

ORIGINAL PAPER

Immunohistochemical study of skeletal muscle in rheumatoid myositis

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Abstract

Introduction: Rheumatoid myositis (RM) represents a poorly characterized entity, immune mechanism, assessment and management remaining still unclear. The aim of this study was to investigate endothelial and inflammatory cells activation in RM muscle biopsy. **Material and Methods:** Prospective study on 23 consecutive rheumatoid arthritis (RA) with muscle involvement as defined by clinical, biological and imagistic parameters. CD4, CD8, CD20, CD3, CD45RO and CD68 markers, HLA-DR, cytokines receptors (IL-2, TNF α , TGFr), pro-apoptotic (CD95) and adhesion molecules (CD54) were assessed by immunohistochemistry in deltoid muscle samples. **Results:** (1) endomysial, perivascular and perimysial inflammatory infiltrates and moderate muscle fibers involvement; predominance of activated (HLA-DR+), memory (CD45RO+) CD3+TCD8+ cells and macrophages surrounding and invading non-necrotic muscle fibers (34.78%) and TCD4+ activated cells in perivascular and perifascicular areas (65.22%); (2) up-regulation of HLA-DR, CD54 and IL-2R on both endothelial cells and lymphocytes (85%); (3) aberrant increased CD95 in endothelial cells without any other apoptotic sign (83%) have been described. **Conclusion:** Increased expression of activation markers, adhesion molecules and cytokine receptors may indicate early endothelial activation in RM pathogenesis, while endomysial TCD8+ activation may account for further development and perpetuation of myositis.

Keywords: rheumatoid myositis, immunohistochemistry, endothelial activation.

□ Introduction

Rheumatoid arthritis (RA) represents a chronic inflammatory autoimmune rheumatic disease characterized by articular and extra-articular, systemic involvement, resulting in important disability and severe impairment of quality of life [1, 2]. TNF α and IL-1, main pro-inflammatory cytokines involved in the development and perpetuation of the rheumatoid process, are in the mean time important determinants of both chronic inflammation and early, irreversible, progressing of articular damage (cartilage and subchondral bone) [1, 2].

Although, commonly reported in RA and recognized from early '60, the concept of muscle involvement in RA, including etiology, clinical spectrum, diagnostic criteria and assessment, is still poorly characterized [3–11]. Several possible causes of skeletal muscle disease secondary to RA have already been proposed, including: inflammation (myositis), vasculitis, drug-related myopathy (corticosteroids, antimalarials, D-penicillamine), impaired muscle plasticity governed by TNF α , a reflex response to pain, psychological factors [12–18].

Evidence of increased prevalence of symptomatic muscle disease in different stages of RA, potential implicated factors, lack of diagnostic criteria and

therapeutic protocol, as well as recent development of new assessment modalities for myositis stand for sufficient arguments information to realize a complex assessment of skeletal muscle involvement (especially inflammatory aspects) in RA [14, 15, 19–21].

The main objective of the current study was to investigate endothelial and inflammatory cells activation in skeletal muscle, aiming to define and understand immune mechanisms of myositis in rheumatoid arthritis patients.

□ Material and Methods

Hundred and twenty (n_1) consecutive patients fulfilling 1987 ACR diagnostic criteria for rheumatoid arthritis were screened for skeletal muscle involvement according to a standard protocol. Assessments were performed on several *muscular parameters*, including (i) *clinical* (muscle pain and weakness), (ii) *biological* (serum muscle enzymes – creatine phosphokinase activity, LDH, aldolase) and (iii) *imaging* (electromyogram, EMG; conventional and color Doppler myosonography; magnetic resonance imaging, MRI), but also rely on *specific RA scores* (disease activity and functional indices).

Among all these RA patients, only a small group ($n_2 = 23$) was characterized by inflammatory muscle

pathology (as defined clinically, biologically and by imaging analysis) and subsequently enrolled in this prospective descriptive study aiming to define immunohistochemical patterns of rheumatoid myositis.

As part of the study protocol, open surgery muscle biopsy was performed in all 23 RA patients with defined muscle involvement; left deltoid muscle was preferred and the biopsy was either ultrasound or MRI-guided. Histological examination (Hematoxylin–Eosin, HE) and immunohistochemistry (IHC) were used to assess biopsy specimens.

