Increased visfatin levels are associated with higher disease activity in anti-Jo-1-positive myositis patients

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Abstract

Objective

The aim of this study was to evaluate serum levels of visfatin in anti-Jo-1-positive myositis patients, its expression in muscle tissue and to investigate potential relationships between visfatin, B-cell activating factor of the TNF family (BAFF), disease activity and anti-Jo-1 autoantibody levels.

Methods

Serum levels of visfatin and BAFF were measured in 38 anti-Jo-1 positive myositis patients and 35 healthy subjects. Disease activity was evaluated by myositis disease activity assessment tool (MYOACT) using visual analogue scales (VAS) and by serum muscle enzymes. Visfatin expression was evaluated by immunohistochemistry in muscle tissue of myositis patients (n=10) and compared with non-inflammatory control muscle tissue samples from patients with myasthenia gravis (n=5).

Results

Serum visfatin and BAFF levels were significantly higher in myositis patients compared to healthy subjects and were associated with clinical muscle activity assessed by VAS. Only serum BAFF levels, but not visfatin levels, positively correlated with muscle enzyme concentrations and anti-Jo1 antibody levels. There was a positive correlation between visfatin and BAFF serum levels in myositis patients but a negative correlation was observed in healthy subjects. Visfatin expression was up-regulated in endomysial and perimysial inflammatory infiltrates of muscle tissue from myositis patients.

Conclusion

Up-regulation of visfatin in myositis muscle tissue and an association between increased visfatin levels and muscle disease activity evaluated by MYOACT in anti-Jo-1 positive myositis patients could support possible role of visfatin in the pathogenesis of myositis.

Key words

myositis, visfatin, BAFF, anti-Jo-1, muscle enzymes

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Introduction

The idiopathic inflammatory myopathies including polymyositis (PM) and dermatomyositis (DM) are characterised by muscle weakness and inflammatory infiltrates in skeletal muscle tissue (1). Other organs such as skin or lungs may be affected as well.

Autoantibodies, some of which are considered to be myositis-specific, are present in 60–80% of patients with myositis (2, 3). The most frequent myositis specific autoantibodies directed against histidyl-tRNA synthetase (anti-Jo-1) are present in approximately 10–30% of myositis patients (4) and are associated with a distinct clinical phenotype (5). Presence of these autoantibodies before the onset of clinical symptoms (6, 7), as well as the role of the target antigen in the breach of immune tolerance (8, 9), suggest a possible role of anti-Jo-1 in the pathogenesis of myositis. Furthermore, a correlation between serum levels of anti-Jo-1 autoantibodies and myositis activity was previously reported (10).

Visfatin, also known as pre-B cell colony-enhancing factor (PBEF) or nicotinamide phosphoribosyltransferase (NAMPT) is ubiquitously expressed in all tissues (11) and was recently described as a new adipokine up-regulated in visceral fat cells (12). Beside the role in energy metabolism; immunomodulatory and pro-inflammatory properties of visfatin were emphasised recently (13). Visfatin enhances activation of leukocytes, synthesis of adhesion molecules and production of pro-inflammatory cytokines, such as interleukin (IL)-1, tumour necrosis factor (TNF) and IL-6 in monocytes (14) and, in addition, protects fibroblasts and neutrophils from apoptosis (15, 16). Its production is increased in lymphocytes and stimulates their proliferation during polyclonal immune response (15, 17). Visfatin acts as a cytokine that promotes B-cell maturation (18).

Increased levels of visfatin were documented in several chronic autoimmune and inflammatory diseases such as systemic lupus erythematosus (19, 20) or rheumatoid arthritis (RA) (21-23). In RA, visfatin levels correlate with clinical disease activity (24) and predict radiographic progression in established (22) as well as in early disease (25). Visfatin and other adipokines are actively produced by local cells of the rheumatoid joint and by the periarticular adipose tissue (24, 26).

