Increased osteopontin in muscle and serum from patients with idiopathic inflammatory myopathies

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Abstract

Objectives

Osteopontin (OPN) is a non-traditional pro-inflammatory cytokine and is involved in muscle regeneration and inflammation. The aim of this study was to investigate the expression of OPN in skeletal muscle and serum of patients with IIMs.

Methods

45 patients with IIMs (27 with PM and 18 with DM) were included in the study. Patients received initial prednisone therapy (1–1.5 mg/kg/day) without other immunosuppressive agents. Muscle biopsies were taken before start of treatment and serum samples were collected from each patient before and after corticosteroid treatment. The expression of OPN in skeletal muscle was using immunofluorescence staining and western blotting. Serum OPN levels were detected by enzyme-linked immunosorbent assays (ELISA).

Results

OPN expression was increased in muscle samples from IIM patients compared to control muscle. The serum level of OPN was significantly higher in IIM patients than in controls. Moreover, the serum concentrations in the DM subgroup were significantly higher compared to the PM groups. Serum OPN levels positively correlated with creatinine kinase (CK), C-reactive protein (CRP) in PM and DM patients, respectively. After corticosteroid treatment, serum OPN levels decreased significantly in steroid responders compared to baseline, but no significant decrease was observed in steroid non-responders.

Conclusion

OPN is increased in patients with DM and PM, both in muscle and serum. OPN may play an important role in the pathogenesis of IIMs. Moreover, serum OPN may be a potential biomarker for this illness, and changes in serum OPN may provide an index of therapeutic efficacy.

Key words

osteopontin, skeletal muscle, idiopathic inflammatory myopathies, polymyositis, dermatomyositis
Increased OPN in muscle and serum of IIMs / F. Xiao et al.

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Introduction

Immune mechanisms are closely involved in the onset and progression of idiopathic inflammatory myopathies (IIMs). Infiltration of inflammatory cells, muscle fibre degeneration, and necrosis are the primary pathological phenomena in polymyositis (PM). Macrophages and T lymphocytes play an important role in muscle fibre damage. Cytokines and chemokines activate, regulate, and control the migration of these cells. Cytokines also play a vital role in IIMs (1), and interactions among various cytokines may enhance inflammatory responses. Interferon γ (IFN-γ) and tumour necrosis factor α are upregulated in dermatomyositis (DM), PM, and inclusion body myositis (IBMs) (2). IFN-α and -β are implicated in innate immune responses in DM (3), and IFN-γ is regarded as an important regulator of IIMs (4, 5).

Osteopontin (OPN) is an acidic glycoprotein expressed in a wide variety of cells including osteoblasts, macrophages, lymphocytes, endothelial cells, skeletal muscle cells, fibroblasts, and tumor cells (6-9). OPN is an important regulator of bone resorption and formation. In recent years, OPN has also been recognised as a non-traditional pro-inflammatory cytokine. OPN upregulates expression of both IFN-γ and interleukin 12 (IL-12) (10-12).

OPN expression is significantly higher in muscles of patients with Duchenne muscular dystrophy and injury (13-15), suggesting that it may be involved in muscle regeneration and inflammation. OPN expression is also elevated in the dermis of patients with DM (16). However, the expression of OPN has not yet been studied in the muscle of IIMs. Thus, in this paper, we evaluated the expression of OPN in skeletal muscle, and the serum concentration of OPN, in patients with IIMs. In addition, we compared OPN serum levels before and after glucocorticoid treatment.