The Streptavidin–Biotin IHC method (LSAB Kit, DAKO) [22, 23] was performed using a panel of 12 antibodies (DAKO) directed against the following molecules: *CD4*, *CD8*, *CD3*, *CD45RO* and *CD20* (lymphocytes markers), *CD68* (macrophage), *CD54* (ICAM-1 adhesion molecules), *CD25* (receptor for IL-2, IL-2R), *CD27* (receptor for TNF α , TNF α -R), *CD105* (receptor for TGF β , TGF β -R), *CD95* (Fas, apoptotic marker) and *HLA-DR* (activation marker). Three morphological targets investigated, (i) inflammatory cells, (ii) endothelial cells and (iii) muscle cells. Either nuclear, cytoplasmic or membrane brown staining was investigated in the above mentioned sites, while the sections stained for CD or HLA-DR were evaluated using scores from 1 to 3: “+” meaning 10–30% positive cells; “++” 30–50% positive cells; “+++” meaning more than 50% positive cells.

Several parameters were taken into account by classical histological analysis, related to muscle fiber involvement (size, individual architecture, internal structure, types, degeneration and/or regeneration signs) and inflammatory infiltrates (composition, distribution). IHC investigation aimed to study inflammatory cells distribution and to investigate endothelial and inflammatory cells activation in rheumatoid myositis patients.

Statistical analysis was performed in SPSS-13 software, $p<0.05$.

All patients have attended Department of Rheumatology in Rehabilitation Hospital of Iassy between 2000 and 2004, while muscle biopsy analysis was performed at “Victor Babeș” National Institute for Research and Development in Pathology and Biomedical Sciences, Bucharest.

The study received ethical approval from the local ethics committee; written informed consent was signed before enrollment.

Results

The main characteristics of both RA ($n_1 = 120$) and rheumatoid myositis ($n_2 = 23$) patients are presented in Table 1.

Conventional HE examination of muscle samples has revealed the following modifications in RM: (i) *muscle fiber damage*, supported by changes in size (25%) and architecture (22.5%), abnormal fiber-type distribution (particularly type IIB) (54.54%) and degenerative/regenerative modifications (72.72%); and (ii) the presence of *mononuclear immune infiltrates* with perivascular (72.72%), perimysial

(45.45%) and endomysial (72.72%) distribution. Moderate inflammatory infiltrates (especially T- and B-cells), with characteristic single layer distribution, were typically found in perivascular region (45%); no signs of either fibrinoid necrosis or vascular wall destruction (accounting for rheumatoid vasculitis) were reported; only minor perimysial and endomysial infiltrates have been noted in the majority of cases.

Table 1 – RA and RM patients: main characteristics

| Parameter* | Rheumatoid Arthritis (RA) | Rheumatoid Myositis (RM) |
|------------------------------|---------------------------|--------------------------|
| ▪ No. of cases | 120 | 23 |
| ▪ Sex distribution | 3:1 | 1:1 |
| ▪ Age at diagnosis* [years] | 52.50 ± 12.27 | 55.12 ± 8.64 |
| ▪ Disease activity (DAS28)* | 6.38 ± 1.10 | 5.48 ± 1.33 |
| ▪ Functional scores (HAQ)* | 1.76 ± 0.56 | 1.66 ± 0.77 |
| ▪ CK* [U/L] | 43.84 ± 26.08 | 199.50 ± 57.17 |
| ▪ LDH* [U/L] | 248.86 ± 64.02 | 392.0 ± 120.35 |
| ▪ EMG (myopathy pattern) [%] | 49.5% | 82.6% |
| ▪ Myosonography: | | |
| – Classical [%] | 57.9% | 82.6% |
| – Color Doppler | 8.4% | 78.2% |
| ▪ IRM | 10% | 69.1% |

*Mean values.