We have recently described increased serum levels of another adipokine resistin in myositis patients compared with healthy controls and their association with systemic inflammation and disease activity, particularly in anti-Jo-1 positive myositis patients (27). However, the role of visfatin in the pathogenesis of myositis has not yet been studied. Therefore, the aim of this study was to compare serum levels of visfatin in anti-Jo-1 positive patients with myositis to levels in healthy individuals and assess their potential relationship with anti-Jo-1 autoantibodies, with myositis activity and with serum levels of B-cell activation factor of the TNF family (BAFF), a crucial cytokine for B-cell maturation and survival, with known association with myositis and anti-Jo-1 autoantibodies (28). We also analysed expression of visfatin in myositis and non-inflammatory muscle biopsies.

Patients and methods

Patients

Thirty-eight anti-Jo-1 positive myositis patients (27 PM and 11 DM) regularly followed at the Institute of Rheumatology in Prague were included in this study. The diagnosis of PM and DM was established based on the Bohan and Peter criteria (29). In 16 patients, longitudinal blood samples obtained during treatment were available (median time between sample collection was 13.4 months, range 7 to 73.6 months). A control group consisted of 35 age- and sex-matched healthy subjects. This study was performed after approval by the local Ethics Committee of Institute of Rheumatology. Written informed consent was obtained from all individuals prior to participation.

Clinical data were collected at the time of serum sampling. Disease activity was evaluated using the Myositis Disease Activity Assessment Tool (MYOACT) according to the International Myositis Assessment & Clinical Studies Group (IMACS), including extra-

muscular, muscular and the physician's score of overall disease activity using visual analogue scales (VAS). Interstitial lung disease (ILD) was defined by either presence of cough or dyspnoea, reduction of lung volumes or changes on radiograph/HRCT as described elsewhere (30).

Laboratory measurements

Serum levels of creatine kinase (CK), myoglobin, lactate dehydrogenase (LDH) and C-reactive protein (CRP) were measured by routine laboratory techniques. Enzyme-linked immunosorbent assay (ELISA) was used for the measurement of serum visfatin (Bio-Vision Research Products, Mountain View, USA) and BAFF levels (R&D Systems, Inc., Minneapolis, USA) according to the manufacturer's protocol. Absorbance was detected using the Sunrise ELISA reader (Tecan, Salzburg, Austria). The sensitivity was 30pg/ml for visfatin and 3.38 pg/ml for BAFF. Quantitative indirect solid phase ELISA assay (Orgentec, Mainz, Germany) was used for detection of IgG class anti-Jo-1 levels according to manufacturer instructions. Normal range declared by producer was <15 U/ml and borderline values were 15–25 U/ml. Anti Jo-1 positivity was confirmed by line blot assay (Myositis-LIA, IMTEC, Berlin, Germany) and myositis western blot using Anti-Myositis-Antigen EUROLINE-WB kit (Euroimmun, Lubeck, Germany).

Table I. Demographic characteristics, clinical and laboratory data of patients at initial evaluation.

	All n=38	Controls n=35	*p*
F : M	26 : 12	24 : 11	0.99
DM : PM	11 : 27	NA	
Age (year)*	52.5 ± 10.8	50.7 ± 12.5	0.53
Years from diagnosis**	1.92 [0.01 to 18.5]	NA	
ILD†	32 (84%)	NA	
Medication - GCS†	35 (92%)	NA	
- DMARDs†	22 (58%)	NA	
- No therapy†	3 (8%)	NA	
Dose of GCS (mg/d)**‡	17.5 [0 - 80]	NA	
CRP (mg/l)**	2.93 [0.1 to 83.0]	1.42 [0.3 to 7.4]	0.03
CK (μcat/l)**	2.44 [0.26 to 94.5]	ND	
Myoglobin (μg/l)**	96.8 [24.8 to 5313]	ND	
LDH (μcat/l)**	4.14 [1.67 to 22.8]	ND	
ALT (μcat/l)**	0.48 [0.09 to 6.7]	ND	
AST (μcat/l)**	0.43 [0.14 to 5.1]	ND	
BAFF (ng/ml)**	1.41 [0.5 to 20.9]	1.0 [0.42 to 2.1]	0.02
anti-Jo-1 (kU/l)**	183.7 [0.03 to 3604]	ND	
Visfatin (ng/ml)**	1.9 [0.13 to 9.9]	1.51 [0.14 to 5.2]	0.01
MYOACT (VAS mm)**	n=37		
Constitutional	3 [0 to 35]	NA	
Cutaneous	0 [0 to 30]	NA	
Skeletal	0 [0 to 42]	NA	
Gastrointestinal	0 [0 to 17]	NA	
Pulmonary	15 [0 to 86]	NA	
Cardiac	0 [0 to 36]	NA	
Other	0 [0 to 29]	NA	
Global Extraskeletal Muscle	12 [0 to 64]	NA	
Muscle	11 [0 to 82]	NA	
Global	16 [0 to 79]	NA	
MYOACT Score	0.55 [0 to 2.3]	NA	

