MACROPHAGES: A MINIMALLY INVASIVE TOOL FOR MONITORING COLLAGEN VI MYOPATHIES

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Accepted 7 December 2010

ABSTRACT: *Introduction:* Collagen VI expression was tested in peripheral blood macrophages from patients with collagen VI–related myopathies and compared with muscle biopsy. *Methods:* RNA and protein studies were performed in blood macrophages from 5 patients previously diagnosed with either Ullrich congenital muscular dystrophy (UCMD) or Bethlem myopathy (BM). The full spectrum of possible genotypes was considered, including both dominant and recessive UCMD and BM cases. *Results:* In the dominant BM patient, no collagen VI alterations were detectable in macrophages or muscle biopsy. In the remaining patients, the protein defect caused by the selected mutations, as well as the transcriptional abnormalities, were readily detectable in macrophages, at levels comparable to those observed in muscle biopsy samples and cultured skin fibroblasts. *Conclusions:* Our data support the suitability of peripheral blood macrophages as a reliable, minimally invasive tool for supplementing or replacing muscle/skin biopsies in the diagnosis and monitoring of collagen VI–related myopathies.
Muscle Nerve **44:** 80–84, 2011

Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) are allelic disorders caused by a defect in collagen VI, a ubiquitous constituent of the extracellular matrix.[1] In UCMD patients, immunohistochemical analysis can be used to reveal partial or complete deficiency of collagen VI in the muscle tissue.[1] Classic dominant BM cases usually show normal collagen VI in muscle biopsy, whereas recessive BM mutations have been shown to reduce collagen VI expression in skeletal muscle[2] by immunohistochemical and biochemical analyses. Collagen VI deficiency is generally also detectable in cultured skin fibroblasts, both in UCMD and BM cases.[3] However, because collagen VI synthesis and secretion may be influenced by culture conditions[4] and as heterogeneity of skin fibroblasts has been reported with respect to extracellular matrix production,[5] the information obtained from these latter cells may be unreliable. Thus, although relatively traumatic for patients, biopsy and analysis of muscle tissue or cultured skin/muscle-derived cells are preferred sources both of protein and mRNA for testing the transcriptional behavior of genomic mutations[6] and for monitoring drug effects, such as in the therapeutic trials currently underway to gauge the effectiveness of cyclosporine and derivatives at repairing mitochondrial function in collagen VI myopathies.

In the pilot version of this study,[7] each of the patients received two muscle biopsies: one at baseline to confirm mitochondrial dysfunction, and the other after treatment to evaluate the effect of the drug. Nevertheless, in full-scale clinical trials involving larger patient samples and more extensive monitoring, the invasiveness of this protocol may seriously affect the willingness of patients, or their families, to participate.

Peripheral blood monocytes and monocyte-derived macrophages (MDMs) produce and secrete abundant collagen VI.[8] Hence, to determine whether MDMs may be a reliable, minimally invasive adjunct or surrogate tool for muscle biopsy in collagen VI–related myopathies, RNA and protein studies were employed to analyze collagen VI expression in these cells from 3 UCMD patients and 2 BM patients, carrying previously characterized *COL6* gene mutations.

METHODS

Patient UCMD-A1 carries the 921–935del15 mutation within *COL6A1* exon 9, in heterozygosity[9]; patient UCMD-A2 is compound heterozygous for recessive *COL6A2* mutations [c.1009+5 G>A/Arg366Stop (patient UCMD2 in the study by Martoni et al.[6])]; and patient UCMD-A3 carries the *de novo* heterozygous c.6210+1 G>A mutation within *COL6A3* intron 16.[10]

Patient AD-BM belongs to a family with dominant transmission of the BM phenotype. He is heterozygous for the missense change Leu1643Arg within *COL6A3* exon 11 (c.4928 T>G–NM_004369.3), a mutation that segregates in the affected father and brother.