Three characteristic patterns of *inflammatory infiltrates* were demonstrated by **IHC analysis** in RM patients:

- *moderate perivascular single layer inflammatory infiltrates*, with predominance of TCD4+ activated memory cells (TCD4+HLA-DR+C45RO+) (40%), TCD8+CD3+ activated memory cells (TCD8+CD3+HLA-DR+CD45RO+) (25%) and rare macrophages (3.5%); B-cells were reported in only patient (20% of cells in perivascular infiltrate);

- *minor endomysial patchy inflammatory infiltrate*, mainly consisting on TCD8+HLA-DR+CD3+CD54RO+ sub-population (50%), invading non-necrotic muscle cells (35.5%) and macrophages (10%);

- *minor perimysial patchy inflammatory infiltrate* with the predominance of TCD4+CD45RO+ cells (up to 45%).

Inflammatory cells activation and analysis of cytokines receptors showed the following: *increased HLA-DR* expression on inflammatory cells (85.5%), mainly perivascular lymphocytes; *up-regulation of IL-2R* (CD25) on perivascular lymphocytes (31.5%), *TNF α -R* (CD27) in all T-cells (22.5%), *TGF β -R* (CD105) limited on perivascular lymphocytes (17.5%).

Perivascular T-cells also expressed increased levels of *CD95*, without any other signs of apoptosis (55%), while interstitial and endomysial T-cells showed in all cases aberrant expression of *ICAM-1*.

We reported the following modifications of *endothelial cell* activation biomarkers, adhesion molecules and cytokine receptors expression: *increased HLA-DR* (55.25%) in both inflammatory and non-inflammatory, normal sites; *up-regulation of IL-2R* (50%) without any differences between inflammatory and normal areas; *up-regulation of TNF α -R* (12.5%), while no *TGF β -R* (CD105) stain; *increased, expression of ICAM-1* (30%); *increased CD95/Fas expression* (75%).

No stain for HLA-DR, cytokines receptors, activation or apoptotic markers has been detected on muscle fibers.

Figures 1 (a–e), 2 (a–e), and 3 (a–f) focus on IHC evaluation of skeletal muscle in rheumatoid myositis patients. While several statistical significant correlations

between clinical, biological and imagistic parameters that define RM and IHC evaluation have been identified ($p<0.05$), they will be detailed in another paper.

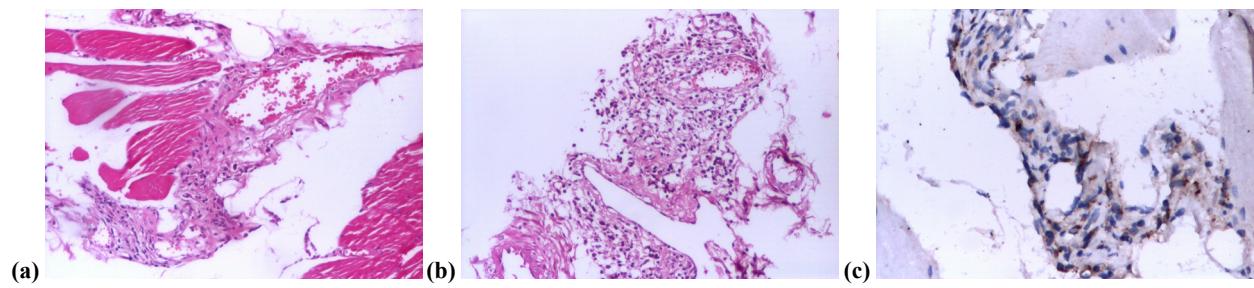


Figure 1 – Skeletal muscle biopsy in RM: moderate single layer perivascular and perimysial inflammatory infiltrate (HE, ob. 10×) (a, b); CD68 expression in perivascular infiltrate (IHC, brown staining, ob. 20×) (c); perivascular (d) and interstitial expression of CD45RO (e) (IHC, brown staining, ob. 20×).

