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***TGFBR2* but not *SPP1* genotype modulates osteopontin expression in Duchenne muscular dystrophy muscle**

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Abstract

A polymorphism (rs28357094) in the promoter region of the *SPP1* gene coding for osteopontin (OPN) is a strong determinant of disease severity in Duchenne muscular dystrophy (DMD). The rare G allele of rs28357094 alters gene promoter function and reduces mRNA expression in transfected HeLa cells. To dissect the molecular mechanisms of increased disease severity associated with the G allele, we characterized *SPP1* mRNA and protein in DMD muscle biopsies of patients with defined rs28357094 genotype. We did not find significant differences in osteopontin mRNA or protein expression between patients carrying the T (ancestral allele) or TG/GG genotypes at rs28357094. The G allele was significantly associated with reduced CD4⁺ and CD68⁺ cells on patient muscle biopsy. We also quantified transforming growth factor- β (*TGFB*) and *TGFB* receptor-2 (*TGFBR2*) mRNA in DMD muscle biopsies, given the ability of *TGFB* and *TGFBR2* to activate *SPP1* promoter region and their role in DMD pathogenesis. The amount of *TGFB* and *TGFBR2* mRNA did not predict the amount of *SPP1* mRNA or protein, while a polymorphism in the *TGFBR2* gene (rs4522809) was found to be a strong predictor of *SPP1* mRNA level. Our findings suggest that OPN mediates inflammatory changes in DMD and that *TGFB* signalling has a role in the complex regulation of osteopontin expression.

Keywords

Duchenne muscular dystrophy; osteopontin; *SPP1*; *TGFB*; *TGFBR2*

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Author contributions

LP, LB and GS conceived and carried out experiments; BFG and MF carried out experiments; LB, GS and AB evaluated patients; ME carried out statistical analyses; EPH, CA and EP conceived experiments and analysed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

No conflicts of interest were declared.

Introduction

Duchenne muscular dystrophy (DMD) is a lethal, progressive muscle disease caused by absence of dystrophin in skeletal muscle [1]. Dystrophin deficiency leads to the loss of the dystrophin-associated glycoproteins [2] that eventually leads to cell death with secondary inflammation and persistent production of a variety of different cytokines [3–7]. There is interpatient variation in disease presentation and progression [8].

Osteopontin (OPN or secreted phosphoprotein 1, encoded by the *SPP1* gene) is a 35–60 kDa glycoprotein expressed by epithelial cells, osteoblasts and osteoclasts, immune cells, skin, endothelial cells and muscle cells, with many physiological functions. It is an intracellular protein but may also behave as an inflammatory cytokine and is implicated in diverse biological processes, including tumour progression, neoangiogenesis, immune response and tissue remodelling [9–11]. The relationship between OPN and skeletal muscle is complex. Myoblasts are an important source of OPN in damaged muscle. OPN released by myoblasts may contribute to both the myogenic and inflammatory processes during the early stage of muscle regeneration promoting muscle repair [12]. In contrast, mice with DMD (*mdx*) and OPN genetic ablation (double mutant mice, DMM) showed that OPN may be an immunomodulator and profibrotic cytokine in dystrophic muscle [13]. DMM mice show a significant reduction in intramuscular neutrophils and NKT-like cells and increased Tregs. These changes in the inflammatory cell subsets result in a net decrease of transforming growth factor- β (TGF β) that correlates with decrease fibrosis in *mdx* diaphragm and cardiac muscle and with increase muscle strength [13]. It is important to note that genetic ablation of OPN removes the protein from all stages of development and homeostasis.

We recently showed that genotype at polymorphism rs28357094 in the *SPP1* gene promoter significantly affects disease progression in DMD. The mutant G allele was associated with greater weakness and younger age at loss of ambulation in two independent cohorts of DMD patients [8]. The less common G allele at the rs28357094 locus has been shown to disrupt gene promoter function in immortalized non-muscle cells *in vitro* (HeLa), modifying the binding affinity for the SP1/SP3 transcription factor and leading to reduced OPN mRNA production [14]. Assuming that the G allele similarly reduces OPN expression in dystrophic muscle, the data from the transgenic mice and DMD patients seem contradictory. In double transgenic mice, less OPN improved the dystrophic phenotype of dystrophin-deficient mice, yet in our two DMD patient cohorts the G allele worsened the phenotype. The transcriptional regulation of the OPN gene appears complex [15,16] and the role of OPN in normal and dystrophic muscle and functional consequences of the rs28357094 polymorphism require further study.

