A Functional Mutation in the Terminal Exon of Elastin in Severe, Early-Onset Chronic Obstructive Pulmonary Disease

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We describe a novel variant in the terminal exon of human elastin, c.2318 G>A, resulting in an amino acid substitution of glycine 773 to aspartate (G773D) in a pedigree with severe early-onset chronic obstructive pulmonary disease (COPD). Transfection studies with elastin cDNAs demonstrate that the glycine to aspartate change compromises the ability of the mutant protein to undergo normal elastin assembly. Other functional consequences of this amino acid substitution include altered proteolytic susceptibility of the C-terminal region of elastin and reduced interaction of the exon 36 sequence with matrix receptors on cells. These results suggest that the G773D variant confers structural and functional consequences relevant to the pathogenesis of COPD.

Keywords: chronic obstructive pulmonary disease; elastin; extracellular matrix; genetics; mutation

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the United States (1). Although cigarette smoking is the major risk factor for COPD, development of chronic airflow obstruction (the defining characteristic of COPD) is markedly variable among smokers, suggesting that additional factors contribute to its pathogenesis (2). Severe α_1-antitrypsin deficiency is a proven genetic risk factor for COPD, but is found in only 1–2% of cases (3). To date, no other genetic determinants of COPD have been proven, although family studies suggest that there is an important genetic predisposition for COPD (4, 5). Linkage analysis in early-onset COPD pedigrees has identified several genomic regions that likely harbor common susceptibility loci (6–8). However, linkage studies are underpowered to detect rare variants of modest effect, which may predispose a smaller subset of the population to develop COPD. One possible approach to identify rare functional variants is to screen genes that have been implicated in monogenic syndromes that include emphysema-like lung pathology as part of their phenotype constellation. This approach has been successfully used to identify susceptibility variants in several complex traits (9–12).

The elastin gene (ELN) represents an excellent candidate gene for COPD, as the destruction of elastin fibers has been implicated in the pathogenesis of the disease (13–15). Furthermore, deletion of the elastin gene in mice results in lungs with emphysema-like lesions consisting of dilated distal airsacs and attenuated tissue septae (16). Frameshift mutations in the distal exons of the human elastin gene have been identified in several families with autosomal dominant cutis laxa (ADCL) (17, 18). Pulmonary complications are the major cause of death in patients with cutix laxa (CL) (19–21), and while pulmonary emphysema is most common in recessive CL, severe early-onset emphysema has also been observed in the autosomal dominant form of the disease (22). Because of the clinical overlap between CL and severe early-onset COPD, and because reduced lung elasticity is a hallmark of emphysema, we hypothesized that elastin gene mutations similar to those seen in ADCL may play a role in the development of COPD. Because our previous linkage studies did not demonstrate evidence for linkage near the ELN gene on chromosome 7, we also hypothesized that any functional variants that were identified would be uncommon. Herein we describe a novel variant in the terminal exon of human elastin in an extended pedigree with severe, early-onset COPD and present in 1.25% of patients with severe COPD and emphysema participating in a clinical trial. *In vitro* evaluation of the mutant elastin protein demonstrated impaired elastic fiber assembly, loss of cell reactivity, and altered protease susceptibility. Together, these findings demonstrate that this variant confers structural and functional consequences relevant to the pathogenesis of COPD.

MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical unless otherwise indicated. Primers sequences and reaction protocols are available on our website (www.innateimmunity.net).

Subjects

The Boston Early-Onset COPD Study was approved by the Human Research Committees of Partners Healthcare and the Brockton/West

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Roxbury VA Hospital. The Genetics Ancillary Study of the National Emphysema Treatment Trial (NETT) was approved by the Institutional Review Boards of each site participating in the study. Participants from the Boston Early-Onset COPD Study and NETT Genetics Ancillary Study provided written informed consent for genetic studies. The genetic studies in the Normative Aging Study (NAS) were approved by the Partners Healthcare Human Research Committee and the IRB of the Veterans Administration Hospitals using anonymized data sets.

Boston Early-Onset COPD Study Cohort

The recruitment and assessment of probands and extended pedigrees with severe early-onset COPD have been described previously (5, 6, 8). Proband ascertainment criteria included: (*1*) $FEV_1 < 40\%$ predicted, (*2*) age < 53 yr, (*3*) absence of severe α_1-antitrypsin deficiency, and (*4*) physician-diagnosed COPD. The arbitrary age threshold of 53 yr was chosen to balance our goal of identifying very young probands with our need to ascertain an adequate number of probands. Available first-degree and older second-degree relatives, and additional affected individuals, were invited to participate. Each participant completed a respiratory questionnaire (5, 23) and performed spirometry in accordance with ATS guidelines. Echocardiogram results were extracted from the medical record, and computed tomographs (CT) of the chest were obtained from the medical archive. The only available chest CT images for the proband with the elastin variant had been obtained with a bone window algorithm, which typically underrepresents emphysematous changes.

NETT Cohort

NETT is a randomized, multicenter clinical trial designed to compare lung volume reduction surgery and conventional medical therapy for severe emphysema. The methods of recruitment and participant characterization and the primary results of the trial have been reported elsewhere (24, 25). DNA was available from 319 willing participants as part of an ancillary study of COPD genetics. One NETT participant who was also enrolled in the Boston Early-Onset COPD Study was not included in the case–control analysis. Baseline phenotypic evaluations including spirometry were completed after pulmonary rehabilitation but before randomization.

