Abstract  The idiopathic inflammatory myopathies (IIM), including dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM), are a group of autoimmune diseases characterized by chronic lymphocytic and macrophagic infiltration in muscle. The mechanism for recruitment of these cells probably involves chemokines. We have previously reported that monocyte chemokine protein-1 (MCP-1), a β chemokine, seems to play a major role in mononuclear cell recruitment especially in DM. Here we have investigated the distribution of the main MCP-1 receptors CCR2A and CCR2B in IIM by polymerase chain reaction (PCR), immunohistochemistry and in situ hybridization. We have shown by reverse transcription-PCR that both CCR2A and CCR2B were expressed at low level in normal muscle and that CCR2A was up-regulated in IIM (P=0.02) and was higher in PM and IBM than in DM (P=0.04). By immunohistochemistry and in situ hybridization we have observed that CCR2 isoforms were expressed by different cell subsets in both normal and IIM muscle. CCR2A was expressed in vessel walls and by some mononuclear cells, especially in cells involved in partial invasion in PM and IBM. CCR2B expression was observed in all satellite cells, in the muscular domain of neuromuscular junctions and in some regenerative fibers of IIM, but not in inflammatory exudates. In conclusion, the present study highlights the major role played by MCP-1 and its counter-receptor CCR2 in the pathophysiology of IIM, and shows that the CCR2 receptors are cell specific. The variation of the total amount of CCR2A and its local distribution according to the type of IIM might be a new path towards the understanding of the constitution of mononuclear infiltrates in IIM.

Keywords  CCR2 · Regeneration · Dermatomyositis · Polymyositis · Inclusion body myositis

Introduction

The idiopathic inflammatory myopathies (IIM) including dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM) are a heterogeneous group of diseases morphologically characterized by chronic lymphocytic and macrophagic infiltration in muscles [4, 10].

In DM, perivascular inflammatory exudates, mainly composed of CD4+ T lymphocytes, B lymphocytes and macrophages, are observed. The main immune effector response appears to be humoral and directed against the microvasculature. Membrane attack complex (MAC) deposits are observed within the intramuscular capillaries [24]. In PM and IBM, cytotoxic CD8+ T cells and macrophages focally surround and invade non-necrotic muscle fibers expressing major histocompatibility complex class I [13, 16, 23].

Numerous studies have shown that cytokines play a major role in autoimmune diseases including IIM. We and others have shown strong proinflammatory cytokine expression, predominant Th1 immune response and up-regulation of cell adhesion molecule expression in IIM [12, 26, 27, 29]. In addition, strong expression of β-chemokines controlling at least monocytes and T cell migration has been reported in IIM [1]. Monocyte chemoattractant protein-1 (MCP-1), the major β-chemokine is highly expressed in IIM [9, 28]. Moreover, we have observed that mRNA expression was highest in DM and that local expression of MCP-1 was different in each IIM. Perivascular inflammatory cells highly expressed MCP-1 mRNA in DM, whereas it was strongly expressed by mononuclear cells partially invading non-necrotic muscle fibers in both PM and IBM. These results suggest that MCP-1 plays a major role in the local accumulation of discrete cell subsets in these diseases [28]. Because chemokines bind and activate specific transmembrane receptors expressed by target cells, it was relevant to search for MCP-1 receptor.
(CCR2) expression in IIM. CCR2 exists as two isoforms CCR2A and CCR2B by alternative splicing of a single gene [7]. CCR2 is closely related to the MIP-1α/RANTES receptor, transduces signals in response to nanomolar concentrations of MCP-1 in a highly specific manner and appears to be the high-affinity MCP-1-specific receptor predicted by the pharmacological studies [7]. CCR2 also binds MCP-2, MCP-3 and MCP-4, but with a lower affinity [18, 20]. CCR2A and CCR2B differ by their C-terminal tails [7]. This probably represents a mechanism to increase the diversity of cellular responses to this important chemokine. To investigate the role of CCR2 in the immune response in human IIM, we have studied the expression of its two isoforms CCR2A and CCR2B by reverse transcription (RT)-PCR in a series of muscle biopsy specimens from patients suffering from IIM. Moreover, we report the cellular distribution of both CCR2A and CCR2B using immunohistochemistry and the focal tissue distribution of CCR2A mRNA by in situ hybridization.

