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Original article

The metastasis promoting protein S100A4 is increased in idiopathic inflammatory myopathies

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Abstract

Objectives. The S100A4 protein is known as a metastasis promoting factor; however, its involvement in non-malignant diseases such as RA and psoriasis has been recently described. The aim of this study was to investigate the expression and possible role of S100A4 in idiopathic inflammatory myopathies.

Methods. S100A4 protein expression was detected by immunohistochemistry in muscle tissue from control individuals ($n = 11$) and patients with PM and DM ($n = 8/6$). IF staining was used to co-localize S100A4 with selected cells. Cytokine expression and protein synthesis in S100A4-treated cells were analysed by RT-PCR and ELISA.

Results. S100A4 protein was significantly up-regulated in muscle tissue of patients with inflammatory myopathies compared with control individuals and was associated particularly with the presence of mononuclear infiltrates. Only few regenerating muscle fibres in PM/DM expressed S100A4. Then we analysed the effect of S100A4 on human myocytes and peripheral blood mononuclear cells (PBMCs). Although S100A4 did not affect myocytes, stimulation of PBMCs with S100A4 significantly induced the expression and synthesis of TNF-α, IL-1β and IL-6, but not of IFN-α. We showed that S100A4 is not directly involved in perforin/granzyme B-induced apoptosis and that it does not modulate the expression of Bax and Bcl2 mRNA in myocytes and PBMCs.

Conclusion. Increased expression of S100A4 in inflamed muscle tissue highlights its potential role in the pathogenesis of inflammatory myopathies. S100A4 may act as a cytokine-like factor indirectly promoting muscle fibre damage by stimulating mononuclear cells to increase the synthesis of pro-inflammatory cytokines.

Key words: S100A4, Polymyositis, Dermatomyositis, Peripheral blood mononuclear cells, Myocytes, Inflammation.

BASIC SCIENCE

Introduction

PM and DM are chronic inflammatory autoimmune myopathies of unknown aetiopathogenesis that are clinically characterized by symmetric proximal skeletal muscle weakness [1]. The main pathognomonic feature of PM/DM is the mononuclear inflammatory cell infiltration associated with muscle fibre necrosis and regeneration. In patients with PM, inflammatory cells often surround and invade non-necrotic muscle fibres and primarily include $CD8^+$ T cells and macrophages, whereas in patients with DM, $CD4^+$ T cells, macrophages and dendritic cells predominate mostly in perivascular areas. In addition, compared with healthy muscles, affected muscle fibres express both MHC Class I and II antigens and may themselves be the site of autosensitization [2]. In chronic myositis, macrophages phagocytose the necrotic muscle fibres and muscles can then be replaced by fibrous connective tissue. Taken all together, T cells, macrophages,

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immunoglobulins and muscle fibres appear to be critical components for final damage of the muscles. There is evidence that not only inflammatory mononuclear cells within the muscle tissue, but also endothelial cells and muscle fibres themselves produce a number of pro-inflammatory mediators contributing to the pathogenesis of PM/DM [3, 4]. Increased expression of IL-1β, IL-6, TNF-α or IFN-α has been demonstrated in muscle tissue of myositis patients [3, 4].

S100A4, also known as metastasin (Mts1), was described several years ago as metastasis-specific protein and subsequently has been demonstrated as a mediator of tumour progression and metastasis [5, 6]. In recent years, it has become apparent that S100A4 also participates in pathogenesis of non-malignant human disorders [7]. We have reported increased amounts of S100A4 protein in blood, SF and synovial tissue of patients with RA [8, 9]. The level of S100A4 in plasma correlates with disease activity of patients with RA [10]. In addition, it has been demonstrated that S100A4 regulates apoptosis and expression of several matrix degrading enzymes in RA synovial fibroblasts [8, 9] as well as expression of TNF-α in monocytes [10]. S100A4 is up-regulated in the upper dermis of psoriatic skin and the protein was shown to be actively involved in the pathogenesis of psoriasis [11]. Association of S100A4 protein with autoimmune inflammatory diseases and with some fibrotic processes has recently been suggested [12, 13]. In the present study, we investigated the expression and local distribution of S100A4 in muscle tissue of patients with idiopathic inflammatory myopathies and studied the role of S100A4 in the pathogenesis of inflammation and muscle tissue damage.