Normative Aging Study Healthy Control Subjects

The Normative Aging Study (NAS) is a longitudinal study of aging established in 1961 by the Veterans Administration of 2,280 men from the Greater Boston area. Since entry, volunteers have reported for periodic examinations that include spirometric tests (26). From this cohort, disease-free control subjects were defined as those subjects with at least 10 pack-years of cigarette smoking who demonstrated normal pulmonary function ($FEV_1 > 80\%$ predicted and FEV_1/FVC ratio $\geq$ 90% predicted based on the prediction equations by Crapo and coworkers [27]) at their last scheduled examination. DNA for 365 disease-free control subjects was available for genotyping.

Mutation Discovery and Genotyping

The six terminal exons of the human elastin gene (corresponding to bovine elastin exons 29–33, and 36) were sequenced using dye-terminator dideoxy sequencing chemistry (PE Biosystems, Foster City, CA). Primers were designed (using Primer 3.0 freeware) to cover the exons, exon-intron junctions, and at least 50 bp of surrounding intron. Sequencing products were analyzed with the ABI 3100 Sequence Detector (Applied Biosystems, Foster City, CA). Phylogenetic analysis was performed with CLUSTAL W (http://www.ebi.ac.uk/clustalw/) (28). The c.2318 mutation was genotyped with the 5′→3′ exonuclease assay as implemented in the TaqMan assay (PE Biosystems, Foster City, CA). Fluorescence was measured with the Prism 7900 Sequence Detector System (Applied Biosystems). Samples that were heterozygous for the mutation were re-sequenced with the exon 36 sequencing primers to confirm the presence of the variant.

Skin Fibroblasts and ELN Transcript Analysis

Skin fibroblasts from two 3-mm full-thickness skin punch biopsies obtained from the posterior aspect of the upper arm were obtained using standard explant techniques. Total RNA was harvested using Trizol Reagent (Invitrogen, Carlsbad, CA) from Day 10 postconfluent cultures (passage 6) and reverse transcribed using oligo$(dT)_{12-18}$ primers and Superscript III reverse transcriptase (Invitrogen). Primers were generated to amplify the terminal eight exons (25–36, including a portion of the 3′UTR) and nested PCR was performed on 0.5 μl of product from the first PCR reaction. PCR products were separated on a 2% agarose gel, excised, and purified with the Qiaquick gel purification kit (Qiagen, Valencia, CA).

Transfection Constructs

Plasmid constructs were derived from a bovine tropoelastin (bTE) cDNA isolated from fetal chrondroblasts (29). WT cDNA was cloned into the pCIneo vector (Promega, Madison, WI) as described previously (30). MU cDNA was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Reaction products were used to transform competent DH5α cells (Invitrogen). Transformants were plated onto LB-ampicillin plates, incubated overnight, and chosen colonies were grown overnight in Circlegro media (Fisher Scientific, Pittsburgh, PA) containing ampicillin. Plasmid DNA was isolated using the Qiagen Mini-Prep kit and subjected to sequencing. A primer to the T3 region of the pCIneo vector was used to sequence across the mutated area to confirm the presence of the mutation. The entire WT and G773D cDNAs were sequenced to ensure that no other significant mutations had been introduced during cloning. Large scale preparation of plasmid DNA was achieved by growing transformed cells in Circlegro media containing Ampicillin, followed by plasmid purification using Qiagen Maxi-Prep kit (Qiagen).

Cell Culture and Production of Stable Transfected Cell Lines

Bovine pigment epithelial (PE) cell cultures (31) were maintained in Dulbecco's Modified Eagle's Medium (Cellgro, Herndon, VA) with 10% fetal calf serum (Sigma, St. Louis, MO), non-essential amino acids (Cellgro), penicillin/streptomycin (Washington University Tissue Culture Center, St. Louis, MO), and L-Glutamine (Gibco). RFL-6 cells (ATCC, Manassas, VA) were grown in HAMS F-12 media (Sigma) supplemented with 20% Cosmic Calf Serum (Hyclone, Logan, UT), nonessential amino acids, L-glutamine, and penicillin/streptomycin. Stably transfected cells were maintained in medium with Geneticin at 500 μg/ml (G418-sulfate; Invitrogen). All cultures were grown in a humidified CO_2 incubator. Stable transfection of PE and RFL-6 cells was performed using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). Briefly, 5×10^5 cells were plated into the wells of a 6-well plate in antibiotic-free media. When cells were 90–95% confluent (24 h), 4 μg of appropriate plasmid DNA and 10 μl of Lipofectamine 2000 were complexed together in Opti-MEM serum-free medium (Invitrogen) and added to the cells. After 24 h, the medium was changed to DMEM or HAMS F-12 containing serum as detailed above. After an additional 24 h, selection medium containing Geneticin was added to the cells. Stable pools of cells were maintained in Geneticin-containing media.