In order to increase our understanding of the role of OPN in DMD muscle, we investigated the role of *SPP1* genotype at rs28357094 in modifying inflammatory cell subtypes, OPN transcript and protein level in DMD muscle biopsies, and correlated these findings with muscle strength. Moreover, given the ability of TGFB and TGFBR2 to activate the *SPP1* promoter region, we investigated whether their amount or genotype correlate with OPN expression.

Materials and methods

Patients

Patients were selected from a cohort of DMD patients followed at the Neuromuscular Center of the University of Padova. All patients had absence of dystrophin in skeletal muscle and/or out-of-frame or nonsense *DMD* mutations. DNA samples were obtained after informed consent according to the requirements of our Institutional Ethical Committee and the Helsinki Declaration of 1975, as revised in 1983. As controls, nine age-matched normal muscle biopsies were used. Patients' muscle biopsies were obtained at the time of diagnosis, prior to any steroid treatment. After diagnosis, patients were treated with steroids with a continuous regimen (0.75 mg/kg prednisolone–0.9 mg/kg deflazacort daily, or equivalent). Six patients (16%) received no steroids. There was no significant difference in the percentage of steroid-treated and -untreated patients with respect to rs28357094 genotype (χ^2 test, $p = 0.69$). For all patients, genotype at rs28357094 of the *SPPI* gene was known [8]. There were 66 TT (57.9%), 38 GT (33.3%) and 10 GG (8.8%).

RNA extraction and cDNA synthesis

Total RNA was isolated from patients' skeletal muscle biopsies following the TRIzol (Invitrogen/Life Technology) standard protocol. Patient and control biopsies were taken from quadriceps femoris muscles. RNA was quantified by UV adsorption in a NanoDrop ND-1000 spectrophotometer (Thermo-Fisher Scientific) and analysed for quality by non-denaturing agarose gel electrophoresis. Aliquots of RNA were reverse-transcribed using oligo dT primer (0.5 $\mu\text{g}/\mu\text{l}$) and SuperScriptTM III Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions.

Real-time PCR

SPPI, *TGFB*, *TGFBR2*, *SP1* and *SP3* gene expression were analysed by quantitative real-time PCR, using SYBR[®] Green (Finnzymes, Finland) and an ABI PRISMTM 7500 Real-time PCR system, as previously described. Briefly, UV absorbance was used to normalize the amount of input RNA in the cDNA reactions. cDNA (25 ng) was amplified with SYBR[®] Green PCR Master Mix (Applied Biosystems, CA, USA) in triplicate, using the following thermal cycling conditions: 94 °C for 5 min, followed by 40 cycles of amplification at 94 °C for 10 s, followed by 60 °C for 20 s to allow for denaturing and annealing–extension. Gene expression was determined from measurements of the increase in fluorescence, corresponding with amplification and incorporation of SYBR Green I dye during the PCR reaction. The amplification efficiency of assay was estimated from standard curve. The cycle number at which the amount of amplified target reaches a manually fixed threshold, set within the linear range of all reactions, represents the cycle threshold (C_T). The mean value of the replicates for each sample was calculated and expressed as C_T . The amount of gene expression was calculated as the difference ($-C_T$) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the reference genes (internal control).

TBP (TATA box binding protein) gene and *GUSB* (glucuronidase- β) gene were used as internal controls. Relative expression (R) was then calculated as the difference ($-C_T$)

between the C_T values of the test samples and of the mean of the control samples (normal muscle) for each target gene. The relative quantitation value was expressed and shown as 2^{-CT} [17]. Only to compare genes expression in DMD muscles versus control muscles, the target gene expression in DMD and controls was expressed as 2^{-CT} (L).

