Immunopathological Characterization of Muscle Biopsy Samples from Immune-Mediated Necrotizing Myopathy Patients

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Background: Immune-mediated necrotizing myopathy (IMNM) is a relatively new proposed category of idiopathic inflammatory myopathies (IMMs), characterized by the presence of abundant necrotic muscle fibers, myophagocytosis, and sparse inflammatory infiltrates. The aim of our study was to analyze the immunopathological characteristics of IMNM by detecting biopsy samples from a cohort of patients, and to delineate the pathways involved in the pathogenesis.

Material/Methods: A retrospective evaluation of muscle biopsy samples, clinical and laboratory data, and immunohistochemical analysis of macrophages MHC-I and MAC, was performed for all patients diagnosed as having IMNM but without a prior exposure to statins.

Results: Immunohistochemical analysis revealed the presence of CD68+ macrophages mainly in the necrotic muscle fibers and the endomysial connective tissue. MHC-I and MAC positively stained not only the necrotic fibers or vessels but also the non-necrotic ones.

Conclusions: Our data describe general immunological features in IMNM patients, which may be helpful in serving as biomarkers, aid in diagnostic decisions, and provide clues into the underlying mechanisms involved in this disease.

MeSH Keywords: Complement Membrane Attack Complex • Macrophages • Major Histocompatibility Complex • Myositis

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Background

Immune-mediated necrotizing myopathy (IMNM) is a heterogeneous group of muscular diseases with specific muscular pathological and immunological features and is considered one of the idiopathic inflammatory myopathies [1–4], including polymyositis, dermatomyositis, and inclusion body myositis. To date, the recognition of IMNM is based on clinical features, pathological characteristics, and specific antibodies.

Although the pathogenesis of IMNM has not been fully elucidated, several studies have suggested some biomarkers and pathological mechanisms indicating the involvement of an autoimmune-mediated process [3–16]. Some studies have shown that major histocompatibility complex class I (MHC-I), the membrane attack complex (MAC, C5b-9), and macrophages were involved in the pathogenesis of IMNM [1,3,4,16,17]. In addition, the presence of IMNM-associated antibodies, such as the signal recognition particle (SRP), 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMGCR), and histidyl-tRNA synthetase (Jo-1) antibodies, have been intensively described for this disease [3,12,16]. However, the underlying disimmunity and the characteristics of IMNM patients in Chinese populations are yet to be described. Here, we aimed to assess muscle tissues in a cohort of 40 Chinese patients with IMNM, focusing on clinical patterns, immunological profiles, inflammatory infiltrates, and the important biomarkers expressed in the biopsied muscles.

Material and Methods

Patients and biopsy specimens

The biopsied muscles, as well as clinical and laboratory data obtained from 40 patients who were diagnosed with IMNM at Tongji Hospital between January 2012 to December 2014 were retrospectively analyzed. The diagnosis of IMNM was made based on the clinical features and muscle pathologic changes, according to the classification criteria proposed by the European Neuromuscular Centre (ENMC) in 2004 [1]. Other differentiat-ed idiopathic inflammatory myopathies (polymyositis, dermatomyositis, and inclusion body myositis), as well as infectious myopathy, were excluded from the study. Informed consents were signed by all the patients after the Ethics Committee of Tongji Hospital approved the study. Magnetic resonance imaging (MRI) was used to scan the muscles in the extremities before a biopsy. All the biopsy specimens were cryopreserved immediately in liquid nitrogen, and then stored at –80°C.

Analysis of clinical data

There was no significant difference in female/male ratio, which was 1: 1 (19: 21). The mean age was 38.1±15.9 years. The disease duration ranged from 3 days to 24 months (mean: 6.4 months). None of the patients had ever been exposed to a

Immunohistochemical analysis

To examine inflammatory infiltrates and study the immunological characteristics, muscle sections were stained with mouse monoclonal antibodies against either CD68, or MHC-I, or MAC, and visualized by IHC and immunofluorescence. The sections were fixed in ice-cold acetone, then treated with 3% H2O2, blocked with 10% fetal bovine serum albumin (BSA), and incubated with the above-mentioned primary antibodies overnight at 4°C. After a washing step, appropriate horseradish peroxidase-binding anti-mouse secondary antibodies or fluorescently labeled antibodies were added and the samples were incubated for 60 min at room temperature. Then, 3, 3’-diaminobenzidine (DAB) was used during a 5-min incubation period, and hematoxylin used to stain the nuclei. Omission of primary antibodies in the control sections resulted in the absence of any positive staining. Finally, the results were observed using a microscope (BX51, Olympus) under light or fluorescent conditions.