ALT: alanine aminotransferase (normal levels <0.75 μcat/l for men and <0.57 μcat/l for women); AST, aspartate aminotransferase (normal levels <0.58 μcat/L for men and <0.52 μcat/L for women); BAFF, B-cell activating factor of the TNF family; CK: creatine kinase (normal levels <2.85 cat/l and <2.42 μcat/l); CRP: C-reactive protein (normal levels <5 mg/l); DM: dermatomyositis; DMARD: disease-modifying anti-rheumatic drug; GCS: glucocorticosteroids; ILD: interstitial lung disease (ever present); LDH: Lactate dehydrogenase (normal levels 0.05 – 4.15 cat/L); MYOACT: Myositis Disease Activity Assessment Tool; Myoglobin (normal levels <92 μg/l, for men and <76 μg/l for women); NA: not applicable; ND: not determined; PM: polymyositis. Data are: *mean ± SD; **median [minimum - maximum] or †number (% from DM, PM or from total number of patients). ‡ Equivalent of prednisolone daily dose.

Immunohistochemistry

Samples for immunohistochemistry were obtained from patients with PM (n=5) and DM (n=5) at the time of diagnostic muscle biopsy, which was guided by positive magnetic resonance imaging (MRI) findings from affected muscles as previously described (31). The biopsies were taken prior to treatment initiation. Muscle tissue samples from patients with myasthenia gravis (MG, n=5) obtained during thymectomy were used as non-inflammatory controls, no lymphocyte infiltrates were present in muscle tissue biopsies from these individuals (32). After the sample collection, 5 μm frozen sections were fixed in acetone and 4% paraformaldehyde and blocked with 0.3% H_2O_2. The sections were incubated with rabbit polyclonal anti- Visfatin antibody (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA, 1mg/ml) at a dilution 1:1000 in Tris Buffered Saline (TBS) buffer for 1 hour. After rinse in TBS buffer, antigen-antibody complexes were visualised with a Histofine detection system (Nichirei Biosciences Inc., Tokyo, Japan) using 3, 3´diaminobensidine as a chromogen. Rabbit IgG (Dako Cytomation, Glostrup, Denmark, 1mg/ml), diluted 1:1000 was used as a negative control. The sections were slightly counterstained with Harris's haematoxylin. All sections were analysed semiquantitatively by two experienced pathologists who were blinded to the clinical data. Visfatin expression was scored semiquantitatively on a four-point scale (- represented negative staining intensity, and scores of + to +++ represented weak, moderate and strong staining intensity) at seven locations (inflammatory infiltrate, perimysial vessels, endomysial capillaries, muscle fibres, regenerating muscle fibres, atrophic muscle fibres and rhabdomyoblasts).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (v. 5.02; GraphPad Software, La Jolla, CA, USA) and SPSS 14.0 (SPSS, Inc., Chicago, Illinois, USA). For analysis of differences between groups, Kruskal-Wallis with