Materials and methods

Immunohistochemical studies

Muscle tissue samples were obtained under local anaesthesia using the open biopsy technique from vastus lateralis (or medialis) muscle. Muscle tissue biopsies were obtained from patients with PM ($n = 8$) and DM ($n = 6$) during diagnostic muscle biopsy from a biopsy site selected using MRI [14]. Patient clinical and demographic data at the time of muscle biopsy are given in Table 1. Patients with PM/DM fulfilled Bohan and Peter diagnostic criteria [15, 16]. Control non-inflammatory muscle tissue was obtained from individuals with myasthenia gravis ($n = 5$) and from individuals ($n = 6$) who underwent diagnostic muscle biopsy, but did not fulfil diagnostic criteria for PM or DM. The study was approved by the local ethics committee (RNDr. Ivana Půtová) and each patient signed an informed consent before biopsy.

Serial cryostat sections of muscle tissue were fixed in acetone and 4% paraformaldehyde and blocked in 0.3% H_2O_2. Sections were washed in Tris-buffered saline (TBS) and incubated with polyclonal rabbit anti-S100A4 antibodies 1 : 5000 [produced at the Danish Cancer society (DCS), Denmark] for 1 h, and rinsed again in TBS buffer. Antigen–antibody complexes were visualized by the Histofine detection system (Nichirei Biosciences Inc.) using 3, 3′-diaminobenzidine as chromogene. Sections were counterstained with Harris's haematoxylin. Isotype-specific antibodies were used as negative controls. The intensity of S100A4 expression was scored using a semi-quantitative four-point scale: 0, represented no staining, while a score of 1–3 represented weak, moderate and strong staining intensity, respectively. The intensity of S100A4 staining was assessed by two pathologists skilled in reading muscle biopsies and mean scores were calculated for statistical analysis. In the case of weak or borderline staining intensity any inter-observer discrepancies were resolved by secondary evaluation until agreement was reached.

For double-labelling experiments we used the same sections. Cryostat muscle sections were fixed in acetone, rinsed in TBS and blocked in TBS supplemented with 5% goat serum. Primary antibodies were incubated overnight in a cold room: polyclonal and monoclonal anti-S100A4 diluted 1 : 2000 (produced in the Department of Molecular Cancer Biology, DCS); monoclonal anti-CD68 (diluted 1 : 200; Dako); anti-CD1a (diluted 1 : 200; Dako); anti-CD3 (diluted 1 : 400; Abcam); anti-vimentin (diluted 1 : 4000; Neo Markers); anti-CD20cy (diluted 1 : 100; Dako); anti-alpha smooth muscle actin (diluted 1 : 4000; Sigma); mast cell tryptase (diluted 1 : 1000); von Willebrand factor (diluted 1 : 500; Dako). Secondary antibodies coupled to Alexa 488 or Alexa 568 were used at a dilution of 1 : 2000 (Molecular Probes, Leiden, The Netherlands). Samples were analysed by confocal microscopy using an LSM 510 microscope (Carl Zeiss MicroImaging, Oberkochen, Germany).

Table 1 Patient characteristics at the time of muscle biopsy

Patient	Age (years)	Sex	Diagnosis	Treatment at the time of biopsy
1	71	F	PM	Pred 40
2	63	M	PM	Pred 60
3	56	F	PM	None
4	49	M	PM	None
5	54	M	PM	None
6	50	F	PM	None
7	57	F	PM	None
8	78	F	PM	None
9	56	F	DM/Scl	Pred 60
10	64	F	DM	None
11	30	M	DM	None
12	74	F	DM	None
13	74	F	DM	Pred 60
14	61	F	DM	Pred 20, MTX

F: female; M: male; Scl: scleroderma; Pred: prednisolone.

Isolation of peripheral blood mononuclear cells, myocyte culture and stimulation

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of patients with idiopathic

inflammatory myopathies ($n = 5$–10) by Ficoll–Plaque density gradient centrifugation. PBMCs were seeded at 5.0×10^5 cells (confluent cells condition) per 1 ml of advanced RPMI medium 1640 (Invitrogen, Carlsbad, CA, USA) in 35-mm diameter culture plates. Commercially available human skeletal muscle cells (Lonza, Basel, Switzerland) were cultivated in DMEM (Invitrogen) supplemented with 10% fetal calf serum at 37°C with 5% CO_2. Cells were seeded at 1.0×10^5 cells per well in 6-well culture plates in 1 ml of DMEM without serum. The cells were treated with a S100A4 multimer (1 μg/ml) and lipopolysaccharide (100 μM) for 6 and 24 h at 37°C with 5% CO_2. The doses of S100A4 and time courses were given according to our previous experiments [8, 9]. The active S100A4 multimer was obtained from recombinant His6-tagged protein by gel filtration as described previously [17]. After 6 h, cells were lysed by RNeasy lysis buffer (Qiagen, Hombrechtikon, Switzerland), cell culture supernatants were collected after 24 h of exposure to S100A4 and stored at −80°C.