Semiquantitative evaluation of immunohistochemical staining

A semiquantitative scoring system reported by Corinna Preusse [4] was adopted with some modifications.

The infiltration with CD68+ macrophages was defined as the number of positive cells in necrotic cells and endomysium visually counted separately in 6 randomly selected high-power fields. Then, the average was graded as follows: <5 positive cells, almost no staining (−); 5–20 positive cells, less staining (+); 21–50 positive cells, more staining (++); and more than 50 positive cells, abundant staining (+++).

Similarly, The MHC-I expression and MAC deposition were defined as the negative staining (−), slightly positive staining (+), moderately positive staining (++), and strongly positive staining (+++) within different areas. All sections were evaluated by 2 independent observers. The averages were calculated.

Statistics

Demographics, clinical data, and results are presented as descriptive statistics. Data are expressed as mean or mean ±SD.

Results

Analysis of clinical data

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| Case (age/sex) | Disease duration (months) | Symptoms of onset | CK value (U/l) | Treatment | Outcome |
|---------------|---------------------------|------------------|---------------|-----------|---------|
| 1 (19/F)      | 12                        | Weakness         | 6344          | PSL, ATP  | Improve |
| 2 (23/F)      | 4                         | Weakness         | 21294         | PSL, MTX  | Remission |
| 3 (13/F)      | 0.3                       | Weakness, myalgia| 6673          | PSL       | Improve |
| 4 (19/M)      | 2                         | Weakness, dysphagia | 14102    | PSL       | Improve |
| 5 (11/M)      | 0.3                       | Weakness, myalgia, dysphagia, dyspnea | 25419 | PSL, TAC | Remission |
| 6 (24/M)      | 1                         | Weakness, myalgia| 271           | PSL       | Remission |
| 7 (47/F)      | 6                         | Weakness         | 7535          | PSL, CTX  | Remission |
| 8 (20/F)      | 2                         | Dyspnea          | 10923         | PSL       | Remission |
| 9 (52/F)      | 24                        | Weakness, amyotrophy | 2430   | PSL, ATP  | Remission |
| 10 (33/F)     | 8                         | Weakness, amyotrophy | 16      | PSL, TAC  | Remission |
| 11 (46/F)     | 2                         | Weakness         | 8252          | PSL       | Remission |
| 12 (54/F)     | 12                        | Weakness, dyspnea| 1099         | PSL, MTX  | Remission |
| 13 (12/M)     | 2                         | Weakness, myalgia| 30836        | PSL       | Remission |
| 14 (49/M)     | 2                         | Weakness         | 14255         | PSL       | Remission |
| 15 (32/M)     | 2                         | Weakness, myalgia| 4803          | PSL       | Remission |
| 16 (64/M)     | 1                         | Weakness         | 3806          | PSL       | Remission |
| 17 (46/F)     | 12                        | Weakness         | 1541          | PSL       | Remission |
| 18 (44/M)     | 12                        | Weakness         | 1104          | PSL       | Remission |
| 19 (39/F)     | 6                         | Weakness, dysphagia, | 638   | PSL       | Remission |
| 20 (26/M)     | 6                         | Weakness,         | 1241          | PSL       | Remission |
| 21 (37/M)     | 4                         | Weakness, amyotrophy | 1088    | PSL       | Remission |
| 22 (28/F)     | 4                         | Weakness         | 12335         | PSL, ATP/TAC | Improve |
| 23 (32/M)     | 12                        | Weakness, myalgia| 10177         | PSL       | Remission |
| 24 (66/F)     | 4                         | Weakness         | 4974          | PSL       | Remission |
| 25 (36/M)     | 24                        | Weakness, amyotrophy | 3202   | PSL, MTX, ATP | Remission |
| 26 (22/M)     | 1                         | Weakness, myalgia| 11057         | PSL       | Remission |
| 27 (30/M)     | 6                         | Weakness         | 1592          | PSL       | Remission |
| 28 (15/M)     | 0.1                       | Weakness, myalgia| 13470         | PSL       | Remission |
| 29 (18/M)     | 2                         | Weakness, myalgia| 1601          | PSL, TAC, IVIG | Fluctuation |
| 30 (55/F)     | 1                         | Weakness         | 4026          | PSL, CTX  | Remission |
| 31 (60/M)     | 0.3                       | Weakness, myalgia| 9187          | PSL       | Remission |
| 32 (31/F)     | 3                         | Weakness, myalgia| 6318          | PSL       | Remission |
| 33 (58/F)     | 12                        | Weakness         | 418           | PSL, IVIG, TAC | Remission |
| 34 (45/F)     | 2                         | Weakness, myalgia, dysphagia | 1790 | PSL, TAC | Remission |
| 35 (42/M)     | 24                        | Weakness, amyotrophy | 32      | PSL       | Remission |
| 36 (65/M)     | 2                         | Weakness, myalgia| 8463          | PSL       | Remission |
| 37 (59/M)     | 1                         | Weakness, amyotrophy | 2281   | PSL       | Remission |
| 38 (21/M)     | 12                        | Weakness         | 18640         | PSL       | Remission |
| 39 (54/F)     | 24                        | Weakness, amyotrophy | 5288   | PSL, CTX  | Exacerbation |
| 40 (50/M)     | 2                         | Weakness         | 3295          | PSL, IVIG, TAC | Fluctuation |

