTE (bottom panel) for 8 days. The networks of elastic fibers in the extracellular matrix were detected by indirect immunofluorescence with MAB2503. FBN-1 fibers were detected with FBN-1. Magnification, × 200. (B) The networks of nTE or fmTE detected with antibodies were quantified by colorimetric assay. The absorbance of both forms (nTE; open box, fmTE; closed box) was detected with MAB2503. The plates were read at a wavelength of 450 nm. The bar indicates means ± SEM, $n = 3$. ###$p < 0.001$ *vs*. nTE.

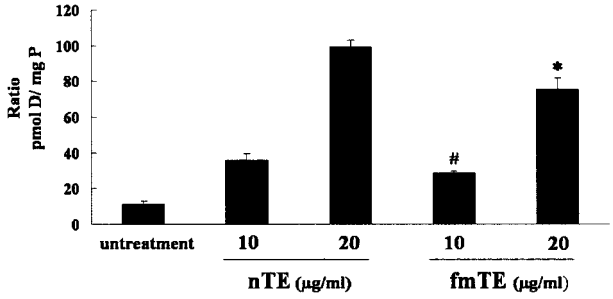

Fig. 3. The Determination of Cross-Linked Amino Acids into the Matrix

ARPE-19 cells were treated with various concentrations of the nTE, the fmTE for 8 days. The cells were then scraped off the plates and hydrolyzed as described in Materials and Methods. Desmosine (pmol D) was detected by radioimmunoassay, and total protein concentrations (mg P) were determined by amino acid analyses. The desmosine levels were normalized by the ratio of desmosine to total protein. The bar indicates means ± SEM, $n = 6$. #$p < 0.05$ *vs.* 10 μg/ml nTE, *$p < 0.05$ *vs.* 20 μg/ml nTE.

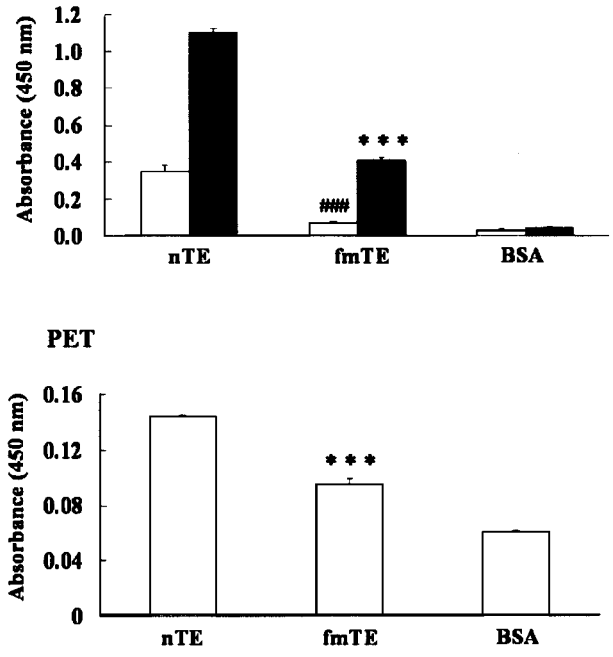

Fig. 4 The Interaction with Microfibril Protein or Elastin Binding Protein in the Solid Phase

The binding assay for each recombinant tropoelastin and fibulin-5 (upper panel) or PET (bottom panel), which is known as the elastin-binding site at the amino-terminal of FBN-1, was performed by solid-phase binding assay. nTE, fmTE, and BSA were coated onto microtiter wells (10 μg/ml) and were incubated with fibulin-5 from conditioned medium in the presence (closed box) or absence (open box) of Ca^{2+} or PET from conditioned medium. The plates were read at a wavelength of 450 nm. The bar indicates means ± SEM, $n = 3$. ***$p < 0.001$ or ###$p < 0.001$ *vs.* nTE in the presence or absence of Ca^{2+}.

that were used (Fig. 5). At all concentrations used there was a significantly lower molecular interaction with fmTE compared to nTE.

DISCUSSION

In the present study, we have investigated elastic fiber formation with nTE and fmTE using an *in vitro* model of elastic fiber assembly. Our results suggest that the C-terminus of tropoelastin plays an important role in the deposition and the maturation of tropoelastin. The fmTE used in the present study has the 37 amino acids at the C-terminus of tropoelastin replaced with a missence translation at a premature stop codon. The replacement results in the loss of the exon 36 of the elastin gene, which contains 2 cysteine residues and an Arg-Lys-Arg-Lys (RKRK) sequence. It is considered that the only two cysteine residues and the RKRK sequences in tropoelastin participate in the formation of an antiparallel beta-structured-loop and the floor of a positively charged pocket in the C-terminus of tropoelastin.[16,17] Previous studies have shown that the amino-terminal domains of FBN has an important role in the direct molecular interaction between acidic microfibril proteins and tropoelastin.[2] Moreover, recent studies showed that fibulin-5, which is known as a calcium-dependent elastin-binding protein, plays an essential role in the development of elastic fiber assembly.[18,19] In the studies present here we have considered the possibility that a decrease in the molecular interaction of tropoelastin with microfibril proteins such as fibulin-5 and FBN would be attributable to a mutation at the C-terminus of tropoelastin.