RT-PCR analysis

Total RNA from myocytes and PBMCs was extracted using the MagNa Pure Compact RNA isolation kit for MagNA Pure Compact Instrument (Roche Diagnostics GmbH, Germany). Complementary DNA was obtained by reverse transcription using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). PCR was performed according to a standard protocol. Pre-developed primers (Applied Biosystems) were used to detect IL-1β, IL-6, TNF-α, IFN-α, Bax and Bcl-2 mRNAs. 18 S ribosomal RNA was used for correcting the results with the comparative threshold cycle (C_t) method for relative quantification, and the C_t of 18 S ribosomal RNA was subtracted from the C_t of studied genes, giving the ΔC_t values. The 7900HT Fast Real-time PCR system was used to perform real-time PCR (Applied Biosystems).

ELISA

Protein levels were measured in the cell culture supernatants using ELISA kits according to the manufacturer's protocol: TNF-α, IL-1β, IL-6 (Ray Biotech, Inc., Norcross, GA, USA), IFN-α (PBL InterferonSource, Piscataway, NJ, USA), granzyme B and perforin (Abcam, Cambridge, UK). The analyses were performed using ELISA reader SUNRISE (Tecan, Salzburg, Austria) at a wavelength of 450 nm.

Statistical analyses

Differences in the expression of S100A4 between myositis and control muscle samples were determined using the median test and Pearson's chi-square test. Changes in mRNA expression and protein synthesis were performed using the Student's paired *t*-test. *P*-values were considered statistically significant when <0.05.

Results

Expression of S100A4 protein in muscle tissue of inflammatory myopathies

S100A4 expression in muscle tissue was detected in all patients with PM/DM and the staining intensity was significantly enhanced compared with control muscle tissue (Fig. 1). There was consistent expression of S100A4 within the inflammatory infiltrates in the muscle tissue, although its intensity varied from mild to very strong (Table 2). Compared with control muscle tissue, S100A4 was up-regulated in mononuclear cells within the inflammatory infiltrates in the affected muscle tissues (Fig. 1), particularly in those surrounding necrotic muscle fibres. Moreover, few muscle fibres—mostly regenerating muscle fibres (8/14) in PM/DM expressed S100A4 (supplementary figure 1, available as supplementary data at *Rheumatology* Online), whereas this was not seen in normal muscles. A similar pattern of S100A4 staining intensity was demonstrated in endothelial cells of large perimysial vessels and endomysial capillaries between control and PM/DM muscle tissues. The S100A4 staining intensity was similar between PM and DM muscle specimens.

Cell-specific co-localization of S100A4 protein in muscle tissue

To determine cell-specific localization of S100A4 protein in muscle tissue samples of patients with idiopathic inflammatory myopathies, we performed double IF staining using antibodies against S100A4 and cell-specific markers for various cell types. Except for B-lymphocytes (CD20), S100A4 protein was ubiquitously expressed in inflamed muscle tissue (Fig. 2). We demonstrated clear co-localization of S100A4 with markers specific for T lymphocytes (CD3), macrophages (CD68), dendritic cells (CD1a), non-epithelial cells of mesenchymal origin, mostly fibroblasts (vimentin), alpha-smooth muscle actin-positive cells (myofibroblasts, pericytes and some others), endothelial cells (von Willebrand factor) and mast cells (mast cell tryptase). These data indicate that S100A4 expression patterns are comparable to those found in our previous findings in RA and psoriasis [9, 11].

S100A4 enhances expression of pro-inflammatory cytokines in mononuclear cells, but not in myocytes

Taking into consideration that S100A4 is up-regulated at local sites of inflammation, we further investigated the role of S100A4 in the process of inflammation. We demonstrated that treatment of PBMCs with the S100A4 protein significantly up-regulates the expression of IL-1β ($P < 0.001$), IL-6 ($P < 0.001$) and TNF-α ($P < 0.01$), but not IFN-α, mRNAs after 6 h compared with untreated cells (Fig. 3A). Consistently with the mRNA expression profile, production and release of IL-1β [9.972 (0.895) *vs* 1.084 (0.149) pg/ml; $P < 0.005$], IL-6 [42.960 (3.666) *vs* 3.973 (1.684) pg/ml; $P < 0.005$] and TNF-α [31.970 (5.054) *vs* 4.902 (1.754) pg/ml; $P < 0.01$], but not IFN-α [1.479