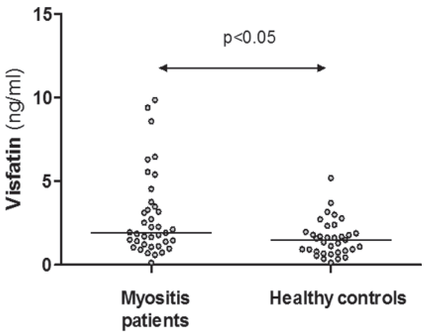

Fig. 1. Serum levels of visfatin are higher in myositis patients compared to healthy individuals. Horizontal bars represent medians; *p*-value of Kruskal-Wallis test is expressed.

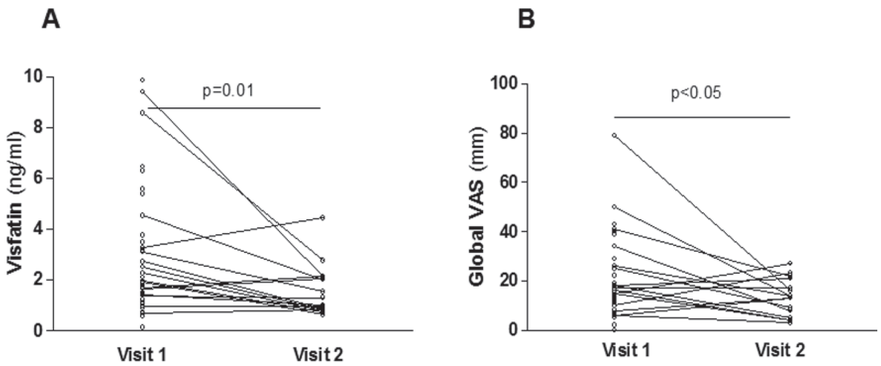

Fig. 2. Visfatin serum levels in paired serum samples decreased between two different time points, median between the two blood withdrawals was 13.4 [7 to 73.6] months (**A**), which was accompanied by global disease activity improvement (**B**). Wilcoxon's paired sample test *p*-values are expressed.

Dunn's post hoc test and non-parametric Mann-Whitney U-test or paired *t*-test with respect to normality of data, were performed. Spearman's rank order test (rs) was used for correlations of parameters. Contingent tables were evaluated with Fisher's exact test. Changes in paired samples were evaluated with Wilcoxon's signed rank test. A *p*-value less than 0.05 was considered to be statistically significant. Unless otherwise stated, data were presented as median [minimum–maximum] values.

Results

Patient characteristics

The demographic characteristics of patients and healthy controls are shown in Table I. Altogether, 35 (92%) patients were treated with glucocorticoids (median dose of prednisone equivalent: 17.5 mg/day; range 0–80 mg/day). 22 patients (58%) were treated with immunosuppressive drugs: 16 received methotrexate, 4 azathioprine, 2 methotrexate in combination with azathioprine, 1 patient used cyclosporine A. Three patients (8%) had no treatment at the time of serum sample collection. There were no significant differences in demographic characteristics, laboratory and clinical disease activity, or treatment between PM and DM patients.

Increased visfatin serum levels in myositis patients

The serum levels of visfatin were significantly higher in myositis patients compared to healthy controls (1.94 [0.13–9.86] *vs*. 1.51 [0.14–5.20] ng/ml; $p<0.05$) (Fig. 1). There were no significant differences in visfatin levels between patients with DM and PM (1.91 [0.13–9.41] *vs*. 1.96 [0.71–9.86] ng/ml; $p=0.89$) or between males and females (1.73 [0.13–6.48] ng/ml vs. 2.28 [0.6–9.86] ng/ml; $p=0.23$). Similarly, no difference was found between visfatin levels in patients double positive for anti-Jo1 and anti-Ro52 (1.96 [0.92–6.31] ng/ml) and patients without anti-Ro52 (1.91 [0.13–9.86] ng/ml; $p=0.68$). The levels of serum visfatin did not correlate with age (r=-0.06, $p=0.75$) or disease duration (r=-0.08, $p=0.63$). Furthermore, visfatin serum levels did not significantly differ between patients treated with glucocorticoids for less or more than one month. Although, serum visfatin levels were comparable between myositis patients with and without conventional immunosuppressive therapy (1.91 [0.1–9.9] *vs*. 2.09 [0.7–6.5] ng/ml; $p=0.89$), the levels of serum visfatin in longitudinal samples significantly decreased during treatment (from 2.12 [0.7–9.4] to 1.16 [0.6–4.45] ng/ml; $p=0.01$) which was accompanied with disease activity improvement in global VAS (from 17.5 [0–79] to 13.5 [3–27]; $p<0.05$) (Fig. 2).