Detection of Transgene Products by Immunofluorescence Microscopy

Bovine tropoelastin was visualized with BA4 antibody, a mouse monoclonal antibody that recognizes bovine and human elastin, but does not cross-react with mouse or rat elastin. Bovine recombinant tropoelastin (BRT) antibody is a polyclonal antibody made against recombinant bovine tropoelastin. Mouse or rat tropoelastin was visualized with mouse recombinant tropoelastin (MRT) antibody, a rabbit polyclonal antibody generated against recombinant mouse elastin exons 6 through 17. To detect tropoelastin in stably transfected cell culture extracellular matrix, cells were plated at a density of 8×10^4 cells/well in a 4-well chamber slide (Tissue-Tek, Sakura Finetek, Torrance, CA) and allowed to reach confluence. At a predetermined number of days in culture, the medium was removed and cells were washed in PBS, fixed with cold methanol, and incubated with antibody solutions as described previously (30). Microscopy was performed on a Zeiss Axioscope (Zeiss, Thornwood, NY) and digital images obtained using an Axiocam camera with Axiovision software. All images were photographed at ×40 magnification unless otherwise specified.

Metabolic Labeling and Immunoprecipitation

Conditioned media and cell lysates were collected from 7 d postconfluent monolayers of stably transfected PE cells and nontransfected PE cells that were metabolically labeled with 50 μCi/ml of L-[4,5-^{3}H]leucine (1 mCi/ml) as described previously (32). Tropoelastin was precipitated after preclearing with mouse IgG and protein-G by adding the BTE antibody. Immune complexes were pelleted and washed before resuspending in Laemmli sample buffer with 100 mM dithiothreitol. Samples were subjected to SDS-PAGE and then gels were fixed and treated with Autofluor (National Diagnostics, Atlanta, GA). After drying for 2 h, gels were exposed to X-Omat AR film (Eastman Kodak, Rochester, NY) at −80°C.

Peptide Generation and Protease Susceptibility

Peptides with sequences corresponding to exons 33–36 of WT and MU human elastin were synthesized on an ABI-431A synthesizer using FastMoc chemistry and purified using reverse phase HPLC chromatography (Vydak 218TP1022 C-18 column using a 0–50% acetonitrile gradient; Hesperia, CA). Peak fractions were dried by rotary evaporation, dissolved in MilliQ water (Billerica, MA), and then lyophilized. Electrospray mass spectrometry performed by the Protein and Nucleic Acid Chemistry Labs at Washington University Medical School verified the identity and purity of the isolated peptides. The concentration of peptide solutions was determined by amino acid analysis using a Beckmann 6300 amino acid analyzer (Fullerton, CA).

The purified peptides (20 μg) were subjected to enzymatic cleavage by incubation with 1 μg neutrophil elastase or cathepsin G (Elastin Products Co., Owensville, MO) for 1 h at 37°. The enzyme digest was then analyzed using MALDI-TOF mass spectrometry (Protein and Nucleic Acid Chemistry Laboratory at Washington University) and peptide fragments were identified using the Protein Analysis Worksheet (PAWS; Rockefeller University, New York, NY, freeware; www.proteometrics.com). From these data it was possible to unambiguously identify the cleavage sites for each protease.

Cell Adhesion Assay

For cell adhesion assays, FBC-170 cells were detached from the plate using 10 mM EDTA in DMEM, washed, and suspended in DMEM with 1 mg/ml BSA (DMEM-BSA). The adhesion protocol was that described by Koo and colleagues (33). Peptides were diluted in DMEM-BSA, then added with ~ 20,000 cells per well to a 96-well ELISA plate (Costar Corporation, Acton, MA) and incubated at 37°C for 1 h. The plates were sealed with tape, inverted, and nonadherent cells removed with 300pN/cell force for 10 min. Adherent cells were quantified using hexosaminidase as a reporter according to the method of Landegren (34).

RESULTS

Mutation Discovery in Severe Early-Onset COPD Probands

The six terminal exons and surrounding intronic sequence of human elastin (exons 29–33 and 36 according to bovine reference sequence) were sequenced in 116 probands with severe early-onset COPD. Four variants were identified, including three intronic polymorphisms (IVS31–15 A>C, IVS32–34 C>T, and IVS35–29 C>G) not predicted to alter normal splicing. The fourth variant identified was located at the first base position of exon 36: a G>A transition at nucleotide c.2318 predicted to substitute aspartate for glycine at amino acid 773 (G773D; Figures 1a and 1b). Phylogenetic analysis of the tropoelastin protein revealed complete conservation of the glycine 773 residue across six species available for comparison (Figure 1c). No additional mutations in elastin exons 1–28 were identified in the G773D carrier, and three previously described ADCL elastin mutations were not observed among early-onset COPD probands.

The proband with the G773D mutation was a 41-yr-old woman with an FEV_1 of 18% predicted and diffuse emphysema of moderate severity despite only 19 pack-years of cigarette smoking. An echocardiographic evaluation revealed normal cardiac chamber and aortic outlet dimensions, and normal pulmonary vascular pressures. The subject died at the age of 43 yr due to complications of her respiratory disease.

The G773D mutation was observed among seven additional family members of the proband's extended pedigree (Figure 1d). Six of these seven additional carriers had evidence of airflow limitation (FEV_1 39–79% predicted); all six were current or previous cigarette smokers. The one carrier with normal pulmonary function (FEV_1 107% predicted), the proband's daughter, was of young age (17 yr old) and was a lifelong nonsmoker. Airflow obstruction was also noted in five of nine pedigree members who did not carry the variant, consistent with the notion that COPD is influenced by multiple genetic and environmental factors.