F – female; M – male; CK – creatine kinase; PSL – prednisolone; ATP – azathioprine; MTX – methotrexate; CTX – cyclophosphamide; TAC – tacrolimus; IVIG – intravenous immune globulin.
myofiber toxic drugs or statins or had previously been diagnosed with a tumor. In addition, there were 11 patients who already presented a chronic course (longer than 1 year) before they came to our hospital. Limb weakness was the initial presentation in all the patients. Among them, 14 patients complained of myalgia, 6 patients had muscle wasting and 4 had severe dysphagia and/or dyspnea. Furthermore, co-existing diseases were identified in some cases, including connective tissue disease (9 cases), interstitial pulmonary disease (2 cases), hepatic dysfunction (5 cases), chronic renal insufficiency (1 case), and myasthenia gravis (1 case). Laboratory investigations revealed that creatine kinase levels were elevated in 38 patients (mean: 7045.4, min: 16, max: 30836 U/l), except for 2 cases who displayed the normal range (3–190 U/l). Half of the patients showed elevated levels of antinuclear antibodies (such as anti-SSA and anti-RNP) and myositis-associated antibodies (such as anti-Ro-52). There were 39 patients who responded well to the treatments (prednisolone or in combination with other immunosuppressants), and only 1 case experienced exacerbating symptoms, possibly due to renal failure. The demographics and clinical data for the 40 patients are summarized in Table 1.

Predominant infiltration with macrophages in the muscle biopsies

The aim of this study was to distinguish IMNM from other myopathies by routine histochemical staining (Figure 1A–1D) in combination with clinical data analysis. Many researchers believe that IMNM is characterized by sparse infiltration of B cells and T cells, but infiltration with macrophages has been frequently observed in muscle biopsies [1]. In our study, the CD68+ macrophages were mainly accumulated in the necrotic muscle fibers and the endomysial tissue (Figure 1E, 1F). In contrast, in more than three-quarters (31: 9) of the samples displayed CD68+ macrophages in the necrotic fibers, and over three-fifths (27: 13) of the samples in the endomysial tissue (Figure 1G). In addition, a few CD4+ T cells were seen in the perimysium (16/40), while CD8+ T cells were scarce (1/40) but without CD20+ B cells in these samples.

Prominent expression of MHC-I and obvious deposition of MAC on capillaries and necrotic fibers in muscle biopsies

In our samples, we observed an upregulation of MHC-I expression in both the vessels and non-necrotic fibers.
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Table 2. Illustration of a semiquantitative scale based on immunohistochemical results of MHC-I and MAC.

| MHC-I | − | + | ++ | +++ |
|-------|---|---|----|-----|
| Necrotic fibers | 13 | 14 | 7  | 5   |
| Regenerative fibers | 26 | 9  | 5  | 0   |
| Normal fibers sarcolemmal | 3  | 31 | 4  | 2   |
| Perimysium vessels | 33 | 3  | 2  | 2   |
| MAC | 2 | 3 | 1 | 5   |

The deposition of membrane attack complex (MAC, C5b-9) was another important pathological feature of IMNM. The MAC deposition was evident on the small blood vessels (Figure 2G, 2I, 2J), consistent with previous reports [1,4]. Notably, the same IMNM cases displayed substantial MAC deposition on the necrotic fibers (Figure 2G), as well as the non-necrotic ones (Figure 2H). The detailed information for all the muscle biopsies is shown in Table 2.