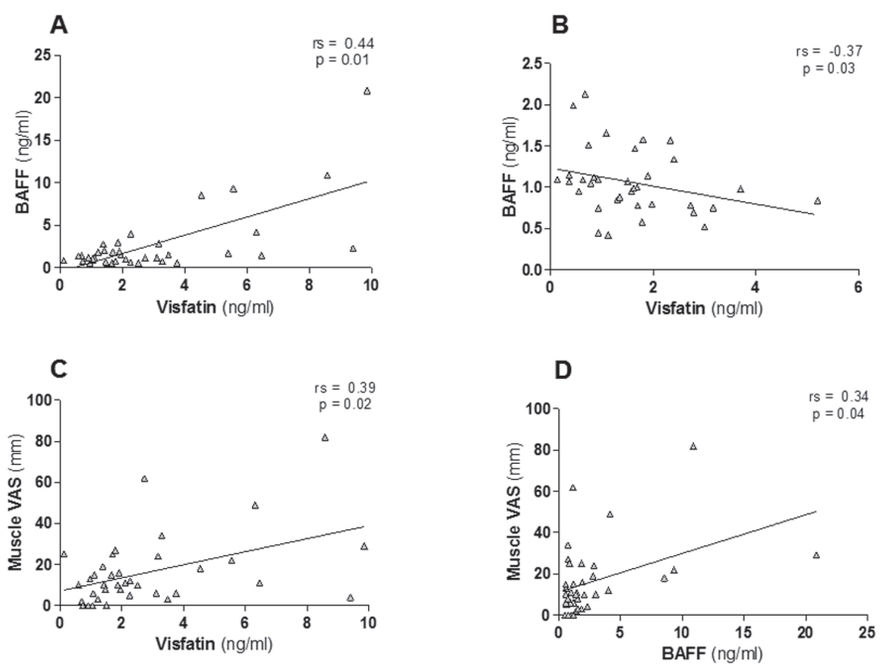

Fig. 3. Correlation between serum visfatin and BAFF levels in myositis patients (**A**) and healthy subjects (**B**). Both serum levels of visfatin and BAFF correlated with muscle disease activity in patients with myositis (**C**, **D**). Spearman's correlation coefficients with p values and linear regression lines are shown.

Association between visfatin, BAFF, autoantibodies and myositis activity

The levels of visfatin (rs=0.39, p=0.02), as well as of BAFF (rs=0.34, p=0.04) correlated with clinical muscle activity assessed by VAS (Fig. 3C and 3D), but did not correlate with laboratory measures of myositis disease activity. There was a trend for correlation of visfatin and BAFF with the global disease activity VAS (rs=0.28, p=0.09 and rs=0.33, p=0.05).