The proband's two daughters (the 773D carrier, IV-B, and her older sister, IV-A, a noncarrier) were available for detailed dermatologic examination, and were evaluated for manifestations of CL by a clinical geneticist (J.S.) unaware of the subjects' carrier status. The 773D carrier had evidence of mild CL: she was older appearing than her older sister, with a subtle bloodhound appearance, and demonstrated exaggerated hyperextensibility of the skin overlying the neck and forearms, hyperextensibility of her scapulae and elbow joints as well as the cartilage of her ears and nose. Her older sister (the noncarrier) had normal examination of the skin and joints. Histologic assessment of the skin from both subjects demonstrated normal architecture and the presence of elastin fibers of normal appearance.

The G773D Elastin Allele in Severe Emphysema and Control Populations

We evaluated the presence of the G773D variant in 318 subjects with severe COPD and radiographically confirmed emphysema who participated in the NETT, and in 365 men followed longitudinally with at least a 10 pack-year smoking history without evidence of airflow obstruction. The mutation was identified in 1.25% of the NETT participants compared with 0.55% in the normal control subjects (Fisher's exact test of proportions P value = 0.42).

The G773D Transcript Is Expressed and Undergoes Normal Alternative Splicing

ELN mRNA transcripts from cultured skin fibroblasts from a mutation carrier (subject IV-B) were evaluated by RT-PCR. Because the c.2318 mutation occurs at the donor splice site of exon 36 and may interfere with normal splicing, proximal and distal nested PCR primers were designed to improve the likelihood of detecting misspliced transcripts. The resultant PCR product sizes for the control and mutant cells were identical and were of the expected size for correctly spliced transcripts containing exon 36 (277 bp or 430 bp using reverse primer to coding region or 3′UTR, respectively). Sequencing of the PCR products confirmed c.2318 G/A heterozygosity in the patient sample transcripts, indicating that mRNA from both the wild-type (WT) and mutant (MU) alleles is transcribed and is stable (Figure 2). The presence of a stable mRNA transcript with the single base pair mutation suggests that a protein product with a single amino acid substitution (G773D) is made along with WT protein.

Expression and Matrix Incorporation of MU and WT Protein

The C-terminus of elastin encoded by exon 36 is a 14–amino acid cationic sequence containing three lysine and two arginine residues. Computer structure prediction programs (e.g., PredictProtein [35]) show that replacing glycine 773 with an acidic aspartic acid residue will adversely affect peptide structure and,