OPN western blot

OPN protein was assessed by western blot and densitometry analysis. Fifteen to twenty 20 μ m sections of muscle samples from DMD and normal muscles were solubilized in 100 μ l lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol). The phosphatase inhibitor cocktail 1 and 2, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) were added. Protein concentration measurements were done using BCA Protein Assay Reagent (Thermo-Scientific, Portsmouth, NH, USA); 40 μ g protein was loaded into each well of 4–12% gradient acrylamide gel (NuPAGE[®] Novex[®] Bis-Tris Mini Gels; Invitrogen, Carlsbad, CA, USA) and processed as previously described, using the following antibodies: goat polyclonal anti-OPN antibody (O3389, Sigma-Aldrich; 1 : 1000) and a mouse monoclonal anti-actin antibody (Millipore, Billerica, MA, USA; 1 : 4000). Secondary antibody HRP-conjugated bovine anti-goat IgG (Sigma-Aldrich) and anti-mouse IgG (GE Healthcare, Piscataway, NJ, USA) were used at a dilution of 1 : 4000. The amount of OPN for each patient was quantitated from X-ray films, using GelPro Analyser software (Media Cybernetics, MD, USA). We defined the OPN signal as the 55 kDa isoform. The area corresponding to OPN was scanned and areas were converted into OD units after subtraction of background. To control for muscle protein content in each lane the actin B signal (47 kDa) was also scanned and the OPN content was adjusted to actin B protein amount. Each patient sample was subjected to electrophoresis adjacent to normal control lanes and quantitation was done relative to control. As the positive control in each gel, we used 15 ng human recombinant OPN protein (Sigma-Aldrich).

Muscle pathology

Muscle pathology was investigated assessing degenerating and regenerating fibres, dystrophin-positive revertant fibres, fibro-fatty infiltration and inflammatory infiltrates. The same optical fields were identified in serial sections. The total number of fibres (at least 1000) were counted on each section and used to calculate the percentage of dystrophin, OPN and fetal myosin-positive fibres. Degenerating fibres were counted on a haematoxylin and eosin (H&E)-stained section and defined as hyaline fibres, hypercontracted fibres or fibres invaded by inflammatory cells.

Immunohistochemistry

Eight serial sections, 8 μ m thick, were obtained from each muscle biopsy. Cryostat sections were mounted onto Superfrost Plus slides (Thermo-Scientific), hydrated in phosphate-buffered saline (PBS) and incubated with appropriate primary antibodies diluted in PBS. Seven different antibodies were used, as follows: CD4⁺ helper/inducer T cells (Biologend, San Diego, CA, USA; 1 : 50); CD8⁺ cytotoxic/suppressor T cells (Biologend; 1 : 50); CD68⁺ macrophages (Dako, Glostrup, Denmark; 1 : 100); MHC class I molecules (W6/32; Dako; 1 : 100); fetal myosin heavy chain (Novocastra, Newcastle upon Tyne, UK; 1 : 100);

dystrophin (Novocastra; 1 : 100); and OPN (Sigma-Aldrich; 1 : 100). After triple washing in PBS, specific labelling was developed by immunofluorescence, using anti-mouse or anti-goat cyanine-3 conjugated immunoglobulin (Caltag, Burlingame, CA, USA; 1 : 100). For OPN immunostaining only, the tissue was pre-incubated for 30 min with blocking solution [0.1% Triton, 1% BSA, 10% fetal calf serum (FCS) in PBS]. Sections were mounted with anti-fading medium and examined with a video-confocal microscope (ViCo, Nikon Instruments, Melville, NY, USA).

The intensity of reaction for CD4⁺, CD8⁺, CD68 and MHC class I molecules was examined by visual inspection by three independent observers and graded as follows: normal (–), slight increase (+/–), mild increase (+), moderate increase (++), severe increase (+++). Similarly, fibrosis was scored on a trichromestained section.

Single nucleotide polymorphism (SNP) genotyping at rs4522809 of the *TGFBR2* gene

rs4522809 was genotyped in 109 DMD patients by restriction fragment length polymorphism analysis, using *AvrII* restriction digestion enzyme (New England Biolabs, Beverly, MA, USA).