Patient AR-BM is compound heterozygous for the recessive *COL6A2* mutation [p.Asp983_Val984del (c.2947_2952del6–NM_001849)] and carries a

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; BM, Bethlem myopathy; CGH, comparative genomic hybridization; FITC, flourescein isothiocyanate; ICC, intracellular cytokine staining; MDM, monocyte-derived macrophage; RT-PCR, reverse transcription–polymerase chain reaction; UCMD, Ullrich congenital muscular dystrophy

Key words: Bethlem myopathy, collagen VI, monocyte-derived macrophages, protein expression, RNA behavior, Ullrich congenital muscular dystrophy

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Published online 12 April 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mus.21999

deletion within intron 1A, identified by array comparative genomic hybridization (CGH) analysis.[11]

Unfixed frozen sections of the tibialis anterior muscle from all patients and controls (2 adults and 2 children) were labeled with anti–collagen VI antibody (MAB1944; Chemicon) and then by flouorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (DAKO).[5]

Mononuclear cells were isolated from 10 ml of peripheral blood on a Ficoll gradient (Sigma) and cultured in RPMI 1640 medium (Sigma-Aldrich) at a density of $10^6/cm^2$. After 3 days, non-adherent cells were removed. The medium, supplemented with 50 μg/ml ascorbic acid, was changed every 2 days.[8]

After 14 days of culture, collagen VI expression was assessed by immunofluorescence, as previously reported.[6] Anti-fibronectin antibody (Sigma Co.) was used as an internal marker.

RNeasy (Qiagen, Chatsworth, California) was utilized to isolate total RNA from MDMs from patients UCMD-A2, UCMD-A3, and AR-BM, which was reverse transcribed as described elsewhere.[6] Primers utilized for reverse transcription–polymerase chain reaction (RT-PCR) analysis were as follows: COL6A2ex5for: gtgagctgcctggaaatccctg; COL6A2ex14rev: gcccttggctcctttcacacc; COL6A3ex 12for: cctgaacaagttcagacagtcctc; and COL6A3ex23-rev: caggtactccatctctccctg.

To quantify the levels of *COL6A2* transcript in UCMD-A2 MDMs, commercially available TaqMan expression assays (Applied Biosystems) were employed for *COL6* genes (*COL6A1*: Hs00242448_m1 Ex 20-21ëƒ *COL6A2*: Hs00242484_m1 (Ex 27–28) and *COL6A3*: Hs00915102_m1 (Ex 23–24) and for actin as a reference gene (*ACTB* endogenous control). Real-time PCR was performed as previously described.[6] cDNA from control MDMs served for calibration.

RESULTS

Immunofluorescence analysis of collagen VI on muscle sections from the 3 UCMD patients revealed markedly reduced expression, as compared with controls, whereas collagen VI was moderately reduced and unevenly distributed in the AR-BM muscle biopsy sample. In contrast, normal expression of collagen VI was detectable in the skeletal muscle from the dominant BM case (AD-BM) (Fig. 1, left lane).

Using the same antibody, greatly reduced levels of collagen VI were detected in MDMs cultures from all UCMD patients with respect to controls. Collagen VI was also found to be moderately reduced in MDMs from patient AR-BM, whereas it was detectable at normal levels in MDMs from the dominant AD-BM case (Fig. 1, middle lane). Fibronectin, used as an internal marker of the secreted extracellular matrix, displayed a pattern comparable to that of normal MDMs in all patients (Fig. 1, right lane).

Furthermore, results of RNA behavioral analysis of the two splicing mutations in patient MDMs paralleled those previously reported in fibroblasts.[6,10] Patient UCMD-A2 exhibited two *COL6A2* transcripts, one with normal exon 8–9 splice junction and the other with intron 8 retention (originating from the +5 mutation) (Fig. 2A, left; see also Martoni et al.[6]). Real-time PCR revealed an even more pronounced reduction in COL6A2 messenger level, related to nonsense-mediated decay of the Arg366Stop allele,[6] than those previously documented in fibroblasts (Fig. 1B). RT-PCR in MDMs from patient UCMD-A3 revealed exon 16 skipping (Fig. 1A, right), as universally reported in this common *COL6A3* mutation.[10]

In patient AR-BM, sequence analysis of the *COL6A2* transcript showed an imbalanced representation of the *COL6A2* alleles, as previously documented in fibroblasts, related to a regulatory mutation identified within intron 1 by CGH analysis.[11]

DISCUSSION

Presently, the major endpoint of hereditary muscle disease–related trials is the restoration (as yet partial and with varying efficiency) of the defective protein(s) in muscle tissues. Monitoring this process involves repeated muscle biopsies, and thus the validation of novel minimally invasive tools for diagnosis and follow-up is a desirable goal.

