Antisense-Induced Messenger Depletion Corrects a COL6A2 Dominant Mutation in Ullrich Myopathy

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Abstract

Collagen VI gene mutations cause Ullrich and Bethlem muscular dystrophies. Pathogenic mutations frequently have a dominant negative effect, with defects in collagen VI chain secretion and assembly. It is agreed that, conversely, collagen VI haploinsufficiency has no pathological consequences. Thus, RNA-targeting approaches aimed at preferentially inactivating the mutated COL6 messenger may represent a promising therapeutic strategy. By *in vitro* studies we obtained the preferential depletion of the mutated COL6A2 messenger, by targeting a common single-nucleotide polymorphism (SNP), cisgenic with a dominant COL6A2 mutation. We used a 2'-O-methyl phosphorothioate (2'OMePS) antisense oligonucleotide covering the SNP within exon 3, which is out of frame. Exon 3 skipping has the effect of depleting the mutated transcript via RNA nonsense-mediated decay, recovering the correct collagen VI secretion and restoring the ability to form an interconnected microfilament network into the extracellular matrix. This novel RNA modulation approach to correcting dominant mutations may represent a therapeutic strategy potentially applicable to a great variety of mutations and diseases.

Introduction

ULLRICH MUSCULAR DYSTROPHY (UCMD-MIM#254090) and Bethlem myopathy (BM-MIM#158810) are congenital disorders caused by defects in collagen type VI, a multimer of various α (VI) chains (Bertini *et al.*, 2011). These chains assemble intracellularly as monomers (1:1:1 ratio), which unite to form dimers of antiparallel monomers, and subsequently tetramers; these are stabilized by disulfide bonds, secreted into the extracellular space, and aligned end-to-end as microfibrils, providing structural and mechanical stability by interacting with various connective tissue basement membrane constituents (Allamand *et al.*, 2011). As collagen VI is a multimer, an abnormal mutated chain retaining the ability to be assembled into growing supramolecular structures will sequester normal chains into nonfunctional complexes, thus exerting a dominant negative effect (Baker *et al.*, 2007). Indeed, dominant *de novo* COL6A gene mutations occur in more than 50% of patients with UCMD (Allamand *et al.*, 2010, 2011), with both missense changes in glycines within the α (VI) chain triple-helix domains and intron-splicing mutations being com-

mon (Allamand *et al.*, 2010). This suggests that a significant proportion of collagen VI-related phenotypes could benefit from therapeutic approaches that selectively reduce/abolish the expression of COL6A gene transcripts carrying dominant negative mutations. This view is reinforced by the evidence that collagen VI haploinsufficiency, which occurs in the parents of children with recessive UCMD, who carry large deletions or premature truncating mutations leading to non-sense-mediated decay (NMD), is not associated with any pathological phenotype (Bovolenta *et al.*, 2010; Foley *et al.*, 2011).

We set out to test this hypothesis, using a novel molecular approach based on exon skipping, designing a 2'-O-methyl phosphorothioate (2'OMePS) antisense oligonucleotide (AON) to target a common COL6A2 single-nucleotide polymorphism (SNP) occurring *in cis* with the *de novo* dominant intron 9 c.954+17_954+22del28 UCMD mutation. Transfecting patient fibroblasts with this AON induced preferential skipping of the mutated exon via SNP recognition, and caused COL6A2 messenger out-of-framing, thereby depleting the mutated transcript via NMD. Skipping quantification confirmed AON efficacy, an RNA transcript count

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evidenced the preferential mutated transcript depletion, and protein and morphological studies showed reduced expression of the mutated COL6A2 allele. Both histology and electron microscopy studies revealed promotion of collagen VI integration into the extracellular matrix (ECM) and focal recovery of the microfibrillar network, thus confirming the feasibility of mutated messenger depletion, and thereby dominant mutation correction, via out-of-frame exon skipping.