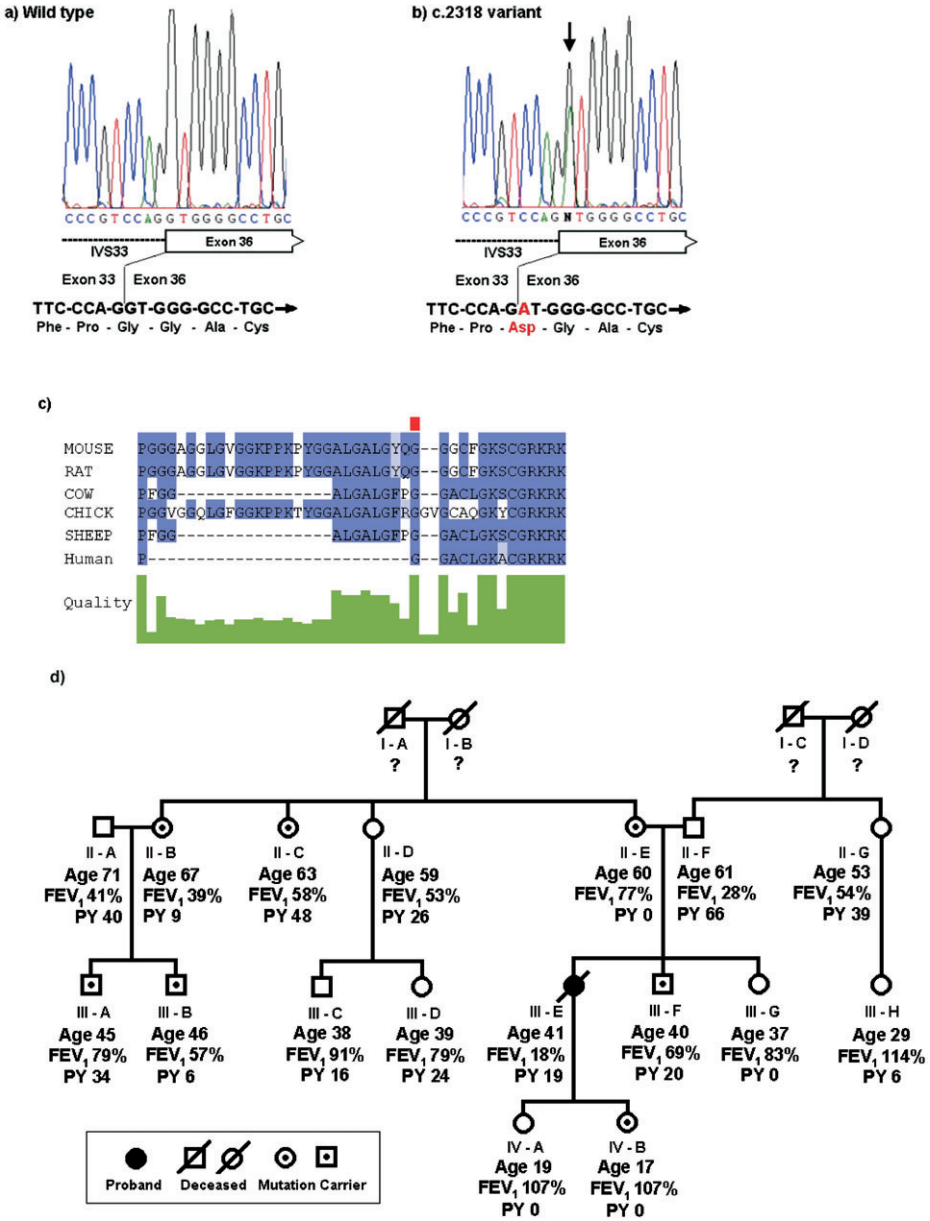

Figure 1. c.2318 G>A mutation in human elastin. DNA sequencing chromatograms from a normal subject (*a*) and a heterozygous mutation carrier (*b*). (*c*) Phylogenetic comparisons of C-terminal region of the elastin protein from six species: cow (NP_786966), sheep (P11547), chicken (P07916), mouse (NP_031951), rat (XP_237869), and human (NP_000492). Comparisons were performed with BLOSUM 62 matrix. Quality block displayed below translation reflects degree of amino acid conservation. Note high degree of conservation of amino acid residue 773 in human tropoelastin (indicated by *red bar*) and C-terminal region of human elastin. (*d*) Severe early-onset COPD pedigree with c.2318 mutation. *Shaded circle* represents proband. *Central dot* denotes c.2318 mutation carrier status. Where available, phenotype data at the time of ascertainment are listed below *circle* (female) or *square* (male) in the following order: age (yr), FEV_1 (% predicted), pack-years of smoking history (PY).

possibly, the function of this important region of the protein. To assess the effect of the G773D mutation on the ability of tropoelastin to assemble into a functional elastic fiber, we introduced the G773D amino acid change into a cDNA encoding bovine tropoelastin and transfected the WT and MU constructs into two well-characterized mammalian cell expression systems optimized to assess elastin assembly: Bovine PE cells and rat RFL-6 fibroblasts (30, 36). We chose to study the bovine instead of the human protein because there is no human cell line that has been as well characterized as PE and RFL-6 cells to study elastin assembly. Furthermore, previous studies have shown that bovine elastin, when expressed in rodent cells or as a transgene in mice, incorporates efficiently into mouse or rat elastic fibers (30, 36). Another argument for using the bovine protein is that it contains all 36 exons and, thus, has the same total exon number as elastin made by the nonhuman cells used in the assay system. Although the human elastin gene lacks exons 34 and 35, the sequence immediately upstream of exon 36 in the human protein (i.e., exon 33) is hydrophobic and similar in composition to that encoded by exon 35 in nonhuman elastins. Thus, the overall physical properties of the human and bovine proteins are analogous in this area and should be affected similarly by the G773D amino acid change.

When transfected into rat RFL-6 cells (a cell line that deposits an abundant elastic fiber network into its extracellular matrix [ECM]), both WT and MU bovine tropoelastin co-localized with rat elastin as evidenced by double labeling with species-specific elastin antibodies (Figure 3A). This result shows that the MU protein can participate in, and does not interfere with, fiber assembly in the presence of WT protein. In contrast to what was found with RFL-6 cells, there were qualitative and quantitative differences in fiber formation when the WT and MU elastin cDNA constructs were transfected into PE cells. PE cells do not synthesize elastin but do produce the microfibrillar proteins required for tropoelastin assembly. Thus, only assembly-competent tropoelastin capable of interacting with microfibrils will be localized to the ECM and form fibers when transfected into this cell line. Figure 3B shows normal amounts of tropoelastin that organized