Macrophages derived from MDMs have recently been shown to express collagen VI mRNA and to secrete abundant protein.[8] Hence, we conducted RNA and protein analyses to evaluate the effects of *COL6A* gene mutations on collagen VI expression in MDMs, and thereby sought to determine whether these cells could represent suitable cells for monitoring collagen VI–related myopathies.

Collagen VI expression was therefore investigated in MDMs from a group of patients representative of the full spectrum of UCMD/BM genotypes and phenotypes. Three UCMD patients were selected, all carrying mutations in different *COL6* genes, as well as 2 BM cases, 1 with dominant and the other with recessive inheritance.

Two UCMD patients, the first carrying one splicing and one nonsense mutation in the *COL6A2* gene[6] and the second a splicing mutation within the *COL6A3* gene, were selected on the basis of their previously characterized transcriptional behavior in cultured fibroblasts. Encouragingly, the transcription pattern of both splicing mutations was detected to a similar degree in macrophages, even though one of the selected mutations was a non-canonical

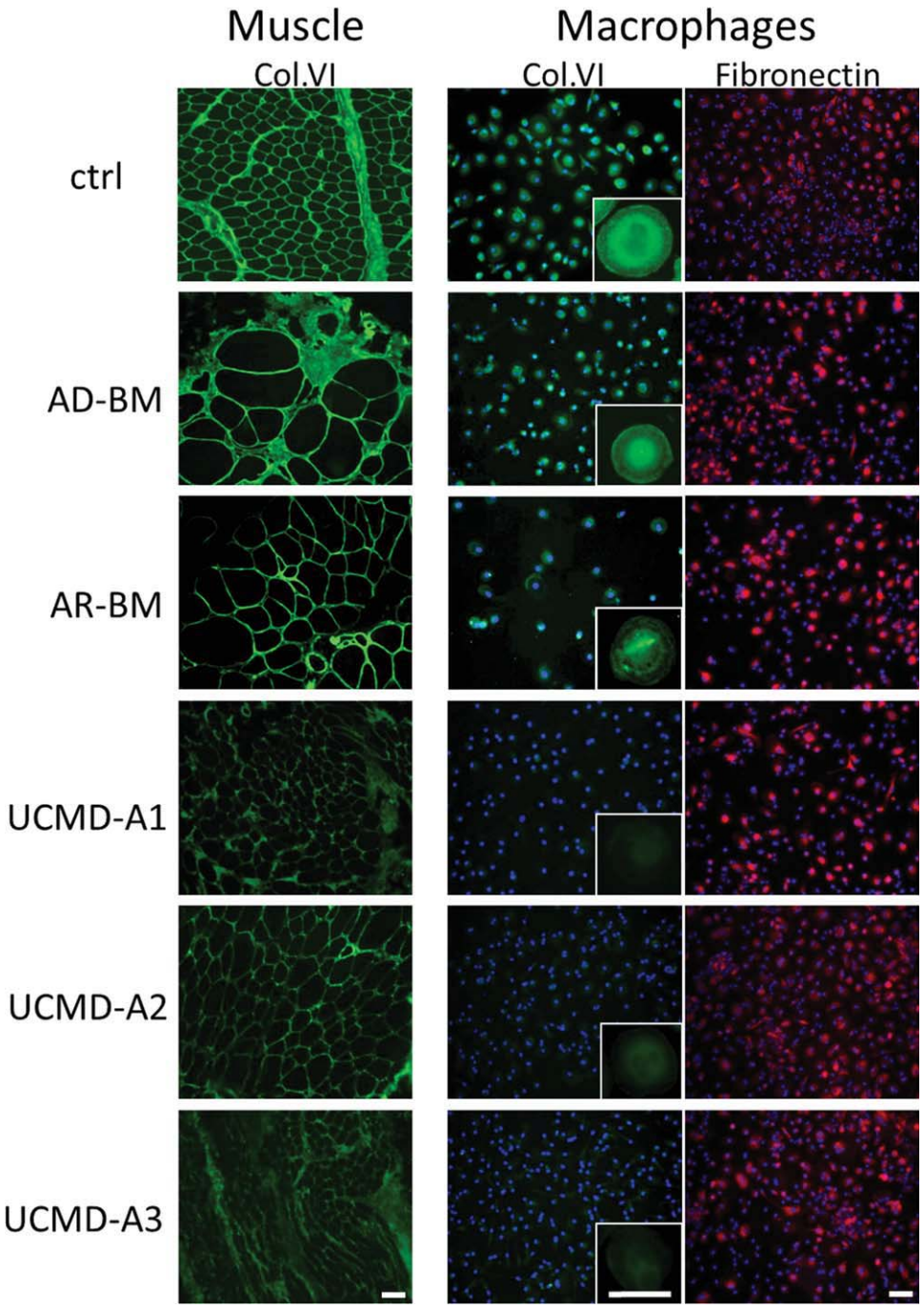

FIGURE 1. Immunofluorescence analysis of collagen VI (green—left and middle lanes) and fibronectin (red—right lane) on muscle sections (left lane) and MDMs (middle and right lanes) from a healthy subject (Ctrl) and patients AD-BM, AR-BM, UCMD-A1, UCMD-A2, and UCMD-A3. On muscle sections from the healthy subject, collagen VI is expressed in the endomysium around muscle fibers. In AD-BM muscle, collagen VI shows a pattern very similar to that of a normal subject, whereas for AR-BM it appears reduced and unevenly distributed. All UCMD patients show a marked reduction around muscle fibers and in the perimysium. Using the same antibody, collagen VI is expressed at the cell membrane of control MDMs. Matching the muscle pattern, collagen VI appears to be normally represented in patient AD-BM, moderately reduced in patient AR-BM, and greatly reduced in MDMs cultures from patients UCMD-A1, -A2, and -A3. Fibronectin, used as an internal marker, yielded a pattern comparable to that of normal MDMs in all MDM patient cultures. Insets show representative higher magnifications. Bar = 40 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

splicing change (+5), which caused intron retention in part of the transcript and would have been expected to display a different recognition pattern in different cells.

The nonsense-mediated decay of mutated *COL6A2* mRNA observed in fibroblasts[6] was also seen in macrophages. Furthermore, in the recessive BM patient, the transcriptional impairment of