Materials and methods

Patients and controls

We studied 45 patients with IIMs who had been admitted to our hospital between July 2010 and June 2014. They were diagnosed with definite PM or DM according to the criteria proposed by Bohan and Peter (17). Individuals with overlapping connective tissue disease, mixed connective tissue disease, or malignancy were excluded. None of the patients had received immunomodulatory treatment prior to hospital admission. All patients underwent routine clinical and laboratory assessments, blood parameters including creatinine kinase (CK), C reactive protein (CRP), erythrocyte sedimentation rate (ESR) were obtained. Because PM and DM mainly affect the proximal limbs, in order to facilitate the analysis, we examined, recorded and analysed the muscle powers of the biceps brachii, the deltoids in the upper limbs and quadriceps femoris in the lower limbs according to Medical Research Council (MRC) scale. We averaged the muscular strengths of the biceps brachii, the deltoids and quadriceps femoris in the same patient, and regard it as the proximal muscle strength of the patient in the study. Global disease activity of patient was evaluated by the visual analogue scales (VAS) score of the Myositis Disease Activity Assessment Tool (MDAAT) [18] before treatment. They received initial prednisone therapy (1–1.5 mg/kg/day) without other immunosuppressive agents. We also used MDAAT (18) 2 months after corticosteroid treatment to assess the efficacy of treatment. Patients who showed obvious improvement in muscle strength (a change of at least one grade based on the MRC scale) and MDAAT scores (decrease in disease activity of ≥10 units) were considered to be responsive to corticosteroid treatment (19).

For muscle specimen controls, we selected ten patients (four men and six women; aged 14 to 60 years) suspected of having a neuromuscular disease, but having normal results for serum CK, muscle histology, histochemistry, immunohistochemistry, and ultrastructural morphology. We selected another 45 gender- and age-matched healthy individuals as controls for serum measurements.

The study was conducted in accordance with the Helsinki Declaration and was approved by the Research Ethics Committee of the First Affiliated Hos-
pital of Chongqing Medical University. All participants provided informed consent.

Sample collection and storage
All patients and controls underwent a diagnostic musculus biceps brachii biopsy. Each sample was divided into two portions. One portion was frozen in liquid nitrogen-cooled isopentane for cryostat sectioning and stored at -80°C until use. Cryostat sections were cut at 8 μm thick. The other portion was stored directly in liquid nitrogen for immunoblotting analysis.

Venous blood samples (5 ml) were collected from each patient before and after corticosteroid treatment. Blood samples from controls were obtained only once. Serum samples were allowed to stand for 30 min at room temperature, centrifuged at 3000 x g for 15 min to collect the serum, and then frozen at -80°C until use.

Immunofluorescence
Immunofluorescence was performed on consecutive 8 μm-thick cryostat muscle sections. To block nonspecific binding, sections were preincubated in 10% goat serum (Zhongshan Golden Bridge) for 30 min at room temperature. Sections were then incubated with monoclonal rabbit anti-osteopontin (1:50, Abcam, ab91655) at 4°C overnight. Sections were then washed with phosphate-buffered saline (PBS) and incubated with fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (1:200, Zhongshan Golden Bridge) in the dark for 60 min at 37°C. Finally, sections were washed with PBS and mounted in 1:1 glycerol/PBS. Fluorescence intensity was examined by laser scanning confocal microscopy (Leica Microsystems) on an Olympus IX 70 inverted microscope (Olympus) equipped with a Fluoview FXV confocal scan head.

Western blotting
Ten samples each from the DM group, PM group and control group were used. Samples were lysed for 15 min in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% NP-40, and complete protease inhibitor cocktail [Roche Diagnostics GmbH, Mannheim, Germany]). The debris was pelleted by centrifugation at 4°C (16,000 x g for 10 min), and clear supernatants were transferred to new tubes. Protein concentration was measured with a bicinchoninic acid protein assay kit (Dingguo Beijing, China). Protein samples were boiled for 5 min in the presence of 5x SDS-PAGE loading buffer. Equal samples (50 μg protein/lane) were subjected to 12% SDS-PAGE and then electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h in Tris-buffered saline (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% non-fat milk powder and then incubated for 1 h with rabbit anti-osteopontin (1:1000, Abcam) or rabbit anti-β-actin (1:5000, Santa Cruz Biotechnology). Membranes were washed and then incubated for 1 h with horseradish peroxidase–labeled goat anti-rabbit or anti-mouse antibodies (1:5000, Santa Cruz Biotechnology) for 1.5 h at 37°C. Images were captured directly using a Gel FX5 chemiluminescence and fluorescence imaging system (Vilber, France). Band density was quantified using Quantity One software (Bio-Rad, Hercules, CA, USA), and osteopontin signals were normalised to β-actin. Experiments were repeated three independent times using the same conditions. The final optical density values were averaged from three independent experiments.