Statistical analysis

Normally distributed variables were analysed using the *t*-test for independent groups. Ordinal variables were analysed using the Mann–Whitney U test, while for categorical variables the χ^2 test was performed. The linear correlation between two variables was tested using the Spearman's rho. Age at loss of ambulation, or age at last follow-up for ambulant patients, were analysed using the Kaplan–Meier method; 95% CIs were calculated using the associated estimated SEs. In monovariate analysis, the log-rank test was used to test significance. Multivariate analysis was performed using the Cox proportional hazards model. Significance level was set at $p < 0.05$.

Results

SPP1 rs28357094 genotype does not predict OPN mRNA and protein in DMD muscle

***SPP1* mRNA real-time PCR**—Thirty-nine DMD patients were selected, based on their genotype and the availability of frozen muscle biopsy. Of these patients, 24 patients carried the TT, 13 the GT and two the GG haplotype. As controls, nine age-matched normal muscle biopsies were used. Mean age in control muscles was 4.7 ± 2.7 (range 1–9) years and 4.9 ± 2.0 (range 1–9) years in DMD. Quantitation of *SPP1* mRNA showed a 13-fold increase in OPN expression in DMD skeletal muscles ($n = 39$) compared to controls ($n = 9$) (8.18 ± 12.03 vs 0.64 ± 1.01 ; $p = 0.03$) (Figure 1A). The amount of *SPP1* mRNA did not correlate with patients' age at biopsy ($R = 0.19$, $p = 0.23$); however, most biopsies were taken at the time of diagnosis over a relatively narrow age range (3–9 years) and OPN mRNA expression was quite variable.

To verify whether the G allele at rs28357094 of the *SPP1* gene is associated with less OPN transcription [14], the 39 DMD patients were stratified based on their genotype at rs28357094. Data were analysed according to a dominant model for genotypes because of

the low sample number for the homozygous mutated genotype. DMD muscles carrying the G allele ($n = 15$) expressed less OPN (3.65 ± 1.97) compared to the muscles carrying the T allele ($n = 24$) (4.09 ± 2.14); however, this difference was not significant using the Mann–Whitney U-test ($p = 0.52$) (Figure 1B).

OPN Western blot—In order to correlate OPN RNA and protein expression, OPN immunoblot analysis was conducted in 28/39 DMD patients (Figure 1E). Protein levels were significantly higher in DMD compared to controls, consistent with mRNA level ($p = 0.0004$) (Figure 1C). The amount of OPN was in the range 3–32-fold of controls ($n = 8$). Similar to our findings regarding rs28357094 genotype effect on OPN mRNA, we did not see any effect of genotype on OPN protein expression. The G-allele muscle biopsies showed 1.09 ± 0.67 OPN absolute value compared to the T-allele muscles 0.98 ± 0.85 and the difference was not significant (Figure 1D).

OPN mRNA expression and muscle strength—To test whether the amount of *OPN* mRNA correlates with muscle strength in DMD, we studied the composite MRC score for upper (deltoid and triceps) and lower (iliopsoas and quadriceps) limbs and age at loss of ambulation, as previously described [8]. Spearman's rho test showed no linear correlations between OPN and both upper (Spearman's rho -0.09 , $p = 0.58$) and lower limb MRC (Spearman's rho -0.02 , $p = 0.90$). Moreover, when Spearman's rho test was restricted to steroid-untreated patients, no correlations were found. However, when age at loss of ambulation was considered, the Cox linear model, including OPN expression and steroid treatment as independent variables, showed a significant association ($p = 0.03$) only with OPN expression, suggesting that the DMD patients with less OPN mRNA lost ambulation at older age. However, the relative risk was low (1.2, CI 95% 1.04–1.4). Given the multiple statistical tests carried out in our phenotype/genotype comparisons, this positive association should be considered cautiously.