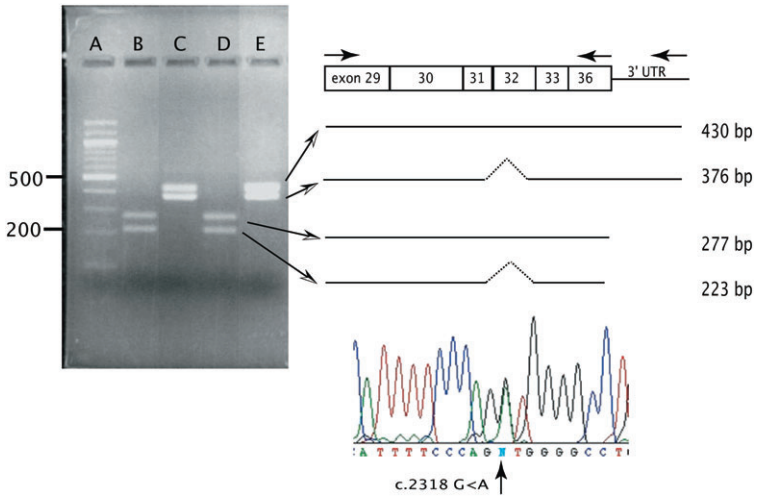

Figure 2. Analysis of elastin mRNA transcripts produced by cultured c.2318 heterozygous fibroblasts. RT-PCR of elastin transcripts from a normal control (*lanes B* and *C*) and the c.2318 carrier (*lanes D* and *E*). RT-PCR products using exon 36 specific primers (*lanes B* and *D*) and 3′ UTR targeted primers (*lanes C* and *E*) were similar for the normal and c.2318 carrier and were of the expected size for correctly spliced transcripts containing exon 36 (277 bp using reverse primer E36r, or 430 bp using reverse primer 3′UTRr). A second PCR product, ~ 50-bp smaller (220 or 376 bp) arising from alternative splicing of exon 32, was observed in both control and c.2318 carrier cells. Sequencing of RT-PCR products confirmed that exon 32 had been spliced out. Primer design and predicted RT-PCR product lengths are shown on the right. DNA sequencing chromatograms (*bottom right*) of PCR products from c.2318 heterozygous fibroblasts show the G to A mutation at the first codon of exon 36 (c.2318 G<A).

into typical fibers when PE cells were transfected with the WT construct. Analysis of cells transfected with the MU construct, however, identified intracellular staining but only occasional foci of matrix-associated elastin that had a globular appearance as opposed to the fiber-like structures seen with the WT construct (Figure 3B). The low levels of MU protein in the ECM were not due to a defect in protein secretion, as immunoprecipitation studies showed comparable levels of tropoelastin in the medium of WT and MU cells (Figure 3C). Staining with antifibronectin antibody demonstrated abundant and similar ECM deposition in both transfected cell lines, confirming that abnormal fiber formation observed for the MU protein was not caused by a general defect in ECM assembly. These results suggest that the MU protein, by itself, is inefficient at organizing into a fiber (PE cell data) but can associate with existing fibers in the presence of WT protein (RFL-6 cell data).

The G773D Sequence Shows a Different Susceptibility to Proteolysis

Degradation of lung elastic fibers has been linked with the pathogenesis and progression of emphysema in humans and in animal models of the disease. To assess whether the G to D mutation alters the susceptibility of the C-terminal region of elastin to proteolysis, synthetic peptides corresponding to MU and WT sequences encoded by the two C-terminal exons of human elastin were subjected to digestion with neutrophil elastase and cathepsin G. Mass spectrometry fragment analysis identified minor cathepsin G cleavage sites in both WT and MU peptides after F at position 8 and after I at position 13 (Figure 4), consistent with the chymotryptic-like specificity of this protease. The major cathepsin G cleavage site in the MU peptide, however, was after A at position 18. No cleavage at this position was evident for the WT peptide.

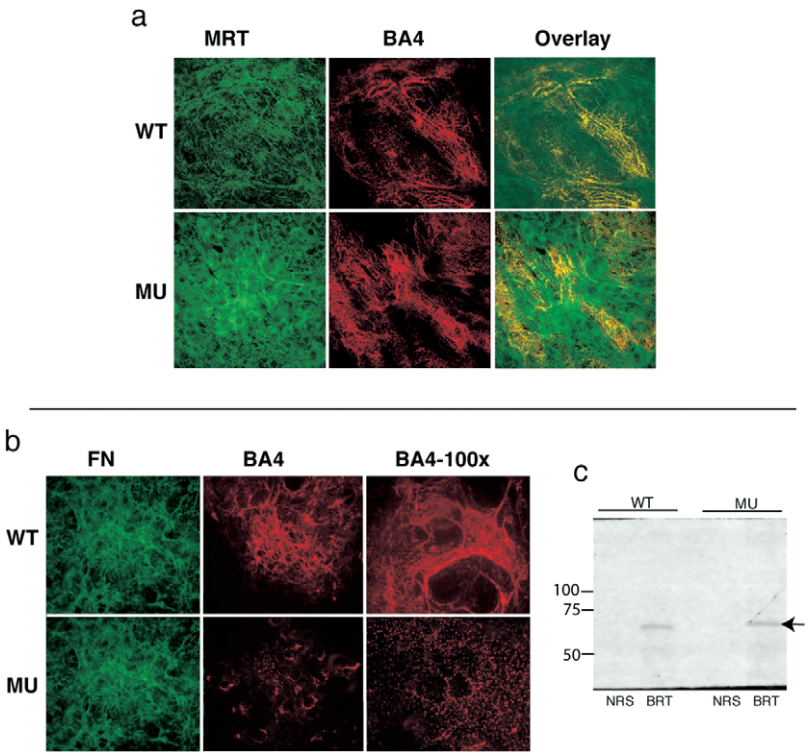

Figure 3. Elastic fiber formation in cultures of RFL-6 and PE cells stably transfected with WT or c.2318 G<A elastin cDNAs. (*a*) RFL-6 cells stably transfected with bovine cDNAs encoding wild-type elastin (WT) or elastin with the c.2318 G<A mutation (MU) were stained with the MRT antibody (*green*) to detect endogenous rat elastin and BA4 (*red*) to detect transgenic bovine elastin. Both MU and WT proteins formed fibers in the ECM and merged images (Overlay) show co-localization of the two proteins (*yellow*) with rat elastin. (*b*) Stably transfected PE cells expressing WT or MU elastin stained with the same antibodies. WT protein was able to form fibers in this cell system but analysis of cells expressing the MU construct showed only intracellular staining (*bottom middle*) and occasional foci of elastin globules in the ECM (*bottom right*). Also shown is the pattern of fibronectin (FN) deposition in the ECM of MU and WT PE cells visualized with a fibronectin antibody. All images were photographed at ×40 except where indicated (BA4 ×100). *c* is an autoradiogram of immunoprecipitation products from the culture medium of stably transfected PE cells expressing WT or MU bovine elastin. Normal rabbit serum (NRS) or bovine recombinant tropoelastin polyclonal elastin antibody (BRT) was used for protein precipitation. *Arrow* indicates the position of full-length bovine tropoelastin.

