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Role of the chemokine receptors CXCR3, CXCR4 and CCR7 in the intramuscular recruitment of plasmacytoid dendritic cells in dermatomyositis☆

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ABSTRACT

To explore the possible mechanism implicated in the recruitment of plasmacytoid dendritic cells (pDCs), we investigated the expression of the chemokine receptors CXCR3, CXCR4, and CCR7 on intramuscular and circulating pDCs from patients with dermatomyositis (DM). Using immunohistochemistry, preferential expression of CXCR3, CXCR4 and CCR7 was identified in the perivascular inflammatory infiltrates within the perimysium in DM muscle. Western-blot analysis showed marked up-regulation of expression of CXCR3, CXCR4 and CCR7 in muscle homogenate from patients with DM compared with that in non-diseased controls. Co-localization of CD303 + pDCs with these chemokine receptors was further examined by double immunofluorescence staining, which showed extensive co-localization of CD303 with CXCR3/CXCR4/CCR7 in DM biopsies. Flow cytometry was then used to investigate the proportion of pDCs among the total PBMCs and the expression of CXCR3, CXCR4 and CCR7 on circulating pDCs. The proportion of CD123 + CD303 + pDCs in peripheral blood from DM patients was markedly decreased compared to that from polymyositis (PM) patients and normal controls. Significantly increased expression of CXCR3, but not CXCR4 or CCR7, was further identified on circulating pDCs in DM. Correlation analysis showed that the expression of CXCR3 correlated inversely with the frequency of pDCs in peripheral blood. Our findings indicate that the chemokine receptors, CXCR3, CXCR4 and CCR7 may be involved in the recruitment of pDCs from peripheral blood to muscle tissues in DM via different mechanisms, and in which CXCR3 may play an important role under DM conditions.

1. Introduction

Dermatomyositis (DM) is an acquired systemic inflammatory disease characterized by subacute onset of symmetric and progressive proximal muscle weakness, specific skin rash, inflammatory infiltrates within the muscle and pathological perifascicular atrophy. To date, the immunopathogenesis of DM has not been definitely elucidated.

For a long time, DM has been modeled as a humoral immune disease in which autoantibodies directed against the endothelium cause intramuscular vascular injury, leading to ischemic myofiber damage (Dalakas and Hohlfeld, 2003). However, the pathogenic autoantibodies, which were postulated to target a specific endothelial antigen, remain elusive. In the past few decades, many studies have suggested that type I interferons (IFN-I), a class of molecules including interferon-α (IFN-α) and interferon-β (IFN-β), also play an important role in the pathogenesis of DM (Baechler et al., 2011; Lundberg and Helmers, 2010) as well as several other autoimmune diseases (Galicia and Gommerman, 2014; Ganguly et al., 2013). Plasmacytoid dendritic cells (pDCs), characterized as CD4 + CD68 + CD123 + CD303 + CD304 + CD11 − (Blomberg et al., 2003; Grouard et al., 1997), were detected in muscle (Greenberg et al., 2005) and skin (Wenzel et al., 2006) from DM patients. With the inherent capacity to produce over 1000 times the amount of IFN-α of any other known cell type, pDCs are also known as natural IFN-α producing cells (NIPCs) (Siegal et al., 1999). Therefore, the abnormal accumulation of pDCs in the perimysium and endomysium was considered to be a primary intramuscular source of IFN-I in DM.

In spite of these advances, the origin and recruitment mechanism of intramuscular pDCs remain unknown. A study of systemic lupus erythematosus (SLE) found that the frequency of circulating pDCs from SLE patients was reduced 70-fold compared to control individuals, which suggested that the pDCs in peripheral blood may be activated and recruited to tissues under SLE conditions (Cederblad et al., 1998). A

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later study supported this hypothesis by detecting a large number of pDCs in dermal lesions of SLE patients (Blomberg et al., 2001).

Various chemokine receptors and their corresponding ligands play an important role in the maturity and migration of pDCs (Sozzani, 2005). In a study analyzing the expression of chemokine receptors on circulating dendritic cells, CXCR3 and CCR7 were highly expressed on pDCs, and CXCR4 was expressed both on pDCs and myeloid dendritic cells (mDCs) (Penna et al., 2001). However, the role of CXCR3, CXCR4 and CCR7 in the recruitment of intramuscular pDCs in DM needs further investigation.

In this study, we explored the possible origin and recruitment mechanism of pDCs within the muscle tissues of DM patients by analyzing the expression of CXCR3, CXCR4 and CCR7 on intramuscular and circulating pDCs.

2. Materials and methods

2.1. Patients

Muscle specimens were obtained retrospectively from October 2010 to May 2015 at the Neuromuscular Institute of Qilu Hospital affiliated to Shandong University. Twenty-six muscle specimens including 12 newly-diagnosed adult DM patients (eight females, four males) and 16 controls were selected, of which 10 patients diagnosed with definite polymyositis (PM) (six females, four males) were taken as disease controls. Six biopsies were taken from patients who were initially suspected of having neuromuscular disorders, but showed normal histological findings and these were taken as non-disease controls (four females, two males).