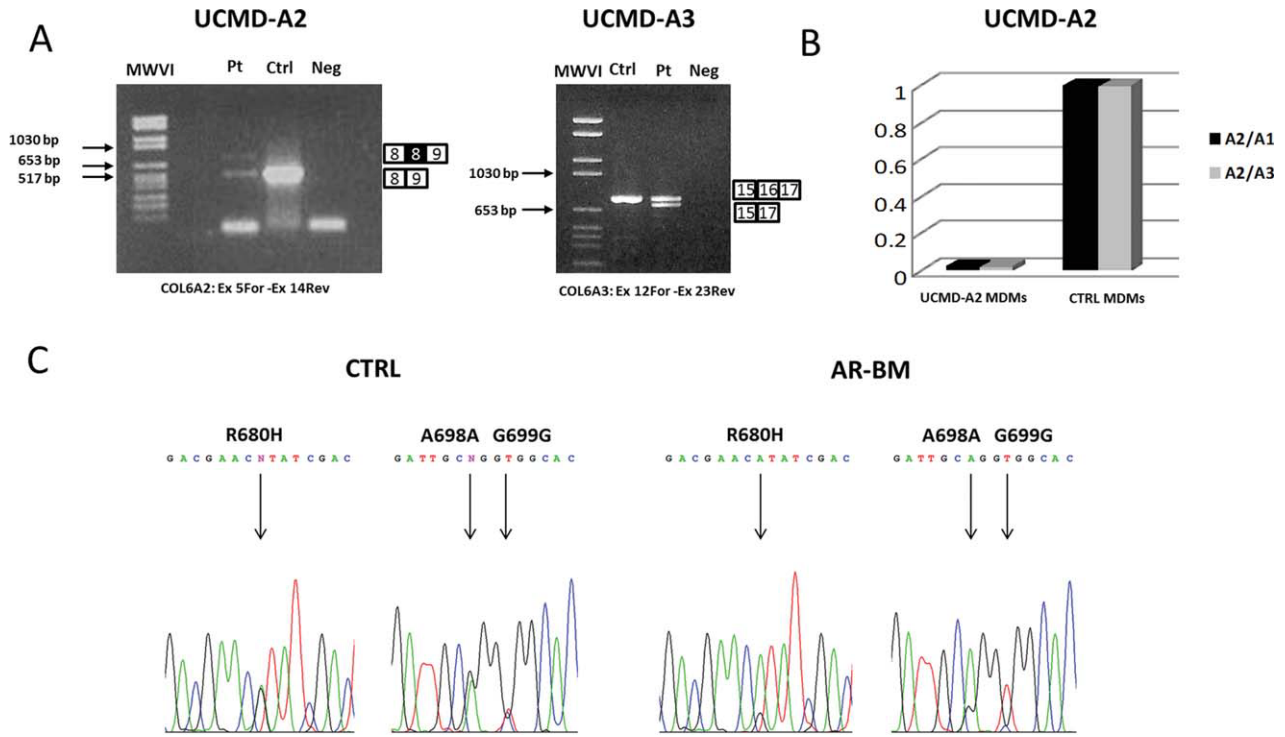

FIGURE 2. RNA behavior of selected UCMD and BM mutations in MDMs. **(A)** RT-PCR analysis in MDMs from patients UCMD-A2 and UCMD-A3. Left*:* RT-PCR with primers within *COL6A2* exons 5 and 14 shows two fragments, with correct exon 8–9 junction and intron 8 retention. Right: RT-PCR with primers within *COL6A3* exons 12 and 23 shows two fragments, with correct exon 15–16–17 junctions) and with exon 16 skipping. **(B)** Real-time PCR quantification of the ratio between steady-state levels of mutated COL6A2 messenger and wild-type COL6A1 or COL6A3 transcripts in MDMs from patient UCMD-A2. The *COL6A2/COL6A1* and *COL6A2/COL6A3* ratios in control MDMs are denoted 1. In UCMD-A2, *COL6A2* transcript ratio was reduced to 0.017 and 0.019 for *COL6A1* and *COL6A3*, respectively. **(C)** Sequence chromatograms of cDNA from control (CTRL) and AR-BM MDMs. Both the control and the AR-BM patient were heterozygous at the genomic level for three polymorphisms within *COL6A2* exon 28 (c.2039 G>A–R680H; c.2094 G>A–A698A; c.2097 C>T–G699G). In cDNA, the two alleles of each polymorphism are uniformly represented in the control (left), whereas an imbalanced transcription can be seen in the AR-BM patient (right). This behavior parallels that previously observed in fibroblasts.[11] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

a *COL6A2* allele carrying an intronic regulatory mutation,[11] previously documented in fibroblasts, was mirrored in MDMs. This observation suggests that regulatory mechanisms of *COL6* gene expression are common to the two cell systems.