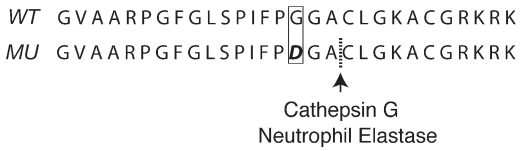

Figure 4. Protease digestion of WT and MU elastin peptides. Synthetic peptides encoded by exons 33–36 of WT and MU elastin were incubated with neutrophil elastase or cathepsin G. After 1 h, enzymatic cleavage sites were identified by characterizing peptide fragments in the enzyme digests using MALDI-TOF mass spectrometry. The *arrow* and *dotted lines* show a new cleavage site in the MU peptide that was used by both proteases. The *box* indicates the position of the G773D mutation.

Analysis of the peptides produced by neutrophil elastase digestion identified the same two minor cleavage sites at F (position 8) and I (position 13) that were observed with cathepsin G. Two additional minor sites (after V at position 2 and A at position 3) were detected in both peptides, consistent with the specificity of neutrophil elastase for hydrophobic side chains (37). As was found with cathepsin G, a prominent cleavage site was detected after Alanine at position 18 that was not found in the WT peptide. Together, these results show that the G773D mutation creates a novel protease cleavage site near the beginning of exon 36.

The G773D Mutation Modifies the Interaction of the C-Terminus of Tropoelastin with Cells

Recent studies have documented an interaction between sequences at the C-terminus of tropoelastin and receptors or binding proteins on the cell surface (38). These interactions appear to be mediated by the clustering of positively charged arginine and lysine residues in and around the sequence encoded by exon 36 (our unpublished results). To assess whether substituting a negatively charged aspartic acid residue for a glycine residue at the beginning of exon 36 would alter the ability of this region to interact with cells, we used cell adhesion assays to compare the biological activity of the MU and WT synthetic peptides described above. A peptide of identical amino acid composition to the WT peptide, but with scrambled sequence, served as a negative control.

The results in Figure 5 show a striking difference in the ability of WT and MU peptide sequences to promote cellular adhesion. The WT peptide showed concentration-dependent adhesive activity beginning at ~ 5 μM. The ability of the MU peptide to promote cellular adhesion, in contrast, was markedly reduced, demonstrating virtually no adhesiveness except at concentrations 10- to 20-fold higher than the WT peptide. A scrambled version of the WT sequence had no adhesive activity over the concentration range tested, confirming that the specific peptide sequence and not just peptide charge is responsible for the adhesive activity of this region of the protein. These results suggest that the G773D mutation modifies the biological properties of the C-terminal region of tropoelastin so that it no longer interacts efficiently with cells.

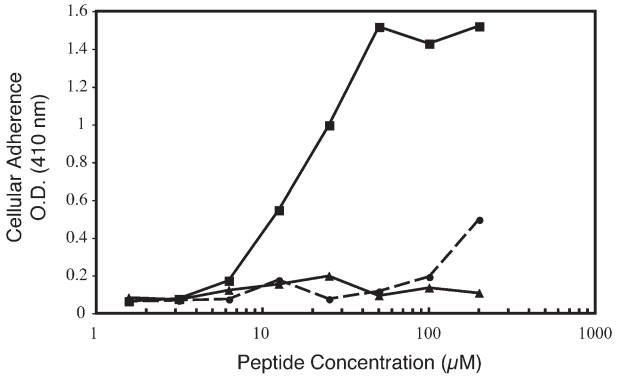

Figure 5. Cell adhesion to WT and MU elastin peptides. Adhesion of FBC cells to synthetic peptides encoded by exons 33–36 of human WT (*squares*) and MU (*circles*) elastin (*see* sequences in Figure 4). A peptide with the scrambled WT sequence served as a control (*triangles*). The number of bound cells was measured using a colorimetric assay for hexosaminidase in cell lysates (O.D. 410 nm).

DISCUSSION

Elastin is the primary protein determinant of connective tissue elasticity and plays a critical role in the development of the cardiovascular and respiratory systems (16, 39, 40). A broad spectrum of elastin gene mutations with varied phenotypic consequences have been described, including mutations producing elastin hemizygosity resulting in SVAS and Williams Beuren Syndrome (40) and distal frameshift mutations producing ADCL (17, 18). To this spectrum of mutations and phenotypes we add the G773D allele, and suggest that it confers a mild CL phenotype, and may increase risk for smoking-induced COPD. We have demonstrated that this variant can incorporate into elastic fibers in the presence of WT protein but is deficient in its ability to interact with microfibrils and to initiate fiber assembly by itself. In addition, susceptibility of the C-terminal region of tropoelastin to proteolysis is enhanced in peptides bearing this variant and the G to D change also alters the ability of the exon 36 sequence to interact with cells. This range of functional consequences represents distinct characteristics that potentially contribute to the pathogenesis of emphysema, either by impairing normal lung development due to diminished elastin deposition or by increasing the susceptibility of lung elastin to proteolytic digestion.