OPN genotype modulates inflammatory cell content in DMD muscle

Muscle pathology and immunohistochemistry—It has recently been shown that OPN in the *mdx* mice is an immunomodulator and profibrotic cytokine [13]. To verify whether fibrosis, degeneration, regeneration and inflammation are modulated by OPN and/or *SPP1* genotype in DMD muscle, 10 muscle biopsies of DMD patients carrying the G allele and 10 muscle biopsies of patients carrying the T allele were studied (Tables 1, 2). Spearman's rho did not show any significant correlation between the amount of *SPP1* mRNA and degeneration, regeneration and fibrosis, even after stratification of patients according to genotype at rs28357094 (Table 1).

Fetal myosin-positive muscle fibres, representing regenerating muscle fibres, were clustered in small groups of 10–15 elements in patients' muscle biopsies and were in the ranges 12–42% in DMD patients with the TT allele and 11–49% in the TG/GG allele patients. Degenerating fibres, including hyaline, hypercontracted and necrotic fibres undergoing phagocytosis, were in the range 11–28% in the TT muscles and 10–22% in the TG/GG muscles. Rare revertant fibres were observed in both genotypes (Table 1). Fibrosis was a prominent pathological feature in DMD muscles, ranging from mild to severe in both

genotypes. There were no significant differences in degeneration, regeneration, fibrosis or number of dystrophin-positive revertant fibres in the two genotype groups of patients (Table 2).

A mild or moderate increase in HLA class I was observed in all biopsies in scattered mononuclear cells, but also in regenerating fibres and on the surface of non-necrotic and non-regenerating fibres. Mononuclear cells, either scattered or organized in small aggregates, were localized in the perimysium and endomysium and in the perivascular space, sometimes surrounding and invading single muscle fibres. Macrophages formed the most abundant mononuclear cell subset and were seen either surrounding or invading muscle fibres or localized in the interstitium. T cells were both of the CD4⁺ and CD8⁺ types (Table 2).

Spearman's rho did not show any significant correlation between *SPP1* mRNA and amount of CD4⁺, CD8⁺, CD68⁺ cells and HLA class I molecules expressed at the myofibre plasma membrane or in the interstitium. However, HLA class I molecules expressed in the cytoplasm of muscle fibres showed significant correlation with *SPP1* mRNA (Spearman's rho = 0.88; $p = 0.00068$). This result may be due to the multiple statistical tests carried out.

No differences were observed between TT and TG/GG genotype patients for HLA class I molecules or CD8⁺ cells, while both macrophages (CD68⁺) and CD4⁺ T cells were significantly more abundant in TT muscles ($p = 0.04$ and 0.05 , respectively) (Table 2).

***TGFBR2* genotype modulates OPN expression in DMD muscle**

***TGFBR2* (rs4522809) genotype and *SPP1* mRNA expression**—OPN ablation in mdx mice has been shown to decrease TGFβ [13] and TGFβ has been demonstrated to activate the promoter region of the *SPP1* gene [18]. A polymorphism (rs4522809) in the TGFβ receptor-2 gene (*TGFBR2*) was shown to be associated with OPN serum level in healthy men [19]. We investigated the role of *TGFB* and *TGFBR2* rs4522809 in OPN expression in DMD, since genotype at *SPP1* SNP rs28357094 failed to predict OPN expression at both mRNA and protein level.

TGFB and *TGFBR2* mRNA were quantified in 13 DMD patients (age range 1.9–17 years). Spearman's rho test showed no linear correlations between *SPP1* mRNA and both *TGFB* (Spearman's rho 0.19, $p = 0.53$) and *TGFBR2* (Spearman's rho 0.39, $p = 0.18$). Gene frequencies at *TGFBR2* rs4522809 in our DMD cohort were: 12.3% for the ancestral CC, 34.2% for TC and 53.5% for the TT genotype. Of the 39 patients studied, those carrying the CC genotype showed the highest level of OPN expression, and patients with the mutant TT genotype the lowest ($p = 0.000001$) (Figure 2). To verify whether genotype at *SPP1* rs28357094 and at *TGFBR2* rs4522809 have an additive effect on OPN expression, two-way analysis of variance was done, which failed to show significant interaction between *TGFBR2* rs4522809 and *SPP1* rs28357094 vs level of *SPP1* mRNA.