Materials and Methods

UCMD mutation and P221(T)AON design

The UCMD mutation is a *de novo*, heterozygous 28-bp deletion within intron 9 of the COL6A2 gene (c.954+17_954+22del28) causing exon 9 skipping and predicting a triple-helix domain internal deletion (Gly310_LYS318del). In patients with UCMD, the mutation is cisgenic, with the T allele of the common COL6A2 c.663C>T SNP (P221P) occurring in the out-of-frame exon 3 (Fig. 1A). A 2'OMePS-modified RNA AON [referred to as P221(T)AON] was designed, according to published guidelines (Aartsma-Rus, 2012), to target this T allele (Supplementary Fig. S1A; supplementary data are available online at www.liebertonline.com/hum); synthesis and purification were as previously described (Spitali *et al.*, 2009). Various AON doses and timings were tested (see Supplementary Table S1).

Cell culture and transfection

Primary human skin fibroblasts (obtained for diagnostic procedures with ethics committee approval no. 7/2009) were cultured at 37°C and 5% CO₂ as described (Martoni *et al.*, 2009). Cells were transfected for 4 hr with either AON (200 nM) plus AON ExGen 500 polyethylenimine (PEI) (3.3 µl/µg; MBI Fermentas/Thermo Fisher Scientific, Waltham, MA) or with PEI alone, as described (Evers *et al.*, 2011).

RNA studies

Cells were harvested 24 hr post-transfection, and total RNA was isolated and retrotranscribed as reported (Martoni *et al.*, 2009). RT-PCR was performed with COL6A primers within exons 2–10 and 4–10 (sequence available on request) to quantify exon 3 skipping, and to assess the proportions of wild-type (exon 9-plus) versus mutated (exon 9-minus) COL6A2 transcripts, calculated as the ratio between each type of transcript and the sum of all transcripts, on an Agilent high-sensitivity DNA chip (Agilent Technologies, Santa Clara, CA) (Supplementary Fig. S1B). TaqMan expression assays (Applied Biosystems) were used to quantify COL6A2 and COL6A1 transcripts (Hs00242484_m1 Ex27/28; Hs00242448_m1 Ex20/21) with respect to β -actin, the endogenous control. Real-time PCR was performed as described (Martoni *et al.*, 2009). cDNAs from untreated fibroblasts served for calibration.

Western blot

Sixteen hours post-transfection, fibroblasts were supplemented with 0.25 mM sodium ascorbate for 24 hr. Medium was collected, and the cell layer was scraped and solubilized in radioimmunoprecipitation assay (RIPA) buffer. Protein content was quantified with a bicinchoninic acid (BCA) protein assay kit (Pierce/Thermo Fisher Scientific, Rockford,

IL). Western blotting was performed as previously reported (Martoni *et al.*, 2009). A loading check was performed with anti- β -catenin antibody (diluted 1:1000; BD Biosciences Transduction Laboratories, Lexington, KY) for cell layer extracts and Coomassie staining for culture medium.

Immunostaining

Sixteen hours post-transfection and after 24 hr in sodium ascorbate, cells were fixed with cold methanol and double-stained with anti-collagen VI (Fitzgerald, Concord, MA) and perlecan (Chemicon, Temecula, CA) antibodies, as previously reported (Sabatelli *et al.*, 2001). After nuclear staining with 4',6-diamidino-2-phenylindole (DAPI), samples were mounted with antifade reagent (Molecular Probes/Invitrogen, Carlsbad, CA) and analyzed with an Eclipse 80i fluorescence microscope (Nikon Instruments, Melville, NY).

Immunoelectron microscopy

Cultures were incubated with 3C4 monoclonal antibody (Chemicon), diluted 1:50 in culture medium at 37°C for 3 hr, and revealed with anti-mouse IgG 5-nm gold-conjugated antibody (GE Healthcare, Piscataway, NJ), diluted 1:50 for 1 hr at 37°C. Rotary-shadowing electron microscopy was performed as described (Zhang *et al.*, 2002). Samples were examined under a Philips EM 400 (FEI, Hillsboro, OR) or JEOL JEM 1011 electron microscope (JEOL, Tokyo, Japan) at 100 kV. For quantitative analysis of microfibrillar tetramer composition, 20 fields at $\times 17,000$ magnification were evaluated for each condition.