FIG. 1 Imunohistochemical staining for S100A4 protein in muscle tissues: (**A1**) PM, (**B1**) DM and (**C1**) control muscle tissue. The S100A4 expression was particularly increased in mononuclear cells within the inflammatory infiltrates surrounding necrotic muscle fibres. Several regenerating muscle fibres in an inflammatory myositis expressed S100A4, whereas it was not present in a control muscle tissue. However, endothelial cells of large perimysial vessels and endomysial capillaries were positive for S100A4 in all patients and controls. The S100A4 staining intensity was comparable between PM and DM muscle specimens. Mouse immunoglobulin G was used as a control (**A2**, **B2**, **C2**). Original magnification was ×200.

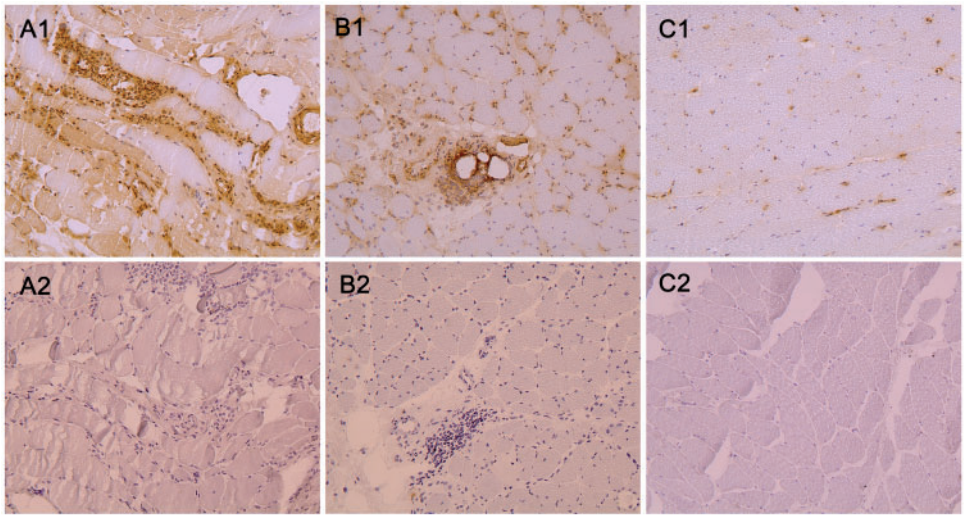


TABLE 2 Expression of S100A4 related to different cellular compartments in muscle tissues from patients with PM/DM and control individuals

Location	PM/DM	Controls	Median test, *P*	Pearson's chi-square test, *P*
Mononuclear cell infiltrates	2 (1–3)	0 (0–0)	0.001	<0.0001
Vessels and capillaries	1.5 (1–3)	1.5 (1–2)	1.0	0.840
Interstitial connective tissue	1 (0–2)	1 (1–2)	0.661	0.301
Muscle fibres	0.5 (0–1)	0 (0–0)	0.003	0.010

The intensity of S100A4 expression was scored using a semi-quantitative four-point scale. Score 0 represented no staining; 1: weak; 2: moderate; and 3: strong staining intensity. The numbers represent median (min–max).

(0.239) *vs* 1.620 (0.294) pg/ml; not significant], by PBMCs was also significantly increased upon treatment with S100A4 compared with untreated cells after 24 h (Fig. 3B). To further define whether S100A4 participates in the process of muscle tissue damage, we analysed expression of apoptotic markers as well as release of granzyme B and perforin from mononuclear cells treated with S100A4. However, the expression of Bax and Bcl2 as well as production of perforin and granzyme B was not changed in PBMCs treated with S100A4 compared with untreated cells (Fig. 3A). Furthermore, expression of IL-1, IL-6 and TNF-α mRNAs remained unchanged in myocytes treated with S100A4 compared with untreated cells (data not shown). These findings suggest that the increased presence of S100A4 in muscle tissue may enhance production of several cytokines participating in the process of inflammation in myositis.

Discussion

This is the first study demonstrating increased expression of S100A4 protein in muscle tissue from patients with idiopathic inflammatory myopathies compared with control individuals. Here, we show that the protein is particularly associated with mononuclear infiltrates and may play a role in the inflammation contributing to muscle fibre damage. When incubated with mononuclear cells, S100A4 promotes the production of several pro-inflammatory cytokines participating in the pathogenesis of inflammatory myopathies.