***SP1/SP3* expression correlates with *SPP1* expression level in patients carrying the T genotype at rs28357094**

Since genotype at rs28357094 of the *SPP1* gene modifies the binding affinity for the *SP1* /*SP3* transcription factors, we checked whether *SP1* /*SP3* level differs in the T and G genotype at rs28357094. We quantitated *SP1* and *SP3* mRNA relative to control muscles in five DMD patients carrying the T (*SP1* mRNA, 0.15 ± 0.10 ; *SP3* mRNA, 3.63 ± 5.09) and in five DMD patients carrying the G (*SP1* mRNA, 1.65 ± 2.55 ; *SP3* : mRNA, 8.39 ± 12.97) genotype at rs28357094 and found no correlations with genotype using the Mann–Whitney U-test. Moreover, no significant difference was detected in *SP1/SP3* expression level between DMD and normal muscles. Spearman's rho, however, showed a significant correlation between *SPP1* mRNA and both *SP1* and *SP3* mRNA, only in the DMD carrying the T genotype (Spearman's rho, $R = 0.90$; $p = 0.037$ for both *SP1* and *SP3*).

Discussion

We have recently shown that genotype at *SPP1* rs28357094 is a determinant of disease severity in DMD [8], suggesting that OPN might have a crucial role in skeletal muscle function. The identification of OPN as the first genetic modifier in DMD raised questions regarding the role of OPN in muscle and the mechanisms by which the rs28357094 polymorphism modulates DMD expression.

Here we have shown that OPN protein is greatly up-regulated in DMD muscle and that this up-regulation is likely driven via transcriptional mechanisms, since both OPN transcripts and protein are similarly elevated. *In vitro* studies have suggested that both proliferating and differentiating myoblasts synthesize OPN and that the increased expression occurs in response to the muscle damage resulting from dystrophin deficiency [12]. However, we failed to show a correlation between OPN expression and the age of the patients at biopsy, degeneration, regeneration and fibrosis. Since in skeletal muscle the sources of OPN production are the muscle cells themselves [9,12,13,19] and the infiltrating inflammatory cells [13], we characterized the muscle infiltrates in DMD patients' biopsies but were unable to find any correlation between OPN mRNA or protein and inflammation or T cell subsets.

We then tested for associations between the genotype at rs28357094 in the *SPP1* promoter region, OPN expression and muscle function. The more obvious *a priori* interpretation, according to the DMM mice model showing a better muscle performance in the mice lacking OPN [13], would be that 'less' OPN is 'good' for muscle. Under this perspective the *SPP1* rs28357094 polymorphism and its association with muscle strength are ideal to verify this assumption, since the G > T variant at the -66 position in the 5' end of the *SPP1* gene has a major effect on promoter activity, the G allele being linked to the less efficient transcription [14]. However, we were unable to validate the previously reported HeLa *in vitro* data: the polymorphism did not predict OPN expression at either the mRNA or the protein level in DMD muscle, although a slight linear correlation between OPN and muscle function was detected, independently of steroid treatment.

Moreover, to further dissect the role of rs28357094 polymorphism on *SPP1* transcription, we quantitated *SP1* /*SP3*, the transcription factors whose binding to *SPP1* promoter region is

modified by rs28357094, in DMD boys stratified according to their genotype. We failed to show any possible compensatory changes in these transcription factors according to rs28357094 genotype. This was not surprising, considering that regulation of the transcriptional activity of *SP1* and *SP3* occurs largely at the post-translational level [21]. However, a significant correlation between *SP1* /*SP3* mRNA and *SPP1* mRNA was detected only in the DMD boys carrying the T genotype, underlining the relevance of *SP1* /*SP3* in the transcriptional regulation of *SPP1*. The lack of correlation in DMD boys carrying the G genotype may be due to the ubiquitous nature of *SP1* /*SP3*, whose amount can hardly be affected by a single polymorphism in a single gene out of multiple target genes of these transcription factors, and again underlying the multiple regulations of *SPP1* gene transcription.