How tropoelastin assembles into functional elastic fibers is not completely understood, although current evidence suggests that the initial step in assembly occurs on the cell membrane where tropoelastin monomers self-assemble into elastin aggregates (30). With time, the elastin aggregates are deposited onto microfibrils, where they fuse to adopt a filamentous appearance. Our findings show that G773D tropoelastin, by itself, does not efficiently form fibers (*see* Figure 3B) but can do so in the presence of WT protein. It is not clear which step in the assembly process is affected by the mutation, but it must be early in the sequence, since little MU protein associates with the ECM in the PE cell assay. The ability of WT protein to rescue this deficiency suggests that the G773D protein is able to use the WT protein as an assembly scaffold by interacting with assembly domains outside of exon 36. This would be consistent with the known role of exon 30, which has been shown in previous studies to encode an important domain that directs association between tropoelastin molecules (30, 36).

It is unlikely that the integrity of elastic fibers is significantly compromised by incorporation of the G773D variant because some individuals with this polymorphism and normal lung function have been identified. Nevertheless, subtle changes in elastin packing and crosslinking resulting from G773D incorporation could make elastic fibers more susceptible to damage and less easily repaired after damage does occur. In previous studies we showed that the deletion of exon 36 or deletion of the RKRK sequence at the end of the sequence encoded by the exon does not inhibit incorporation of tropoelastin into fibers. The fibrils

that form with these truncated molecules, however, have fewer crosslinks than normal elastin indicative of abnormal assembly (30, 36). The soluble elastin precursor molecule, tropoelastin, is highly susceptible to proteolysis by many different classes of proteases that do not degrade the mature elastic fiber (e.g., trypsin- and chymotrypsin-like enzymes). When incorporated into mature elastic fibers, however, ~ 90% of the lysine residues in tropoelastin are modified to form crosslinks, which dramatically restricts the protein's susceptibility to degradation by a small number of proteases with specificity for aliphatic amino acids (e.g., elastases). If crosslinking of the G773D protein is impaired because of abnormal assembly, some lysine residues could remain unmodified rendering the insoluble protein susceptible to proteolytic fragmentation by enzymes that normally do not degrade fully crosslinked elastin. We have previously shown that impairment of crosslinking by copper deficiency can yield functional, insoluble elastic fibers that can be completely degraded by sequential treatment with trypsin and chymotrypsin (41)—proteases that otherwise do not degrade the fully crosslinked protein. In the severe early-onset pedigree harboring the G773D variant, evidence of airflow limitation was most severe among those mutation carriers who smoked, suggesting a gene-by-environment interaction. Given the effects of the G773D variant on elastin structure and susceptibility to proteolysis, it is likely that the destructive effects of proteases induced by cigarette smoke will be enhanced in these suboptimal elastic fibers.

While the experimental data here strongly suggest that the G773D mutation confers functional consequences that could contribute to COPD susceptibility and furthers our understanding of the importance of the C-terminal portion of elastin in elastin fiber synthesis and cellular adhesion, we are not able to make firm conclusions regarding the variant's public health significance. Although we found clinical evidence of mild CL in one 773D carrier, supporting the notion that this variant has phenotypic consequences, only one mutation carrier was available for such detailed examination, thereby precluding generalized conclusions. More importantly, because of the rarity of this variant, neither our pedigree analysis nor the population-based studies are sufficiently powered to provide conclusive evidence that this variant increases susceptibility for COPD. Although all mutation carriers in the extended pedigree who smoked had evidence of COPD, several noncarriers of the G773D variant also had airflow obstruction, suggesting that additional genetic and/or environmental factors may influence the development of COPD, even within this pedigree. Although not reaching statistical significance, the G773D mutation was observed at a carrier frequency two times higher among the NETT severe emphysema cases (1.25%) compared with the male cohort of smokers with normal lung function (0.55%). Given the rarity of the G773D variant, a very large genetic association study would likely be required to demonstrate a significant association. A major limitation of population-based genetic approaches in identifying disease susceptibility genes is their low statistical power to detect effects for alleles with low minor allele frequency (< 5–10%), even when the effects of such rare mutations are strong (42, 43). Although it has been postulated that common complex diseases, like COPD, are influenced by common genetic variants, it is also plausible that rare variants of modest effect could contribute to previously unrecognized subtypes of a complex disease. Although it is perhaps more difficult to prove true disease association with rare variants compared with common variants of similar phenotypic effect, determination of the genetic architecture of complex diseases will require the identification of rare functional genetic variants, using a combination of genetic epidemiologic and molecular approaches. This has recently been demonstrated in several common complex traits (44, 45). Although these rare variants may only be found in a small percentage of affected individuals, they have the potential to provide insight into complex disease phenotypes as well as critical biological processes like elastic fiber assembly.

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