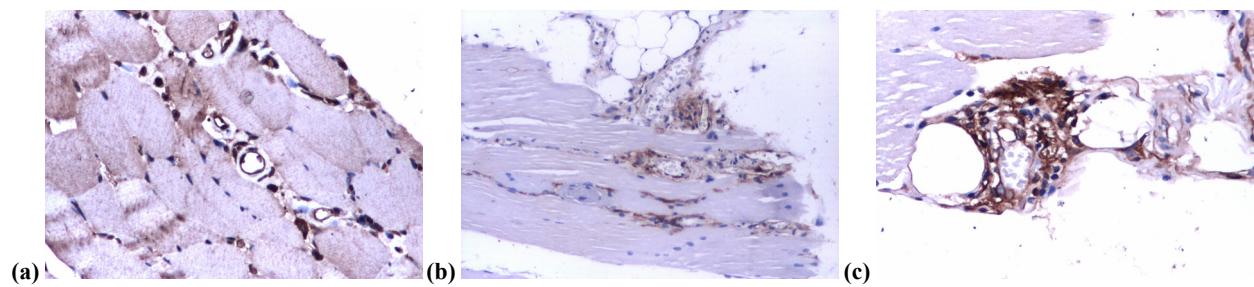


Figure 2 – Skeletal muscle biopsy in RM: CD3+ (a) expression in endomysial and perimysial lymphocytes (IHC, brown staining, ob. 20×); endothelial cell (b), perivascular lymphocytes (c, d) and interstitial lymphocytes HLA-DR expression (e) (IHC, brown staining, ob. 20×).

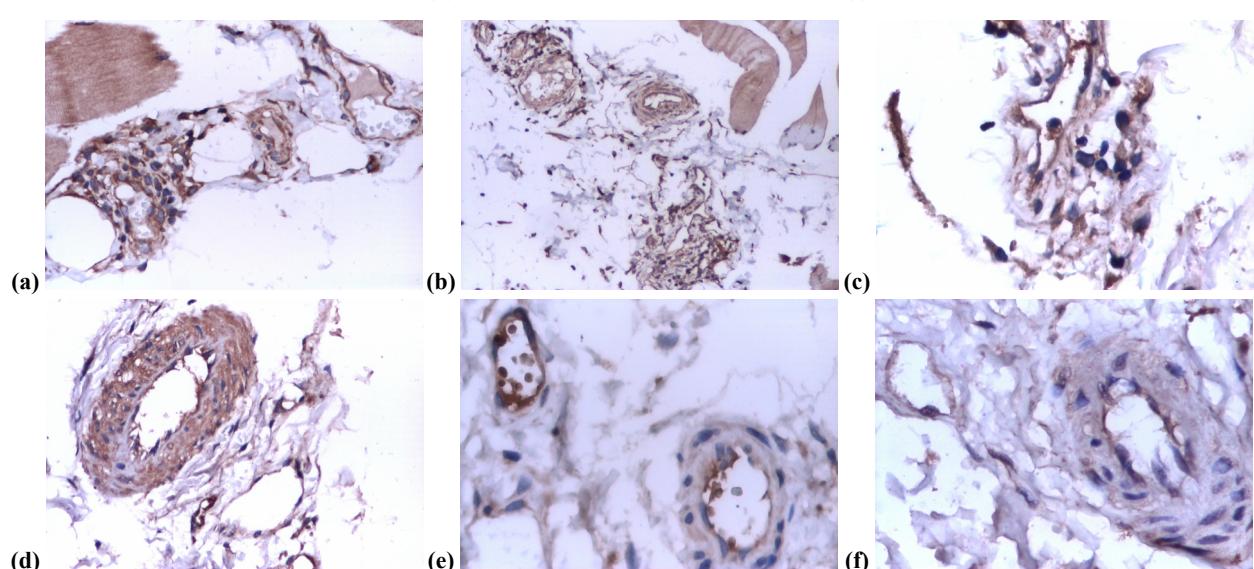


Figure 3 – Skeletal muscle biopsy in RM: CD25 expression on endothelial cells (ob. 20×) and perivascular lymphocytes (ob. 10×) (a, b); CD27 expression on lymphocytes and endothelial cells (ob. 20×) (c, d) (IHC, brown staining); CD54 and CD95 on endothelial cells (ob. 40×) (e, f) (IHC, brown staining).

Discussion

Despite well-known polymyositis-like features including clinical, biological and EMG aspects, little is known about immune mechanisms of skeletal muscle involvement in rheumatoid arthritis.

Muscle biopsy is considered a pivotal test in providing state of the art evidence for myositis [24, 25], particularly in RA patients, especially when MRI and/or ultrasound guided [19–21]. Since rheumatoid myositis is usually moderate and patchy [19, 25], multiple sections analysis was required in order to establish positive diagnosis.