Meanwhile, 10 mL fasting peripheral venous blood was collected from each of the patients in another series of 12 DM patients (eight females, four males). Five definite PM patients (four females, one male) and five healthy volunteers (three females, two males) were taken as disease and normal controls, respectively.

All patients with DM and PM (both biopsy donors and blood sample donors) fulfilled Bohan and Peter's criteria (Bohan and Peter, 1975a, 1975b). The patients whose diseases were complicated with other autoimmune diseases and/or infectious diseases were excluded by clinical and laboratory studies. None of the patients had received immunosuppressive agents within the 3 months prior to the biopsy or blood-sampling.

The study protocol was approved by the institutional review board of Qilu Hospital affiliated to Shandong University. All patients included in the study provided written informed consent.

2.2. Muscle biopsies and immunohistochemistry

Frozen biopsied muscles were sectioned and fixed in ice-cooled acetone, then stained using the avidin-biotin complex technique, with 3,3′-diaminobenzidine as a chromogen (brown) and counterstained with hematoxylin. Primary antibodies were against CXCR3, CXCR4 or CCR7 (Table 1). Negative controls were stained using isotype-specific nonimmune IgG. For all muscle specimens, histological evaluation was carried out using standard hematoxylin and eosin staining.

2.3. Immunofluorescence double staining

For double immunofluorescence, the frozen muscle tissue sections (6 μm thick) were fixed in ice-cooled acetone and incubated with primary antibodies against CD303 in combination with anti-CXCR3, -CXCR4 or -CCR7 antibodies (Table 2), followed by the application of fluorescein isothiocyanate-conjugated secondary antibody. DAPI was used as a nuclear marker. As a negative control, isotype-specific nonimmune IgG was applied as the primary antibody. Images were acquired using DP manager software, version 3.1.1 2008 (Olympus Corp., Tokyo, Japan) and merged using Image-ProPlus software, version 5.1.0.20 (Media Cybernetics, Bethesda, MD, USA).

2.4. Western blotting

Western blotting was performed on muscle homogenates. Specific primary antibodies against CXCR3, CXCR4 and CCR7 were used (Table 2) with β-actin serving as internal standard. Aliquots containing 40 μg protein were loaded onto a 10% SDS polyacrylamide gel for electrophoresis. The signal was detected by enhanced chemiluminescence using a western blotting luminol reagent (Millipore, Darmstadt, Germany). The mean gray values of protein bands were measured by ImageJ 1.46r software (National Institutes of Health, Bethesda, MD, USA).

2.5. Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation over lymphocyte separation medium (Tbdscience, Tianjin, China). For surface staining, suspensions of PBMCs were incubated in the dark for 20 min at 4 °C with anti-CD123 and -CD303 antibodies to identify pDCs, in combination with anti-CXCR3, -CXCR4 or -CCR7 (Table 2). The isotype IgG of same-species origin was used as a control. After staining, cells were analyzed on a Beckman Gallios Flow cytometer (Beckman Coulter, Miami, FL, USA). In all cases, 10,000 events were acquired corresponding to live mononuclear cells as assessed by forward and side light-scatter profile. The expression of CXCR3, CXCR4 and CCR7 was determined as geometric mean fluorescence intensity (GMFI) by further gating on the pDC population (identified by CD123 + CD303 +). Data analysis was performed using Gallios Cytometry List Mode Data Acquisition & Analysis Software (Beckman Coulter).

Table 1
Major antibodies used for IHC, IF, WB and FC.

Primary Ab	Clone	Species	Dilution (IHC/IF)	Dilution (WB)	Dilution (FC)	Company
CD303	Polyclonal	Rabbit	1:100	–	–	PL laboratory
CXCR3	EPR7469(B)	Rabbit	1:200	1:5000	–	Abcam
CXCR4	Polyclonal	Rabbit	1:100	1:2000	–	Abcam
CCR7	Y59	Rabbit	1:200	1:5000	–	Abcam
β-Actin	C4	Mouse	–	1:1000	–	Santa Cruz
CD123	AC145	Mouse	–	–	5 μL/10^6 cells	Miltenyi
CD303	AC144	Mouse	–	–	5 μL/10^6 cells	Miltenyi
CXCR3	CXCR3–173	Mouse	–	–	5 μL/10^6 cells	Biolegend
CXCR4	12G5	Mouse	–	–	5 μL/10^6 cells	Biolegend
CCR7	G043H7	Mouse	–	–	5 μL/10^6 cells	Biolegend

IHC = immunohistochemistry; IF = Immunofluorescence; WB = western blot; FC = flow cytometry.