While, there was a moderate correlation between the level of visfatin and LDH (rs=0.39, p<0.02), it did not correlate with other muscle enzymes and myoglobin levels. On the other hand, BAFF levels significantly correlated with serum myoglobin (rs=0.57, p=0.002), CK (rs=0.51, p=0.001) and also with the levels of anti-Jo-1 (rs=0.85, p=0.001). Although there was a trend towards positive correlation between visfatin and anti-Jo-1 autoantibody levels (rs=0.31, p=0.06), no differences were observed in visfatin levels between patients with and without ILD (1.85 [0.13-9.86] *vs*. 1.91 [0.31-4.55] ng/ml; p=0.96). The levels of serum visfatin in myositis patients did not correlate with CRP levels (rs=0.13, p=0.43). There was a correlation between serum BAFF levels and CRP (rs=0.35, p=0.03). Levels of serum visfatin positively correlated with BAFF levels in myositis patients (rs=0.44; p=0.01), but there was a negative correlation in healthy controls (rs=-0.37; p=0.03), (Fig. 3A and 3B).

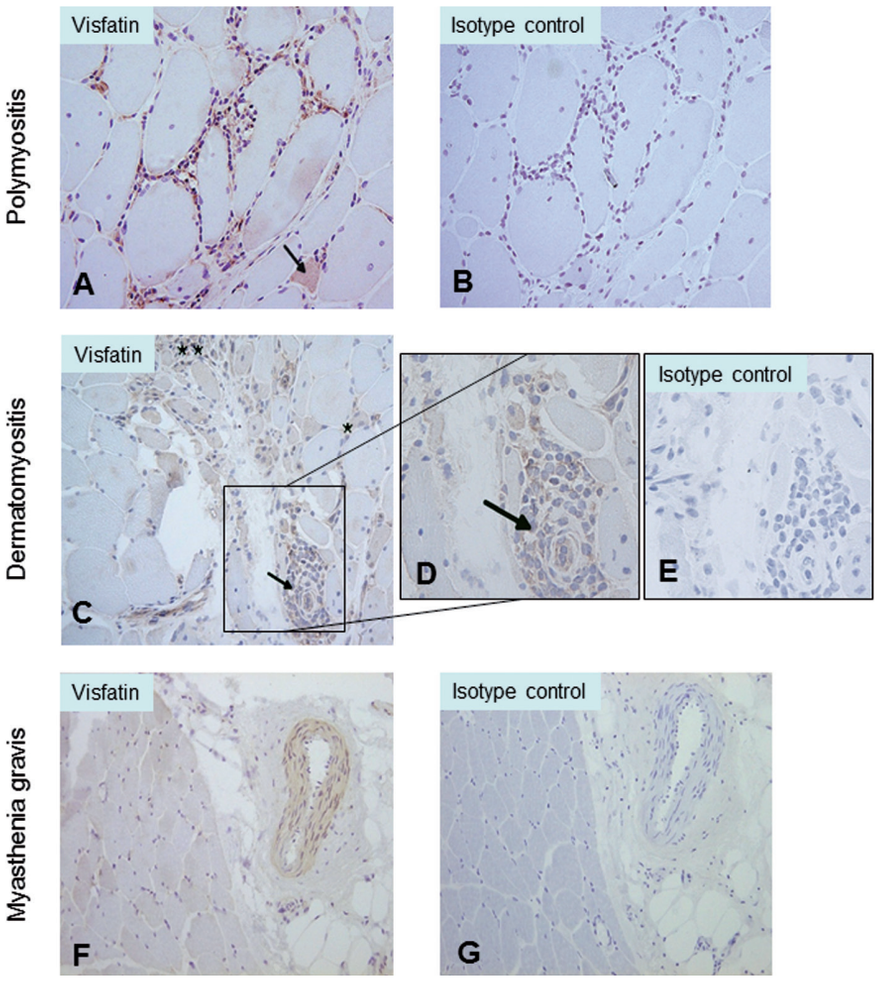

Fig. 4. Endomysial inflammatory infiltrate in a representative case of polymyositis patient express diffuse visfatin immunoreactivity (**A**). Regenerating muscle fibres show slight cytoplasmic immunopositivity (arrow), while muscle fibres of isotype control staining are negative (**B**). A proportion of the perimysial inflammatory cells in a representative case of dermatomyositis (**C**) is immunopositive for visfatin (arrowhead) with detail (**D**). Regenerating muscle fibres are visfatin immunopositive (*). In perifascicular regions, atrophic muscle fibres show cytoplasmatic immunopositivity (**), isotype control staining is negative (**E**). Positivity of visfatin in smooth muscles of perimysial arteries media of a control muscle tissue from MG patient (**F**) compared to isotype control (**G**). Brown colour indicates positive staining. Immunoperoxidase method, counterstain with haematoxylin, magnificaton 200x.