Our study focused on IHC analysis of skeletal muscle in patients with RA and myositis as defined by clinical, biological and imaging tests, aiming to provide detailed information about three main morphological targets (endothelial cells, inflammatory cells, especially lymphocytes, muscle cells).

We were primarily interested in describing *in situ* immune inflammatory response (composition and distribution); three characteristic patterns were identified in RM patients. The predominance of both endomysial TCD8+CD3+ invading non-necrotic muscle fibers and perivascular and perimysial TCD4+ cells reported in our RM patients support data from literature that described as main characteristic feature for myositis in RA, polymyositis-like pattern.

Early, aberrant expression of both class I and II HLA, adhesion molecules (ICAM-1, VCAM-1) and different cytokine receptors, pro- (IL-1 α și β , TNF α , IL-2, IFN- γ) and anti-inflammatory (TGF β , IL-4, IL-10, IL-13) cytokines as well as chemokines (MIP-1 α and - β , MCP-1, RANTES) involvement, both pro- and anti-apoptotic molecules (Fas, Bcl-2, Bcl-XL) expression in muscle, endothelial and inflammatory cells are characteristic immunohistochemical features for idiopathic inflammatory myopathies [24, 25]. Recent studies focus on early vascular activation during polymyositis, suggesting up-regulation of activation markers (HLA-DR), adhesion molecules (ICAM-1, VCAM-1) and pro-inflammatory cytokines (IL-1 α) [19, 24, 25].

Several IHC studies of skeletal muscle have also been performed in patients diagnosed with rheumatoid vasculitis aiming to understand immune mechanisms underlying vascular disease among rheumatoid arthritis cases [12, 15, 16, 24]. Inflammatory infiltrates mainly made of activated (HLA-DR+) lymphocytes as well as increased expression of both ICAM-1 and VCAM-1 adhesion molecules have been reported in skeletal muscle from patients with rheumatoid arthritis and clinically defined vasculitis [14, 16].

Furthermore, fibrinoid necrosis typically found in rheumatoid vasculitis, was not described in our patients; besides, single layer perivascular inflammatory infiltrate not multiple layers have been considered related to RM [12].

No similar pattern has been demonstrated in the absence of rheumatoid vasculitis or in patients suffering from osteoarthritis, indicating that both endothelial changes and inflammatory reaction could be related to rheumatoid vasculitis [12, 14–16].

In the mean time, HLA-DQ and IL-1 α are over-expressed in endothelial cells from skeletal muscle in patients with RA and systemic manifestations, suggesting that systemic endothelial activation is commonly seen in extra-articular involvement in RA [14–16].

Detection of aberrant, increased expression of different biomarkers in endothelial and inflammatory cells may indicate their role in the pathogenesis of rheumatoid myositis. It seems that *endothelial cell activation* plays a central role in the initiation of events in RM. Evidences included increased endothelial cell staining for: (i) HLA-DR, in both inflammatory and normal sites; (ii) cytokine receptors (especially for IL-2, but also for TNF β); (iii) adhesion molecules; (iv) aberrant apoptosis signals (increased CD95/Fas).

T-cells, especially TCD8+ sub-population, play a pivotal role in the development and perpetuation of inflammation in skeletal muscle of RA patients. Evidences included: (i) presence of activated memory T-cells (TCD8+CD45RO+HLA-DR+); (ii) TCD8+CD3+ cells invading non-necrotic muscle fibers; (iii) increased expression of both ICAM-1 and cytokine receptors; the pattern of distribution is comparable to idiopathic polymyositis.

Both paraffin-embedding and thermal procedures used could result in altered evidence of skeletal muscle fibers involvement in our study.

Conclusions

This immunohistochemical study of skeletal muscle in rheumatoid myositis patients has demonstrated the distinctive contribution of three main morphological targets, endothelial cells, inflammatory cells and muscle cells, in the pathogenesis of the disease. Not only cellular immune response (composition and distribution) was assessed, but also increased expression of both activation markers and adhesion molecules, as well as cytokine receptors has been noted. Since endothelial cell activation could be considered as primary event, endomysial TCD8+ cells activation may account for their pivotal role in the development and perpetuation of rheumatoid myositis.

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