Muscle biopsy revealed partial deficiency of collagen VI in all UCMD patients and in the recessive BM case. As MDMs also exhibited a reduced amount of protein at the plasma membrane, they may be considered suitable tools for evaluating the pathogenic effect of *COL6A* gene mutations on protein expression. Moreover, normal expression of collagen VI was detectable in MDMs from the dominant BM patient, as seen in muscle, as expected due to the effect of BM dominant mutations on collagen VI assembly and secretion.[3]

Supporting the predictive value of MDMs as a supplementary or surrogate diagnostic tool in neuromuscular diseases, these cells have already been verified as suitable for a blood-based diagnostic assay in muscle phenotypes caused by dysferlin deficiency.[12] Furthermore, monocytes have proven to be a good source of dysferlin mRNA for mutation analysis,[13] and of protein for immunocytochemistry (ICC) and Western blotting.[14]

Moreover, like other cells, blood monocytes can be cryopreserved after isolation[15] and then cultivated effectively, even after several weeks.

In conclusion, our data reveal that MDMs may represent a reliable, minimally invasive tool for replacing or supplementing the current more invasive diagnostic procedures in collagen VI–related myopathies. Our findings also suggest a use for these easily accessible cells in the hunt for valid neuromuscular disease biomarkers that can demonstrate therapeutic efficacy and efficiency.

The work has been funded by the BIO-NMD Grant (EC, 7th FP, proposal #241665; "http://www.bio-nmd.eu" or "http://www.bio-nmd.eu" www.bio-nmd.eu, to AF as coordinator), by Telethon (Grants GGP08107 to A.F. and N.M.M., GUP08006 to L.M.), and the PRIN Project (Grant 2008PB5S89_003 to A.F.).

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