Increased expression of visfatin in muscle tissue of myositis patients

The expression of visfatin in muscle tissue was observed in all myositis patients and was more prominent when compared to control muscle tissues from patients with MG (Fig. 4). Visfatin staining intensity was comparable between muscle specimens from patients with PM and DM. Expression of visfatin was most evident in the endomysial and perimysial inflammatory cell infiltrates in the muscle tissue, although the intensity varied from weak to strong (Table II). Whereas the vast majority of endomysial inflammatory infiltrate in PM was visfatin positive (Fig. 4A), only part of perimysial inflammatory infiltrate was positive in DM (Fig. 4C). Rhabdomyoblasts, when found, were visfatin positive. In all patients with PM and in four out of five patients with DM, regenerating muscle fibres expressed visfatin, a finding not observed in control patients with MG. Similar intensity of visfatin expression was demonstrated in endothelial cells of perimysial vessels or endomysial capillaries in myositis and control muscle tissues.

Discussion

In the present study, we report an association between higher visfatin serum levels and increased muscle disease activity in anti-Jo-1 positive myositis patients. Furthermore, visfatin expression in muscle tissue from myositis patients was up-regulated compared to non-inflammatory muscle controls. This data support a possible role of visfatin in the pathogenesis of idiopathic inflammatory myopathies.

Visfatin, originally discovered as a growth factor for B lymphocyte precursors, plays a significant role in various pathophysiologic processes including inflammation (14, 33). Our study is the first showing elevated serum visfatin levels in patients with anti-Jo-1-positive myositis. Visfatin serum levels were higher in both PM and DM

Table II. Visfatin expression in muscle tissue of patients with dermatomyositis (DM) and polymyositis (PM) compared to non-inflammatory muscle biopsies from patients with myasthenia gravis (MG).

	No.	Inflammatory infiltrate** -/+/++/+++	Perimysial vessels† -/+	Endomysial capillaries†† -/+	Muscle fibres‡ -/+	Regenerating muscle fibres# -/+	Atrophic muscle fibres[l] -/+	Rhabdo myoblasts§ -/+
PM	1	+	+	+	-	+	-	+
	2	+	+	+	-	+	-	+
	3	+++	+	+	+*	+	-	+
	4	-	+	+	-	+	-	+
	5	++	+	+	-	+	-	+
DM	1	++	+	+	-	+	+	+
	2	+	+	+	-	+	+	+
	3	++	+	+	-	-	+	+
	4	+	+	+	-	+	+	+
	5	-	+	+	-	+	+	+
Control muscle (MG)	1	-	+	+	-	-	-	-
	2	-	+	+	-	-	-	-
	3	-	+	+	-	-	-	+
	4	-	+	+	-	-	-	-
	5	-	+	+	-	-	-	-

**The presence and intensity of inflammatory infiltrate: none inflammatory infiltrate present; +: very mild visfatin positive; ++: medium visfatin positive; +++: intense visfatin positive; †Perimysial vessels: - negative; + endothelial cells and vessel wall positivity; ††Capillaries: - negative; + mild positivity; ‡ Muscle fibres: - negative; + positive; #Regenerating muscle fibres: - not present; + present cytoplasmatic positivity; [l]Atrophic muscle fibres: - negative; + cytoplasmatic positivity; §Rhabdomyoblasts: - not present; + present, cytoplasmatic positivity. *Muscle fibres displaying cytoplasmatic visfatin immunopositivity proved to by ragged red fibres via histochemical methods (succinate dehydrogenase, cytochrom C oxidase).