Fig. 2 Cell-specific expression of the S100A4 protein in muscle tissue using double IF staining. The cells are labelled by cell-specific markers: anti-CD68 (macrophages); anti-CD20 (B-lymphocytes); anti-CD3 (T-lymphocytes); anti-CD1a (dendritic cells); anti-smooth-muscle actin (myofibroblasts and pericytes); vimentin (cells of mesenchymal origin); von Willebrand factor (endothelial cells) and mast cell tryptase (mast cells). The scanning dimensions are for all images: 0.22 μm × 0.22 μm, except for CD20: 0.06 μm × 0.06 μm, CD1a: 0.11 μm × 0.11 μm.

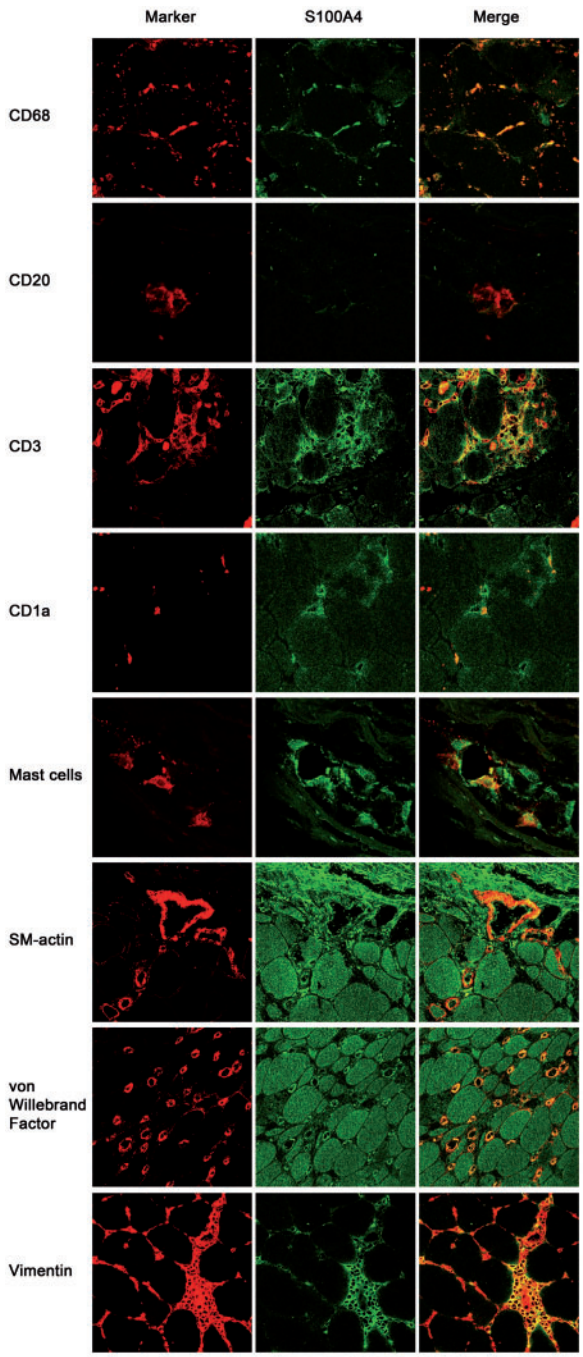
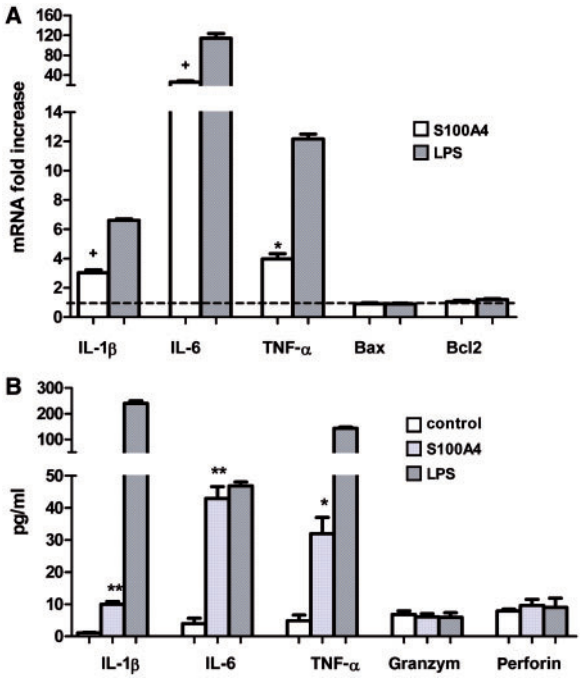