Results

P221(T)AON induces exon 3 skipping, transcript out-of-framing, and COL6A2 transcript degradation

Patient-derived fibroblasts were transfected (in three independent experiments) with 200 nM P221(T)AON, and total RNA was isolated after 24 hr. RT-PCR and DNA chip analysis showed average exon 3 skipping of 30.3% (range, 21.3–39.3%), whereas none was observed in cells treated with control AON (against dystrophin exon 51) (Goemans *et al.*, 2011). After P221(T)AON treatment, real-time PCR showed COL6A2 mRNA depletion to about 60% of baseline (0.62 ± 0.10); COL6A1 expression was not significantly affected (1.07 ± 0.20) (Fig. 1B).

P221(T)AON reduces collagen VI at cell layer and in medium

In parallel with transcript depletion, P221(T)AON treatment caused a decrease in the global amount of collagen VI in UCMD patient cell layer (Fig. 1D, left). P221(T)AON-treated samples showed even more pronounced reduction of collagen VI in the medium fraction (Fig. 1D, right) with respect to basal conditions. As medium extract is composed of proteins neither retained in cells nor deposited on ECM, the difference in collagen VI depletion between the two fractions seems to indicate more efficient post-treatment ECM deposition of the protein.

P221(T)AON affects mutated/wild-type COL6A2 allele ratio

To assess the sequence specificity and efficacy of P221(T)AON, the proportions of mutated versus wild-type

FIG. 1. (A) Schematic representation of the Ullrich muscular dystrophy (UCMD) mutation within the COL6A2 gene. cDNA sequencing showed the intronic c.954 + 17_954 + 22del28 mutation (which causes exon 9 skipping) to be *in cis* with the T allele of the c.663C > T (P221P) SNP within COL6A2 exon 3. (B) Real-time PCR analysis of RNA from UCMD patient fibroblasts after treatment with P221(T)AON and control dystrophin exon 51 (dys51) AON. P221(T)AON transfection had no effect on COL6A1 transcript level, whereas COL6A2 mRNA was reduced to 60% of the pretreatment level (β -actin as reference). Control AON had no significant effect on either COL6A1 or COL6A2 transcript level. The graphs are representative of three independent experiments. Data are given as means $\pm$ SD and the statistical significance has been calculated by the Student *t* test for unpaired data (**p* < 0.005); (C) Relative proportion of mutated versus wild-type COL6A2 transcript before and after P221(T)AON treatment. RT-PCR was performed with COL6A2 primers within exons 4–10, and products were run on an Agilent high-sensitivity DNA chip for relative quantification of the COL6A2 mutated allele (lacking exon 9) versus the wild-type allele (including exon 9). Pretreatment, the mutated allele represents $82.72 \pm 1.86\%$ of the wild-type allele. After P221(T)AON transfection, this ratio was lowered to 72.87 ± 1.18 . Control AON had no effect on the allele ratio. Statistical significance has been calculated by the Student *t* test for unpaired data (**p* < 0.005). (D) Western blot analysis after treatment with P221(T)AON. Samples corresponding to cell layers (20 μ g, left) and culture medium (right), from skin fibroblasts of the UCMD patient [before and after P221(T)AON treatment] and healthy donor (CTRL), were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis onto 8% polyacrylamide gels under reducing conditions. Collagen VI was detected with an antibody recognizing all three α chains. Protein bands were quantified with ImageJ software. Collagen VI quantity was calculated as an absolute value and normalized using β -catenin for cell layer extract and by Coomassie staining for culture medium. Densitometric analysis (histograms) shows a decrease in the amount of collagen VI, after p221(T)AON treatment, in UCMD patient samples, more pronounced in the medium. As medium extract is composed of proteins neither retained in cells nor deposited on ECM, the increase in protein in the medium of untreated samples is probably related to the inefficient deposition of mutated collagen VI, significantly ameliorated by treatment. The blots are representative of three independent experiments. Data are given as means $\pm$ SD and the statistical significance has been calculated by the Student *t* test for unpaired data (**p* < 0.005).

COL6A2 transcripts were compared in treated versus untreated cells. In untreated cells, the mutated COL6A2 allele (missing exon 9) comprised $82.72 \pm 1.86\%$ of the wild-type. After P221(T)AON transfection, this pathological messenger was reduced by 10% to $72.87 \pm 1.18\%$. Control AON did not significantly affect the allele ratio ($80.70 \pm 1.00\%$) (Fig. 1C).

