Activated IL-22 pathway occurs in the muscle tissues of patients with polymyositis or dermatomyositis and is correlated with disease activity

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Abstract
Objective. The aim of this study was to assess the expression of IL-22, IL-22 receptor 1 (IL-22R1), IL-22 binding protein (IL-22BP) and p-STAT3 in muscle tissue from patients with PM and DM.

Methods. Levels of IL-22, IL-22R1, IL-22BP and STAT3 mRNA were quantified by RT-PCR. The expression of IL-22, IL-22R1, IL-22BP and p-STAT3 was also analysed using immunohistochemistry.

Results. Significant modulation of the IL-22 pathway was observed in inflammatory myopathic tissues. In particular, a significant overexpression of IL-22 at the protein but not the mRNA level was observed in PM/DM tissues and was correlated with myositis activity. IL-22R1 aberrant expression was also observed among infiltrating mononuclear cells and necrotic muscle cells. IL-22BP, which inhibits IL-22 signalling, was expressed only in some muscle fibres in PM/DM patients.

Conclusion. Our findings indicate that the IL-22 pathway is activated in inflammatory myopathic tissues and may be involved in the induction of muscle inflammatory processes and muscle necrosis.

Key words: IL-22, IL-22R1, IL-17, p-STAT3, polymyositis, dermatomyositis.

Introduction
PM is an idiopathic inflammatory myopathy that causes symmetrical, proximal muscle weakness with elevated skeletal muscle enzyme levels and characteristic EMG and muscle biopsy findings [1]. Although an autoimmune origin has been hypothesized on the basis of histopathological findings demonstrating the involvement of activated Th1 and Th17 cells in the pathophysiology of the disease [2, 3], the pathogenesis of PM remains to be elucidated.

IL-22 is an important cytokine that may play either a protective or a pathological role in several autoimmune diseases, such as primary SS and RA [4, 5]. IL-22 is primarily produced by activated T cells, mainly Th1, Th17 and Th22 cells, and mediates its effect through two receptors, IL-10Rb, which is ubiquitously expressed, and the heterodimeric receptor IL-22R1, which is restricted to non-lymphoid cells. Upon binding to its receptors, IL-22 can initiate inflammatory immune responses through the induction of STAT3 phosphorylation [6]. However, the functional outcome of IL-22 seems to depend also on the presence of a natural inhibitor of IL-22 activity, the IL-22 binding protein (IL-22BP), which functions neutralizing nearly all IL-22 activity.

As yet, it is not clear whether dysregulation of the IL-22/IL-22R1 axis also occurs in PM patients. The study presented here was designed to examine the expression of IL-22/IL-22R1 and p-STAT3 in patients with PM and to evaluate the eventual association of IL-22 with clinical features. Our results showed that an imbalance between IL-22 and IL-22BP occurs and that IL-22R1 and p-STAT3 were overexpressed in the inflamed muscles of...
PM and DM patients. We also provide a demonstration that IL-22R1 aberrant expression occurred in myeloid cells infiltrating the inflamed muscles, suggesting that the IL-22/IL-22R1 axis may be involved in the pathogenesis of PM.

Patients and methods

Patients

Muscle biopsies were obtained from 10 consecutive patients with myositis (7 with PM and 3 with DM), fulfilling the Bohan and Peter criteria [7]. All the patients with PM/DM had characteristic inflammatory lesions observed on muscle biopsy. As controls, muscle tissue samples from five healthy subjects undergoing joint replacement surgery were obtained. The muscle weakness of patients with myopathy was measured by using the manual muscle test (MMT) (0–5-point scale, selecting 10 muscles including deltoid, biceps, triceps, quadriceps and hamstring, both right and left sides, with a maximum point score of 50, which indicates full muscle strength) [8]. For histology and immunohistochemical analysis, tissues were immediately fixed with 4% formaldehyde and embedded in paraffin until required. The study was approved by the ethics committee of the University of Palermo and written informed consent was obtained from all patients and controls.

Real-time PCR

Immediately after biopsy, samples were stored in RNAlater® solution (Applied Biosystems, Foster City, CA, USA) and processed as previously described [4]. The technique for extraction of RNA was equally efficient for normal versus diseased myofibres. For quantitative TaqMan real-time RT-PCR, sets of primers and probes were obtained from Applied Biosystems. Samples were run in triplicate using the Step-One RT-PCR system (Applied Biosystems). Relative changes in gene expression between controls and patients were determined using the ΔΔCt method as previously described. Final values were expressed as fold of induction.