patients compared to healthy controls. We found that visfatin as well as BAFF serum levels were associated with muscle disease activity and there was a trend for correlation with the global disease activity score. This association is supported by evidence that visfatin is associated with several pathological conditions, including cardiovascular diseases, metabolic disorders, inflammatory diseases or malignancies (34). In addition, visfatin levels correlated with disease activity (24, 35) and structural progression of rheumatoid arthritis (22) and ankylosing spondylitis (36). On the other hand, in systemic sclerosis, visfatin levels were not different from that in healthy controls (37, 38) and in systemic lupus erythematosus, visfatin levels were either elevated (20, 39) or comparable (38) to those of healthy subjects.

Although, immunosuppressive therapy did not seem to influence visfatin levels in a cross-sectional analysis, the levels of visfatin significantly decreased during treatment in myositis patients whose disease activity improved. We and others have reported that visfatin levels decrease following successful therapy in rheumatoid arthritis patients (35, 40), a finding which is not consistent in all studies (41).

In this study a positive association between visfatin and BAFF serum levels in anti-Jo-1 positive myositis patients was observed. Both cytokines are involved in B cell maturation into autoantibody producing plasma cells, which provides a possible explanation of the association between visfatin and particularly BAFF levels with that of anti-Jo-1 antibodies. However, we observed an inverse association between visfatin and BAFF levels in healthy subjects, which may potentially represent a counterbalancing mechanism in B cell function under non-inflammatory conditions. The role of these cytokines in myositis pathogenesis may be further supported by the fact that patients with anti-synthetase syndrome respond better to B-cell depleting therapy with rituximab compared to patients with other forms of inflammatory myopathies (42).

Here, we have also shown that visfatin levels did not correlate with muscle enzymes, with the exception of LDH or inflammatory markers in myositis patients. This may suggest that visfatin is more likely associated with immune inflammatory reaction rather than with muscle damage. In this sense it may be hypothesised that visfatin is a biomarker related to the inflammatory aspect of the disease whereas CK levels are more related to the actual muscle cell injury. Finally, we detected increased visfatin expression in muscle tissues from myositis patients compared to non-inflammatory control muscle specimens from MG individuals. Visfatin was predominantly expressed in endomysial and perimysial inflammatory cell infiltrates and also by regenerating muscle fibres in a majority of myositis muscle tissue samples. Since BAFF together with its receptors are up-regulated in myositis muscle tissue (43), and BAFF increases visfatin gene expression in primary B cells *in vitro* (44), it can be hypothesised that BAFF may participate in visfatin up-regulation in muscle tissue inflammatory infiltrates. Increased visfatin expression has been identified in a variety of chronic inflammatory diseases. In rheumatoid arthritis synovial membrane, visfatin was predominantly expressed by fibroblasts, lymphoid aggregates, and interstitial vessels (24, 26, 45). Visfatin can promote synthesis of chemokines, pro-inflammatory cytokines, angiogenic growth factors, and matrix degrading enzymes (24, 26, 45, 46). The expression of visfatin was also demonstrated in skeletal muscle tissue raising the possibility that it may act as a myokine affecting skeletal muscle growth and metabolism (47).

The strengths of our study rely in the homogeneous population of anti-Jo-1 positive patients and the fact that clinical assessments were performed at the time of serum collection, enabling correlative analyses. The limitation of this study is a relatively low number of patients due to the infrequent occurrence of the disease. With this limitation in mind we can still hypothesise that increased amount of visfatin may have an effect on immune as well as muscle cells and it may participate in the disease process in inflammatory anti-Jo-1 positive myositis.

Conclusion

In conclusion, we demonstrated here elevated serum levels of visfatin, similarly to BAFF, in patients with anti-Jo-1 positive myositis. Both cytokines associated positively with muscle disease activity evaluated by MYOACT. In addition, visfatin expression was up-regulated in muscle tissue from myositis patients; however, the exact role of visfatin in the pathogenesis of myositis remains to be determined in further studies.

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