The addition of exogenous tropoelastin to cell culture media markedly enhanced the accumulation of matrix elastin compared to cell culture matrices without added tropoelastin. The tropoelastin incorporation of fmTE was significantly less than observed with nTE illustrating the importance of this region for microfibrillar binding. What this elastin was incorporated into the matrix and actually formed elastic fibers was documented by the corresponding increase in matrix desmosine. Tropoelastin does not contain these cross-links, which are formed in elastin only after secretion from the cell into the matrix followed by lysyl oxidase oxidation and subsequent interaction with the microfibrillar component of the extracellular matrix. By incorporating a frameshift mutation in exon 32 of the elastin gene we were able

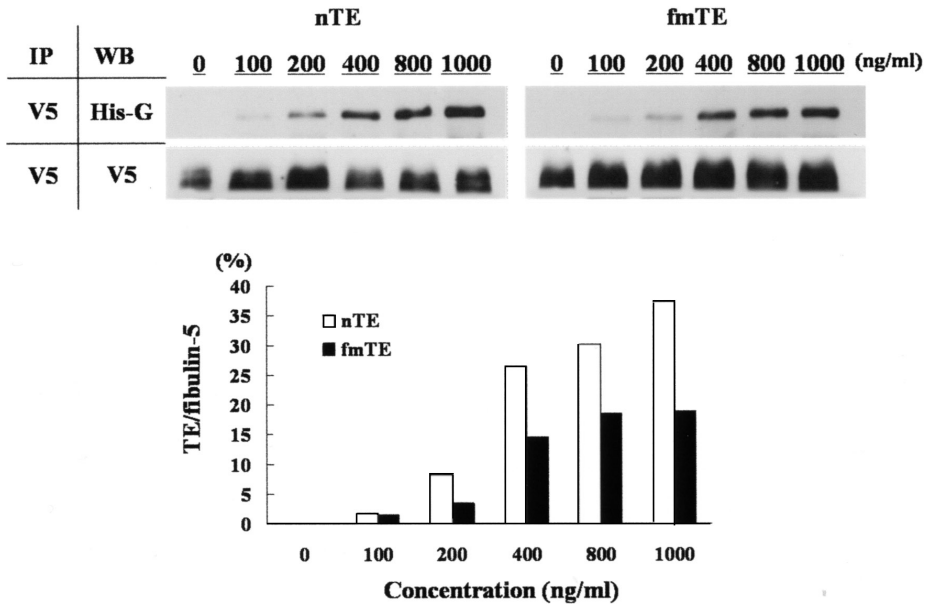

Fig. 5. The Interaction with Each Tropoelastin and Fibulin-5 in the Solution Phase

The molecular interaction between fibulin-5 and nTE or fmTE in the solution phase was examined by immunoprecipitation assay using anti V5-epitope antibody, and then each of the bands were identified by Western blot analysis with anti His-G antibody or anti V5-epitope antibody. Each tropoelastin was adjusted to 0–1000 ng/ml in PBS, and then fibulin-5 from conditioned medium was added to each of the solutions. The area of each of the bands was measured using Kodak-1D Image Analysis Software in order to quantitatively compare nTE and fmTE in the co-immunoprecipitation with fibulin-5.

to show that C-terminal region of elastin is important for initiating the molecular interaction between tropoelastin and fibulin-5 of FBN.

Lysyl oxidases are extracellular copper-requiring enzymes that catalyze the cross-linking of elastin through oxidative deamination of lysine side chains.[20] The allysine side chain is thought to condense spontaneously with another allysine molecule *via* an aldol condensation reaction and with an ε-amino group of an unoxidized lysine residue *via* a Schiff's base reaction to form the cross-links desmosine and isodesmosine.[21] We previously reported that the formation of allysine may play an important role in the first step for elastic fiber assembly. Our previous study showed that the deletion of exon 36 of tropoelastin decreased substrate specificity for LO,[22] and the molecular interaction between FBN-1 and tropoelastin promotes the formation of cross-links.[23] Recently, it was also shown that lysyl oxidase like-1 (LOXL-1) specifically localizes to the site of elastogenesis, and the molecular interaction among tropoelastin, fibulin-5, and LOXL-1 plays a regulatory role in the formation of cross-links.[24] Consequently, it is possible that part of the effect we observed in decreased desmosine and elastic fiber accumulation in fmTE was due to decreased interaction between tropoelastin and LO.

It has been reported that a homozygous missense mutation (T998C) and a heterozygous tandem duplication in the fibulin-5 gene resulted in a severe form of cutis.[25,26] Our present findings showing that mutations in the C-terminal region of tropoelastin decreased molecular interactions between tropoelastin and microfibrils suggests this may be one of the factors leading to skin disorders such as cutis laxa.[18,25–27]

Acknowledgements This work was supported by the Hoshi University Science/Technology Frontier Research Base. The authors are grateful to Madoka Ando, Takako Kodaira, Marie Ishida, Hiroyuki Ooyama, and Risa Nonaka for their technical assistance.

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