Materials and methods

Human muscle specimens

Muscle biopsy specimens were obtained for diagnostic purposes. None of the patients had received corticosteroids or immunosuppressive therapy at the time of muscle biopsy. The diagnoses were based on conventional criteria. Eight cases were classified as DM (four men and four women, mean age 22 years, range 2–42 years), five cases as PM (three men and two women, mean age 48 years, range 22–74 years), and four cases as IBM (two men and two women, mean age 70 years, range 61–79 years). The mean duration of disease before diagnosis was 28 days for DM cases, 4 months for PM cases, and 5 years for IBM cases. All muscle samples were frozen in isopentane, cooled in liquid nitrogen and stored at −80°C until required. Muscle specimens from four patients biopsied for investigation of familial malignant hyperthermia susceptibility and showing negative contracture test results served as negative controls. Human tonsils was used as positive controls for CCR2A and CCR2B expression.

RNA extraction, PCR amplification and radioactive hybridization

Total cellular RNA was extracted from about ten 25-µm sections of each muscle specimen with the total quick RNA cell and tissue extraction kit from Euromedex (Souffelweyersheim, France). RT was performed from 26 (G3PDH) and 35 (CCR2A and CCR2B) amplification cycles, each cycle consisting of 95°C for 10 s, 65°C (G3PDH) or 46°C (CCR2A) for 15 s and 72°C for 8 s (G3PDH) or 10 s (CCR2A and CCR2B). The 311-bp (CCR2A) and 277-bp (CCR2B) products were purified with the Geneclean kit (Bio 101, La Jolla, Calif.) and subcloned into a pGEM-T vector (Promega, Madison Wis.), sequenced to verify specific amplification, and used as probes for RT-PCR Southern blots. PCR products were electrophoresed in 2% agarose gels (Nusieve, TEBU, Le Perray en Yvelines, France) and the bands were visualized with ethidium bromide staining. The size of the PCR products was determined using DNA molecular weight marker VI (Boehringer, Mannheim, Germany).

PCR products were transferred onto the Hybond-N nylon membrane (Amersham, Les Ulis, France) and fixed by UV cross-linking. Probe was [32P]dCTP-labeled (Amersham) using the “Non-Primer Kit II” (Appligene, Illkirch, France). Hybridizations were done at 65°C overnight in a 6x SSC (standard sodium citrate), 5× Denhardt, and 0.5% SDS solution. Washes were performed as follows: 30 min in 0.5× SSC-0.1% SDS and 30 min in 0.1× SSC-0.1% SDS. Signals were detected by exposing the membrane to X-ray-sensitive films (Eastman Kodak, Rochester, N.Y.); intensity analysis of the amplified bands was performed using the “NonPrimer Kit II” (Appligene, Illkirch, France) and the appropriate computer program (NIH image 1.61). Statistical analysis was performed using the Mann and Whitney test to compare subgroups of patients.

The “SureSite II in Hybridization System” kit (Novagen, Oxon, UK) was used for this experiment. For CCR2A, a template consisting of the 311-bp complementary DNA in the expression vector pGEM-T was used for riboprobe synthesis. pGEM-T vector was linearized with the restriction enzymes NdeI and Ncol (Biolabs, Montagny le Bretonneux, France) as recommended by the manufacturer and Sp6 and T7 RNA polymerases were used to synthesize sense and antisense pGEM-TP riboprobe, respectively, according to the manufacturer’s instructions. The probes were labeled with [35S]UTP (Amersham) and purified. They were electrophoresed on formaldehyde gel, transferred onto Hybond-N nylon membrane, and exposed to X-ray-sensitive films to monitor their integrity.

Several cryostat sections, 5 µm thick, were used for in situ hybridization. Slides were coated with 3-aminopropyl-triethoxy-silane, fixed in 4% paraformaldehyde for 20 min, treated with proteinase K (5 µg/ml) for 5 min, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, and rinsed in PBS. Slides were then submerged in prehybridization solutions. Hybridization was carried out overnight at 50°C in a moist chamber with 5×105 dpm per slide. After hybridization, the slides were washed according to the manufacturer’s instructions. Tissue sections were dipped in liquefied NTB-2 emulsion (Eastman Kodak) and exposed at 4°C in boxes containing desiccant for 4 weeks before being developed (Kodak D 19 developer), counterstained with Mayer’s hematoxylin, and coverslipped. Sense probes were used in each experiment to stain adjacent sections under the same experimental conditions.

Immunohistochemistry

Immunohistochemistry was performed on serial 5-µm-thick frozen sections in all muscle specimens. The first one was stained with hematoxylin and eosin. The others were treated for anti-CCR2A (1/50) and CCR2B (1/20) (Santa Cruz, Calif.) or used as a negative control. For that, ten times the blocking peptide (pCCR2A or pCCR2B) was added to 4 μg/ml and 10 μg/ml of the diluted primary antibody, respectively, and incubated overnight at room temperature. Then, instead of the primary antibody, the mixture of the blocking peptide and the primary antibody was applied to the section, which was then processed by conventional immunohistochemistry. In addition, im-