Immunohistochemistry

Immunohistochemistry for IL-22, IL-22RA1 and p-STAT3 was performed on 5-μm-thick paraffin-embedded sections from muscle biopsies and from tonsils (used as positive controls) as previously described [4]. Isotype-matched irrelevant antibodies were used as a negative control. The primary antibodies rabbit anti-human IL-22 (1:100 dilution; Novus Biological, Littleton, CO, USA), rabbit anti-human IL-22R1 (1:100 dilution; Novus Biological), rabbit anti-human p-STAT3 (1:50 dilution; Cell Signaling Technology, Beverly, MA, USA), rabbit anti-human IL-17 (1:100 dilution; R&D Systems, Minneapolis, MN, USA) and rabbit anti-human IL-22BP (1:100; Novus Biological) were added and incubated for 1 h at room temperature. In control experiments, goat IgG or matched mouse IgG isotype was applied at the same concentration as the primary antibodies. The slides were incubated for 30 min with peroxidase-conjugated Dako EnVision polymer and peroxidase activity was visualized using diaminobenzidine chromogen (Dako, Glostrup, Denmark). Slides were lightly counterstained with haematoxylin before dehydration and mounting in DePex (VWR International, Oslo, Norway). The number of IL-22-expressing cells was evaluated by counting the immune-reactive cells on photomicrographs obtained from three random high-power microscopic fields (original magnification 400×) using a Leica DM2000 optical microscope and a Leica DFC320 digital camera.

Statistical analysis

Statistical analysis of quantitative variables was performed using the Mann-Whitney rank sum test. Pearson’s correlation analysis was utilized to quantify expression associations between the genes of interest. Data are expressed as mean (s.d.) and P-values <0.05 were considered significant.

Results

IL-22, IL-22R1 and p-STAT3 expression in myositis tissue

No clinical differences in the entity of muscle involvement were observed between DM and PM patients. The IL-22 axis activation in the muscle of patients with myositis was first examined by RT-PCR. As shown in Fig. 1, the mRNA expression of IL-22 (Fig. 1A), IL-22BP (Fig. 1B) and STAT3 (Fig. 1C) was not significantly up-regulated in inflamed muscle of PM and DM patients compared with normal muscle tissue. In contrast, the inducible subunit of the IL-22 receptor IL-22RA1 was strongly up-regulated in muscle tissue from patients with PM and DM (Fig. 1D).

Immunohistochemical analysis of IL-22 expression in myositis tissues

The expression of the IL-22 axis was also evaluated by immunohistochemistry in the inflamed muscle tissues of patients with myositis and the expression pattern was compared with normal muscle tissues. In spite of the absence of modulation of the transcription levels, intense IL-22 expression was observed in inflamed muscle (Fig. 1E–G). IL-22 immunoreactivity was mainly detected in inflammatory infiltrating mononuclear cells from PM muscle tissue and in the blood vessels located near inflammatory infiltrates (Fig. 1F). In contrast, in DM patients IL-22 expression was detected among infiltrating cells surrounding muscle fibres in DM (Fig. 1G). No significant IL-22 expression was detected in normal muscle tissues (Fig. 1E).

To determine whether the level of expression of IL-22 could be related to myositis activity, we examined the correlation between the number of IL-22-expressing cells and the serum creatinine kinase or MMT score. As shown in Fig. 1, the number of IL-22-expressing cells in patients with PM and patients with DM was significantly correlated with creatinine kinase (r² = 0.9, P < 0.0001) and the MMT score (r² = −0.92, P < 0.0001) (Fig. 1I and J, respectively). Since IL-22 has been demonstrated to be produced by Th17 cells and these cells are increased in
Relative mRNA quantification of IL-22, IL-22BP, STAT3 and IL-22R1 was assessed by quantitative RT-PCR in muscle biopsy specimens from patients with PM/DM and controls. (A) IL-22 mRNA expression in the muscle of patients and controls. (B) IL-22BP mRNA expression in the muscle of patients and controls. (C) STAT3 mRNA expression in the muscle of patients and controls. (D) IL-22R1 mRNA expression in the muscle of patients and controls. (A–D) Data are shown as mean (±D). (E–G) Representative microphotographs showing IL-22 immunostaining in (E) healthy controls, (F) PM and (G) DM patients. (H) Representative microphotographs showing isotype antibody staining of myositis tissue sample. (I–L) Correlation between the number of IL-22-positive cells and (I) creatine phosphokinase levels and (J) manual muscle test (MMT) scores. The $r^2$ and $P$-values were determined with the Pearson correlation coefficient. (K) Representative microphotographs showing IL-17 immunostaining in PM patients. (L) Quantification of IL-22-positive and IL-17-positive cells in muscle of PM/DM patients; data are shown as means. (E–H, K) Original magnification 250×. *$P < 0.001$; **$P < 0.05$. Findings are representative of three independent experiments.
PM/DM patients, we also evaluated the expression of IL-17. IL-17 expression was also considerably overexpressed in myocytes and among inflammatory infiltrates in myositis (Fig. 1K), but there was not a perfect co-localization between IL-17- and IL-22-producing cells, as the percentage of IL-22-producing cells was significantly higher (Fig. 1L).