Since OPN is a predominantly a secreted protein, an alternative attractive hypothesis is that the rs28357094 polymorphism modulates the amount of plasma OPN more than the intracellular level. Unfortunately, we did not collect a plasma sample at the time of biopsy in our cohort of patients to verify this hypothesis. However, two recent publications point to a more complex regulation of secreted OPN. Biros *et al* [19] showed that an intronic polymorphism in the *TGFBR2* gene, and Ermakov *et al* [22] showed that a polymorphism in the promoter region of the integrin-binding sialoprotein gene (*IBSP*) is positively associated with OPN level. Further studies are needed to explore the molecular and physiological mechanisms underlying OPN plasma level. To address this point would be particularly relevant, given the potential usefulness of plasma OPN as a marker for longitudinal correlation with muscle pathology progression.

In the OPN/dystrophin double knockout mouse, the ablation of OPN resulted in a marked reduction in muscle-infiltrating neutrophils and NKT-like cells, and increased Treg [13], pointing to the changes in the muscle inflammatory milieu as a downstream effect of OPN itself. We showed an increase in CD4⁺ and CD68 cells in muscle from patients carrying the T allele compared to those carrying the G allele, suggesting that OPN has a role in the modulation of cellular immune profiles in human muscle, although these results should be taken with caution. First, we used a semi-quantitative histological method to assess immune cell subsets; the limited availability of patient muscle biopsy precluded quantitative flow cytometry methods. Second, we used a relatively limited repertoire of surface cell markers for infiltrating mononuclear cells compared to the previously reported mouse studies. Overall, our human data remain somewhat inconsistent with mouse data, but there are significant (physiological, pathological and genetic) differences between the mouse models and our patients that make direct comparisons problematic. Finally, to further characterize the regulation of OPN in skeletal muscle, we studied TGFβ based on the observation of TGFβ reduction in the DMM mice and the hypothesized role of OPN as a profibrotic cytokine in dystrophic muscle [13]. It is well known that TGFβ has a relevant role in the regulation of the extracellular matrix composition, is a powerful modulator of inflammation and myogenesis [23–27] and plays a major role in dystrophin-deficient muscles, as demonstrated by elevation of *TGFB* mRNA and protein in DMD [28–30]. The OPN promoter is activated by *TGFB* [18] but we were not able to find a linear correlation between *TGFB* or *TGFBR2* mRNA and OPN expression, although we showed that a

polymorphism in the *TGFBR2* gene (rs4522809) is a strong determinant of OPN expression in skeletal muscle, supporting previous data implicating TGF β in OPN production. The exact role of the *TGFBR2* polymorphism in modulating OPN expression is still elusive but it does not seem related to either *TGFB* or *TGFBR2* transcriptional regulation.

The results of this study point toward a multifaceted regulation of OPN and underlie its relevance in DMD. It is well known that OPN may be regulated at different levels: activators or repressors of OPN promoter, alternative splicing mechanisms, post-translational modifications such as phosphorylation, glycosylation and proteolytic cleavage [31], and the net OPN production may be the results of all these mechanisms. In this scenario, *TGFBR2* rs4522809 and *SPP1* rs28357094 polymorphisms play a part which is not fully understood. The identification of these molecular mechanisms is crucial because it may shed light on still unrecognized candidate targets for therapeutic interventions in DMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SPP1 mRNA and OPN are over-expressed in DMD but are not predicted by *SPP1* genotype. Both real-time PCR and immunoblots confirm the increased expression level for OPN in DMD compared to controls (A, C). Data for *SPP1* mRNA are presented as expression level *L* (A) or relative expression *R* (B); data for OPN are expressed as average. Stratification of DMD according to genotype at rs28357094 does not show any significant difference in osteopontin mRNA or protein between DMD boys carrying the T or G alleles (B, D). Error bars represent SE. (E) Example of an immunoblot analysis of osteopontin using the polyclonal anti-OPN antibody (O3389) shows a variable increased in osteopontin in DMD muscles compared to controls (ctr). ACTB corresponds to actin B used to normalized myofibre protein content. In the first lane human recombinant OPN was loaded as the positive control.

Figure 2.

TGFB2 genotype predicts *SPP1* mRNA expression in DMD. Shown is the association of *TGFB2* genotype with muscle OPN in DMD skeletal muscle. Data are presented as relative expression (R); error bars represent SE.