P221(T)AON partially recovers collagen VI-based ECM

In untreated UCMD patient fibroblasts, collagen VI secretion appeared severely affected, with little protein deposited in the ECM in a spotlike arrangement, indicating a decreased ability of secreted mutant tetramers to associate into microfibrils. P221(T)AON treatment appeared to increase the amount of collagen VI associated with the ECM (Fig. 2A); the secreted protein was organized in a microfibrillar network and colocalized with perlecan labeling, as per control (Fig. 2B). Electron microscopy (EM) analysis showed improved tetramer–tetramer association and enhanced ability to form a microfilament network (Fig. 3A). In untreated UCMD culture, 83% of microfibrils were constituted by 2 to 10 tetramers, whereas microfibrils with more than 20 tetramers were absent. After treatment, about 80% of fibrils contained more than 20 tetramers (Fig. 3B). No effects were

seen in fibroblasts treated using AON directed toward dystrophin exon 51 (data not shown).

Discussion

Correcting dominant mutations by splicing modulation is appealing, because the only way to intervene in these cases is by specifically silencing the mutated allele, a feat that has been achieved using various genetic methodologies (Wood *et al.*, 2007; Le Roy *et al.*, 2009). In particular, the exquisite sequence specificity of siRNAs has been used with encouraging results for allele-specific knockdown in several non-muscular diseases *in vitro* and in animal models (Wood *et al.*, 2007), and a phase 1b trial based on mutation-targeted siRNA, locally injected within skin lesions of pachyonychia congenita, is underway (Leachman *et al.*, 2010). Nevertheless, concerns regarding delivery, interference with endogenous RNAi processing, and nonspecific off-target effects have thus far hampered a wider clinical exploitation of siRNA-based therapies (Wood *et al.*, 2007; Wang *et al.*, 2010). In contrast, AON-based treatment has reached phase 1–2a clinical trials in neuromuscular diseases. In patients with Duchenne muscular dystrophy (DMD), 2'OMePS-AONs and phosphorodiamidate morpholino oligomer (PMO) against dystrophin exon 51 have been systemically used to induce in-frame exon skipping,

FIG. 2. (A) Immunofluorescence analysis of collagen VI in fibroblast cultures of a UCMD patient before (UCMD pre-treatment) and after treatment with P221(T) AON (UCMD post-treatment). Before treatment, collagen VI shows an intracellular localization and only rare deposits can be detected in the ECM (arrow). After treatment, collagen VI secreted in the ECM appears to be increased (arrows) and displays a fibrillar arrangement (*inset*, arrowheads). Nuclei are stained with DAPI. Scale bars, 20 μ m. (B) Immunofluorescence analysis of collagen VI (green) and perlecan (red) in fibroblast cultures of a control subject (CTRL) and the patient with UCMD before (UCMD pre-treat) and after treatment with P221(T)AON (UCMD post-treat). In normal cells, collagen VI develops a dense microfibrillar network that overlaps with perlecan labeling, as showed by yellow staining in the merge image. In the culture of the UCMD patient fibroblast under basal condition (UCMD pre-treat), collagen VI appears accumulated mainly in the cell cytoplasm; some spotlike collagen VI-positive structures can be detected in the extracellular matrix. After treatment with P221(T)AON, the amount of protein secreted and associated with the ECM is increased and shows a fibrillar arrangement. The colocalization of collagen VI with perlecan is also recovered, as shown in the merge image. Nuclei are stained with DAPI. Scale bar, 10 μ m.