Fig. 3 S100A4 protein significantly increased the expression (**A**) and release (**B**) of TNF-α, IL-1β and IL-6, but not IFN-α, in PBMCs. Bax, Bcl2 mRNA (**A**), and granzyme B and perforin proteins (**B**) were not changed in PBMCs treated with S100A4 protein. Expression of mRNA was determined after 6 h and protein levels in cell culture supernatants were measured after 24 h. All the results are expressed as mean and S.E.M., $^{*}P < 0.01$; $^{**}P < 0.005$; $^{+}P < 0.001$.



Recent studies have shown that S100A4 can be associated not only with cancer, but also with some inflammatory and fibrotic diseases (for review see [12, 13]). We have previously demonstrated increased S100A4 expression in RA compared with OA synovial tissues with the protein being abundantly produced by synovial fibroblasts, several immune cells and endothelial cells [8, 9]. Very similar patterns of S100A4 distribution were documented in the dermis of psoriatic skin, where S100A4 was also up-regulated [11]. The results in this study are consistent with previous findings. We found increased expression of S100A4 in mononuclear cells particularly at sites of inflammatory infiltrates surrounding necrotic muscle fibres of patients with inflammatory myopathies. These results are in agreement with the study of Seeliger *et al.* [18] that described increased expression of other S100 family calcium-binding proteins S100A8 and S100A9 in inflamed muscle tissue of patients with inflammatory myopathies. S100A8/9 dimer was expressed by activated macrophages, but did not appear to be expressed in myotubes. In contrast, we have detected S100A4 protein also in some regenerating muscle fibres. Whereas S100A4 protein was not found in the normal mature cardiac myocytes, it is present in myocytes within the infarct border zones and a role for S100A4

protein as a factor eliciting hypertrophic response and pro-survival activities has been suggested [19]. In this context, one can hypothesize that S100A4 might be engulfed by regenerating myofibres, thus counter-parting mechanisms involved in the process of muscle fibre injury in myositis. Endothelial cells of large perimysial vessels and endomysial capillaries exert similar patterns of S100A4 expression between control and inflamed muscle tissue suggesting more general roles for S100A4 in human physiological and pathological functions.

There is clear evidence that S100A4 protein is increased at sites of inflammation [8–11]. Recently, several pro-inflammatory cytokines have been demonstrated to be responsible for the increase in S100A4 production [20]. However, when released from the cells, S100A4 also exerts cytokine-like activities and modulates the expression of several genes that are involved in the process of tissue damage [6–12, 20, 21]. The functional activity of extracellular S100A4 protein is attributed to the multimeric forms of the protein [10, 17, 22]. Since we found increased amounts of S100A4 protein in interstitial connective tissue surrounding necrotic muscle fibres, we hypothesized that the protein might be implicated in the observed muscle fibre damage and examined the effects of extracellular S100A4 on these cells. Mononuclear cell infiltrates, particularly cytotoxic T cells and NK cells, participate directly in the process of muscle damage by releasing granzyme B and perforin [23], which however, were not induced by the treatment with S100A4. Although S100A4 protein did not modulate the expression of pro-inflammatory cytokines or apoptotic mediators in myocytes, it significantly increased the expression and release of IL-1β, IL-6 and TNF-α from mononuclear cells. The diversity of S100A4 effects can be explained by the fact that mononuclear cells, but not myocytes, express TLR-4 ([24], supplementary figure 2, available as supplementary data at *Rheumatology* Online), which based on our unpublished observation, participate in S100A4-induced cell activation. Thus, it could be hypothesized that locally up-regulated S100A4 protein participates in the muscle fibres damage indirectly, by activation of mononuclear cells to synthesize pro-inflammatory cytokines that have been shown to contribute to the pathogenesis of inflammatory myopathies [3, 23–27].

In summary, our study shows increased expression of S100A4 protein in inflamed muscle tissue of patients with inflammatory myopathies. The protein originates from inflammatory and resident tissue cells and in an autocrine and/or paracrine manner may increase the synthesis of pro-inflammatory cytokines in mononuclear cells, and thus indirectly participate as a factor contributing to the pathogenesis of inflammatory myopathies.

Rheumatology key messages

- S100A4 protein is significantly up-regulated in inflammatory myopathies.
- S100A4 induces the production of pro-inflammatory cytokines in mononuclear cells.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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