**Discussion**

As a newly identified clinical entity, the immunopathological profiles of IMNM have not been fully elucidated [4,17,18]. The

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**Figure 2.** Expression of MHC-I and MAC deposition in IMNM. MHC-I is upregulated in vessels (A) and normal fibers (B) marked by arrows, necrotic fibers and regenerative fibers (C, D) compared with the normal muscle sample (E, F). MAC is deposited on endomysial vessels (arrows) and necrotic fibers (G), normal fibers (arrows) (H) and perimysium vessels (arrows) (I, J). MAC deposition is shown in red. Asterisk indicates necrotic fibers; arrows designate positive immunoreactivity; the triangle, indicates the regenerative fibers. Scalebar: 50 μm for panels A, C, E, G, I; and 20 μm for panels B, D, F, H, J.
findings in the cohort of 40 IMNM patients without exposure to statins suggest that in the development of IMNM, not only the complement system is activated, but that macrophages may also play an important role.

Because of the abundant myofiber necrotic debris, myophagocytosis is a scavenging process often seen in multiple myopathies, such as in necrotizing myopathy, autoimmune myopathies, toxic and paraneoplastic myopathies, and in some muscular dystrophies. Therefore, immunostaining with MHC-I and MAC is of further diagnostic value. The abnormal overexpression of MHC-I and the MAC deposition on different areas in muscle tissues such as in the non-necrotic, necrotic fibers, and endomysial vessels indicate the possibility of an immunemediated inflammatory process [4]. Furthermore, macrophages, as the predominant cells of infiltration in IMNM, were widely detected in the necrotic fibers and endomysial tissue, while the predominant inflammatory cells in other idiopathic inflammatory myopathies were T cells or B cells or/and macrophages [19]. In addition, the majority of these macrophages had a proinflammatory phenotype, as indicated by the presence of the classically activated CD68⁺ M1 macrophages. Interestingly, endophagocytosis of the normal-looking myofibers was frequently observed (9/40) (Figure 2H), suggesting the very early involvement of macrophages, before myofiber necrosis occurs. However, the detailed pathogenesis of the activated macrophages in IMNM, whether they simply act as scavengers of the necrotic debris resulting in imbalanced homeostasis, or whether they play a key role in upstream pathways leading to necrosis, needs to be investigated more thoroughly in future studies.

The up-regulation of MHC-I in inflammatory myopathies has been extensively reported, with various results in different studies [3,4,9]. Although some researchers did not report MHC-I overexpression in the sarcolemmal tissue of IMNM patients [3], others have shown very intense immunostaining against MHC-I on non-necrotic fibers [4,9], with the latter being supported by our latest observations. In addition, several muscle biopsy specimens in our study have shown an obvious up-regulation of the molecules on the perimysium vessels and regenerative fibers, which has not been described before. The pivotal role of up-regulation of MHC-I has already been suggested in a mouse model, showing that the self-sustaining autoimmune myositis can be successfully induced by conditional up-regulation of MHC-I, in combination with the production of myositis-specific antibodies [20]. It is important to further evaluate the involvement of MHC-I on regenerating muscle fibers in IMNM.

The deposition of MAC in IMNM samples has also been reported on a variety of tissue structures in different studies, including the typical deposition on myosial capillaries [1]. A previous study has observed an increased deposition only in necrotic myofibers [3]. However, there was an indication that MAC deposition on the sarcolemmal surface of non-necrotic fibers constituted a common feature of anti-HMGCR myopathy [9]. In contrast, our study showed that MAC was not only deposited strongly on the vessels, but also on the necrotic fibers and to a lesser extent on non-necrotic fibers. The different patterns of deposition sites of MAC in different reports may be due to sampling from different subtypes of IMNM. In the development of myofiber necrosis, the involvement of MAC suggests activation of the complement system, which could be elicited by proinflammatory cytokines [21–25]. Inhibiting activation of the complement system may be a method to reduce necrosis and to reduce the number of refractory cases in disease treatments, such as in the eculizumab treatment of dermatomyositis [26,27].

Our study did not establish any association between the muscle pathology and the myositis-specific antibodies in the cohort of patients with IMNM. We speculate that our cohort included patients with different subtypes of IMNM and different immunopathological profiles, such as the SRP-abs or other myositis-specific antibodies. Given the heterogeneity of the immunological features, the pathways or cell types involved in the necrotic process might be distinct. In future studies, enrollment of IMNM patients with similar immunological characteristics is the key to minimizing variability in the results.

Conclusions

In summary, our data describes the general immunopathological features of IMNM, the abnormal expression of MHC-I, and the deposition of MAC on the vessels, necrotic fibers, and non-necrotic fibers. Further studies on the role of macrophages are needed to elucidate the mechanism(s) of IMNM development.

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