Table 1

Muscle pathology data in DMD patients according to genotype at rs28357094

Patient no.	rs28357094 genotype	<i>SPPI</i> mRNA *	Age at biopsy (years)	Revertant fibres	Regenerating fibres (%)	Degenerating fibres (%)	Fibrosis
6993	TG	0.723	4	3	22	15	+
4445	TG	4.870	2.8	12	15	10	+
6014	TG	6.711	5	0	26	19	+
6390	TG	0.895	4	3	19	17	++
6718	GG	2.499	5	0	11	11	++
2620	TG	2.775	1.9	0	29	22	+
2064	TG	1.019	1.9	2	37	21	++
2432	TG	3.865	7	7	35	21	+++
2663	TG	5.266	4.1	5	34	22	+
2512	TG	3.238	6.7	4	49	17	+++
Mean ± SD		3.19 ± 2.01	4.7 ± 1.6	3.6 ± 3.7	27.67 ± 0.11	17.66 ± 0.04	1.7 ± 0.82
3639	TT	2.087	5	0	18	15	+++
3852	TT	3.782	6	8	22	18	+++
3320	TT	6.068	5.3	0	12	13	+
3258	TT	3.012	5.9	6	14	11	++
3709	TT	3.460	9	1	18	16	+
2486	TT	4.687	6.8	2	21	14	++
2609	TT	2.912	3.2	5	36	16	++
2676	TT	5.975	8.3	0	22	15	++
2467	TT	4.341	8.9	7	36	20	++
2568	TT	5.419	8	41	42	28	++
Mean ± SD		4.17 ± 1.35	6.9 ± 2.0	7.0 ± 12.3	24.02 ± 0.10	16.68 ± 0.04	2.00 ± 0.66
<i>p</i> =		n.s.	0.02	n.s.	n.s.	n.s.	n.s.

n.s., not significant.

* Amount was normalized to control muscle.

Table 2

Immunohistochemical data in DMD patients according to genotype at rs28357094

Patient no.	rs28357094 genotype	CD4 ⁺ cells	CD8 ⁺ cells	CD68 ⁺ cells	OPN	HLA class I		
						Membrane	Cytoplasm	Interstitial [*]
6993	TG	+	+	+	+/-	-	+++	+/-
4445	TG	-	+	++	++	+	++	++
6014	TG	-	-	+/-	-	+/-	-	+/-
6390	TG	-	-	+	+/-	+/-	+++	+
6718	GG	-	+/-	++	+/-	-	++	+
2620	TG	+/-	+++	++	+/-	+	++	++
2064	TG	++	++	++	n.d.	++	++	++
2432	TG	++	-	++	+/-	+/-	++	++
2663	TG	+	+	+	++	+	+	+
2512	TG	++	+	+/-	+	++	++	++
Mean ± SD		0.85 ± 0.9	0.95 ± 1.0	1.5 ± 0.7	0.95 ± 0.8	0.85 ± 0.7	1.9 ± 0.9	1.5 ± 0.7
3639	TT	+++	++	+++	+	+/-	++	++
3852	TT	+++	++	+++	+	+	++	++
3320	TT	++	++	+++	+	+/-	+++	+
3258	TT	++	+	+++	++	n.d.	n.d.	n.d.
3709	TT	+/-	-	+++	+	n.d.	n.d.	n.d.
2486	TT	+/-	++	+	+	+	++	++
2609	TT	++	+	++	-	++	+/-	++
2676	TT	++	+	++	+	+	++	+++
2467	TT	++	+/-	+	+/-	+	+	+
2568	TT	+/-	++	++	++	+/-	++	++
Mean ± SD		1.75 ± 1.0	1.35 ± 0.7	2.3 ± 0.8	1.05 ± 0.6	0.93 ± 0.5	1.81 ± 0.7	1.87 ± 0.6
p =		0.05	n.s.	0.04	n.s.	n.s.	n.s.	n.s.

n.d., not determined; n.s., not significant.

^{*} Including connective tissue, vasal and mononuclear infiltrating cells.