Enzyme-linked immunosorbent assay (ELISA)
OPN concentrations in serum were measured by sandwich ELISA using a human Osteopontin ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Each serum sample was diluted 1:50 and measured in duplicate. Optical densities were quantified with a Multiskan Spectrum Microplate Spectrophotometer (Thermo Fisher Scientific, USA) at 450 nm. Serum OPN levels were compared at baseline and 2 months after corticosteroid treatment. In addition, serum levels were compared in patients according to gender and age (≥50 years vs. <50 years).

Statistical analysis
All data are expressed as mean ± standard deviation or median and interquartile range where appropriate. Comparisons of more than two groups were performed by one-way analysis of variance (ANOVA). Differences between two groups were assessed using the independent-samples t-test. To differentiate steroid responders from non-responders, statistical comparisons of serum OPN levels before and after prednisone treatment in each patient were analysed using the paired t-test. The correlations between serum OPN levels and clinical features or laboratory markers were evaluated with Spearman’s correlation test in patients with PM and DM, respectively. Chi square test was used to compare sex ratio of PM and DM. All p-values were derived from two-sided tests, and p<0.05 was considered to be statistically significant. Statistical analyses were performed using Statistical Product and Service Solutions version 17.0 software (SPSS Inc., Chicago, IL, USA).

Results
Patient characteristics
Twenty-seven patients were diagnosed with definite PM, and 18 patients were diagnosed with definite DM, according to the Bohan and Peter criteria. Patients included 15 men and 30 women, with a mean age of 41.0±15.57 years (range, 15 to 66 years). Table I summarises the clinical characteristics and laboratory tests of the patients. At 2 months after the start of therapy, 37/45 patients (82.2%) had responded well to steroid treatment, and eight patients showed a poor response.

OPN expression in muscle
In cryostat sections from DM and PM pathology samples, OPN was present as an intense green fluorescent signal in the cytoplasm of some fibres (Fig. 1A-B). OPN-positive fibres were not seen in control muscle (Fig. 1C).

Western blotting showed that OPN expression was increased in 20 muscle samples from IIM patients (10 samples from DM group and 10 samples from PM group) compared to control muscle (Fig. 2A). Densitometric analysis
Increased OPN in muscle and serum of IIMs / F. Xiao et al.

Table I. Clinical characteristics of patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PM (n=27)</th>
<th>DM (n=18)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>45.74 ± 11.09</td>
<td>33.89 ± 18.70</td>
<td>0.023</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>18/9</td>
<td>12/6</td>
<td>1.000</td>
</tr>
<tr>
<td>Disease duration, months</td>
<td>4.24 ± 3.27</td>
<td>4.75 ± 2.98</td>
<td>0.599</td>
</tr>
<tr>
<td>Median (25–75 percentiles)</td>
<td>4.0(3.0-4.0)</td>
<td>4.0(3.0-4.0)</td>
<td>0.892</td>
</tr>
<tr>
<td>Global disease activity, VAS</td>
<td>5.04 ± 1.58</td>
<td>6.33 ± 1.57</td>
<td>0.010</td>
</tr>
<tr>
<td>CRP, mg/dl</td>
<td>10.59 ± 4.15</td>
<td>13.61 ± 4.30</td>
<td>0.023</td>
</tr>
<tr>
<td>ESR, mm/hr</td>
<td>22.04 ± 8.79</td>
<td>26.56 ± 10.07</td>
<td>0.118</td>
</tr>
<tr>
<td>CK, U/L</td>
<td>2834 ± 2400</td>
<td>731 ± 522</td>
<td>0.000</td>
</tr>
</tbody>
</table>
| DM: dermatomyositis; PM: polymyositis; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; CK: creatine kinase; VAS: visual analogue scale; MRC: Medical Research Council.

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Discussion
OPN can exist as an intracellular molecule or as a secreted form (20, 21). OPN is expressed in and can be secreted by inflammatory cells such as macrophages and lymphocytes, as well as skeletal muscle fibres (6-9). The secreted form of OPN is regarded as a solu-
Increased OPN in muscle and serum of IIMs / F. Xiao et al.

ble molecule with cytokine-like func-
tions. OPN is involved in macrophage and polyclonal B cell activation and activation-induced T-cell death in autoimmune animal models (22, 23). OPN also upregulates expression of IFN-γ (12) and IL-12 (10-12). Skeletal muscle-derived cells produce OPN in response to pro-inflammatory cytokines (9).