FIG. 3. (A) Electron microscopy analysis (rotary-shadowed replicas) of cultured fibroblasts. Fibroblasts from a control subject and the patient with UCMD, under basal conditions (UCMD pre-treatment) and after P221(T)AON treatment (UCMD post-treatment), were immunolabeled with anti-collagen VI and 5-nm colloidal gold conjugates and rotary shadowed. Transmission electron microscopy (TEM) analysis of replicas obtained from control cells reveals the presence of long interconnected microfilaments forming a complex network. In the UCMD patient culture, under basal conditions single short microfilaments can be detected outside the cells. After treatment with P221(T)AON, the length of the isolated microfilaments was increased, as well their ability to develop an interconnected network. Scale bar, 500 nm. F, fibroblast cytoplasmic process. (B) Quantitative analysis of collagen VI tetramer composition. Collagen VI deposited in the extracellular matrix of the UCMD patient fibroblasts before and after P221(T)AON treatment was visualized by immunogold and rotary shadowing techniques, and the ability of the tetramers to associate end-to-end was quantified. The occurrence of microfibrils containing 1–10, 11–20, 21–40, and 41–80 tetramers is shown as a percentage of the total number of microfibrils. Tetramer–tetramer association appears significantly improved after treatment ($p < 0.05$). (C) Schematic representation of the improvement in tetramer composition after P221(T)AON treatment. Under pretreatment conditions the actual ratio between normal and mutant COL6A2 transcripts was 1:0.8 [the ratio between normal and mutant $\alpha_2(\text{VI})$ chains was approximated to 1:1]; after P221(T)AON treatment, this ratio become 1:0.7, with a 10% relative depletion of the mutated transcript and, theoretically, an equal reduction of the mutated chain. The tetramer assembly process accounts for the “magnification effect”: after treatment, the percentage of functional tetramers is doubled despite of the relatively low efficiency of mutated allele depletion.

which can restore dystrophin production, apparently without overt toxic effects, paving the way to extended treatment phases (Cirak *et al.*, 2011; Goemans *et al.*, 2011).

We adopted exon skipping, exploring the efficacy of an SNP-specific 2'OMePS-AON to induce out-of-frame skipping and consequent allele degradation, as a corrective approach for a COL6A2 gene dominant negative mutation. This strategy was based on the following evidence: (1) 2'OMePS-AON sequence specificity and efficacy in inducing exon re-

moval have been largely demonstrated in humans (Spitali *et al.*, 2009); (2) COL6 gene transcripts with nonsense mutations almost invariably undergo RNA nonsense-mediated degradation, and quantification of transcripts has been proposed as a tool for pinpointing the mutated gene (Allamand *et al.*, 2011); (3) COL6 genes are highly polymorphic and several common SNPs are known, which, if cisgenic with pathogenic mutations, can be specifically targeted by AONs to induce transcript depletion in large patient populations;

(4) dominant *de novo* mutations represent the predominant type, being the 67% of the COL6 genes missense mutations, 57% of small deletions, and 44% of splice site mutations leading to in-frame exon exclusion (Allamand *et al.*, 2011). By AON-targeting a common SNP, we were able to target the causative mutation and deplete the mutated transcript, partially correcting the associated severe cellular phenotype. Treatment was not completely specific and caused a global reduction in COL6A2 mRNA level, preferentially involving the mutated allele. We observed that a relatively low efficiency of mutated allele depletion (10% variation in mutated/wild-type allele ratio) was nevertheless able to significantly improve extracellular collagen VI assembly, and restore its ability to develop a microfilament network, in single-dose and short-term treatment. The collagen VI complex assembly process, which explains the dominant negative behavior of mutations leading to aberrant chains, also accounts for this “magnification effect” of protein correction (Fig. 3C).

Progress in unraveling the pathogenic mechanisms of collagen VI-related myopathies (Grumati *et al.*, 2010) has opened intriguing therapeutic perspectives; nevertheless, RNA-splicing modulation is appealing, because it is based on safe and highly reliable drugs in terms of systemic administration and tissue targeting (Cirak *et al.*, 2011; Goemans *et al.*, 2011). Furthermore, exon skipping can be personalized, treating specific mutations using dedicated AONs.

We provide proof of principle that out-of-frame exon skipping by AONs induces allele targeting and corrects COL6 gene dominant mutations, highlighting molecular splicing as a potential treatment for collagen VI-related myopathies. In addition, because our AON design targeted a common SNP linked to the mutation, we envisage a novel AON therapy that may facilitate personalized treatments, targeting “common variations embracing rare mutations.”

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Author Disclosure Statement

No competing financial interests exist.

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