Regarding the role of OPN in skeletal muscle disorders, OPN expression is significantly increased in muscle from patients with Duchenne muscular dystrophy and from mdx mice (13, 14), suggesting that OPN is involved in muscle regeneration and inflammation. OPN expression is induced after skeletal muscle injury, and is involved in inflammatory cell infiltration and muscle fibre necrosis following injury (15). Here, we observed overexpression of OPN in muscle from patients with DM and PM, suggesting that OPN may also play a role in the pathogenesis of IIMs. OPN upregulates expression of IFN-γ (12), which is an important regulator of IIMs (1) and may upregulate major histocompatibility complex I in muscle fibres (1). The pro-inflammatory chemokines CXCL9 and CXCL10, which are induced by IFN-γ, further enhance inflammatory reactions in IIMs (1). Thus, increased OPN expression, produced by the skeletal muscle itself, may promote the inflammatory response in IIMs. After muscle injury, limited acute OPN overexpression is regarded as beneficial in injured muscle, but chronic overexpression of OPN may lead to chronic inflammation, fibrosis, and functional impairment of injured muscles (15).

Our study also found increased levels of serum OPN in patients with IIMs, indicating that OPN synthesised by inflammatory cells and muscle may be secreted into the peripheral blood. Increased concentrations of OPN in serum may reflect the increased expression of OPN in muscle tissue. In patients with clinical remission, the inflammatory reaction was clearly reduced. In our study, serum OPN levels decreased significantly in steroid responders compared to baseline, but this was not seen in steroid non-responders. Therefore, changes in serum OPN levels may indirectly reflect changes in the disease state, and patients who do not show a significant reduction in serum OPN concentration may be considered non-responders to treatment.

Our results suggest that serum OPN concentrations in DM are higher than in PM, even though there was no significant difference in muscle OPN expression between the two groups. The dermis of DM patients contains increased levels of diffusely distributed OPN protein (16). OPN acts as an immobilised matricellular protein that can interact with the hyaluronan receptor CD44 variant 7 to activate intracel-

Table II. Osteopontin concentration in serum.

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (ng/ml)</th>
<th>t (F)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Patient age (years)</td>
<td>≥50 (13)</td>
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<tr>
<td></td>
<td>&lt;50 (32)</td>
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<tr>
<td>Gender</td>
<td>Male (15)</td>
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<td></td>
<td>Female (30)</td>
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<tr>
<td>Groups</td>
<td>DM (18)</td>
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<tr>
<td></td>
<td>PM (27)</td>
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<td></td>
<td>Control (45)</td>
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<td>Responders (37)</td>
<td>Baseline</td>
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<td></td>
<td>Treated</td>
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<tr>
<td>Non-responders (8)</td>
<td>Baseline</td>
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<td></td>
<td>Treated</td>
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* p<0.01 vs. control; * p<0.01 vs. PM. DM: dermatomyositis; PM: polymyositis.

Fig. 3. ELISA of baseline OPN levels in serum. * p<0.01 vs. control; * p<0.01 vs. PM.

Fig. 4. ELISA of serum OPN levels in steroid responders and non-responders before and after prednisone treatment. Serum OPN concentrations decreased significantly in steroid-responders after treatment, but did not change significantly in non-responders. * p<0.01 compared with baseline; * p>0.05 compared with baseline.
24). Because both the skin and muscle are involved in DM patients, and the dermis contains more OPN in DM patients, we hypothesise that synthesis of OPN is higher in DM patients than in PM patients. Because OPN can be secreted into the blood, the serum OPN level was also higher in DM patients. OPN concentrations may indeed be different between DM and PM, but more samples are needed for validation.

In conclusion, our study demonstrates that OPN is increased in patients with DM and PM, both in muscle and serum. Further research into the mechanism of action of OPN in the pathogenesis of IIMs may be worthwhile. In addition, since serum OPN level is strongly correlated with known disease activity markers, such as CRP, CK and global VAS score, it may be a potential biomarker for this disease, and changes in its concentration may serve as an indicator of disease activity markers, such as CRP, CK and global VAS score, it may be a potential biomarker for this disease, and changes in its concentration may serve as an indicator of disease activity.

Acknowledgements

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References