IL-22R1, p-STAT3 and IL-22BP are differentially expressed in myositis tissues

We next evaluated the expression of the receptor IL-22R1 in inflamed muscles. As shown in Fig. 2, increased expression of IL-22R1 was observed in the muscle of PM/DM patients compared with controls. Two different patterns of tissue expression were observed in myositis patients. The first pattern, common to DM and PM patients, was observed on the surface of degenerated, necrotic myocytes (Fig. 2B and C). The second pattern was immunologically more relevant, being characterized by the aberrant expression of IL-22R1 in the inflammatory infiltrates. PM patients displayed IL-22R1 expression on microvessels distributed at the periphery of inflammatory infiltrates (Fig. 2B) and among infiltrating myeloid cells scattered throughout the perivascular inflammatory infiltrates (Fig. 2B). In DM, IL-22R1 immunoreactivity was mainly observed among infiltrating cells surrounding muscle fibres (Fig. 2C). Since the signal of IL-22/IL-22R1 is mediated through the phosphorylation of STAT3 [6], we also studied the muscle expression of p-STAT3. p-STAT3 expression was clearly detected among infiltrating perivascular mononuclear cells of PM (Fig. 2F) and at the periphery of muscle fibres in DM patients (Fig. 2G), but not in control muscle (Fig. 2E), suggesting a functional relevance of the overexpression of IL-22/IL-22R1 in the inflamed muscle of PM/DM patients. Finally, since the biologic activity of secreted IL-22 depends also on the level of IL-22BP, we also evaluated the expression of IL-22BP in muscle biopsies from patients and controls. As shown in Fig. 2, some degree of IL-22BP expression was observed among sporadic intact muscle fibres only in PM patients compared with control subjects and DM patients (Fig. 2J and K), whereas no IL-22BP immunoreactivity was observed in the context of inflammatory infiltrates (Fig. 2J and K).

Discussion

IL-22 is a member of the IL-10 family of cytokines, which has often been viewed as a Th17 cytokine even if it is produced by a wide variety of cells of both the adaptive and innate immune systems, sometimes independently of the Th17 response. Depending on the target tissue and the cytokine milieu, IL-22 may be pathogenic rather than protective. To avoid pathology, IL-22 signalling is tightly controlled, with the IL-22R1 subunit expression being restricted only to epithelial cells and IL-22 signalling further regulated by interactions with the IL-22 soluble binding protein [6].

In this study we demonstrated for the first time significantly altered expression of the IL-22 axis in the muscle of PM/DM patients. IL-22 was in fact significantly up-regulated at the protein level in PM/DM patients and significantly correlated with the degree of myositis activity. The high levels of IL-22 were not counterbalanced by a comparable up-regulation of its natural inhibitor, IL-22BP, suggesting the presence of a severe imbalance of IL-22/IL-22BP in inflammatory myopathies. The high levels of protein expression and the absence of IL-22 mRNA modulation observed may suggest a role of IL-22 in negatively modulating its own expression in inflamed muscle or a post-transcriptional regulation of protein levels. IL-22 has been widely regarded as a Th17 cytokine, even in human CD4+ T cells, and does not correlate with the expression of either IL-17 or RORc [9, 10]. The analysis of the distribution of IL-22-expressing cells in our study demonstrated that despite the presence of a population of IL-22/IL-17 co-expressing cells (presumably Th17 cells), a good percentage of cells expressing only IL-22 was clearly observable in inflamed muscles, suggesting that the IL-22 response may not be completely related to the Th17 cells in PM/DM patients.

The results of this study also contribute to the understanding of the immunoregulatory properties of the IL-22 axis in muscle by providing the relevant observation that IL-22R1 was specifically expressed among infiltrating IL-22-producing mononuclear cells. The IL-22R1 subunit is expressed mainly on intestinal and respiratory epithelial cells, keratinocytes and hepatocytes, but not on cells of haematopoietic origin [6]. The aberrant expression of IL-22R1 on cells of haematopoietic origin in inflamed muscles may represent a mechanism of self-perpetuation of inflammation in PM/DM.

Although the expression of IL-22R1 has been found on airway smooth muscle cells, no expression of IL-22R1 has been demonstrated in skeletal muscle cells [11], and in our study IL-22R1 expression was only observable on the surface of degenerated, necrotic myocytes. Cytokines can affect different stages of myocyte development and proinflammatory cytokines are known to mediate muscle atrophy in many chronic disease states [12]. In this regard, the expression of IL-22R1 in myocytes may suggest a role of IL-22/IL-22R in the induction of muscle damage. Finally, intense IL-22R1 expression was observed in endothelial cells located at the periphery of inflammatory infiltrates. Molecular changes of blood vessels have been described in patients with PM/DM and proposed as primary events in the development of myositis [13]. IL-22 and its receptor have been demonstrated to be expressed in inflammatory conditions and IL-22 signalling seems to be able to disrupt blood–brain barrier tight junctions in vitro and in vivo [14]. Thus the increased expression of IL-22/IL-22R1 in endothelial cells may represent an important step in promoting transendothelial migration of inflammatory cells to the inflamed muscles.

Since STAT3 is crucial and most likely sufficient for IL-22 signalling [6], the functional relevance of IL-22R1 expression was studied by evaluating the expression of p-STAT3 in the inflamed muscle of PM/DM patients. Although no modulation of STAT3 was observed by
Paraffin-embedded sections (5-μm thick) of muscle biopsy specimens obtained from DM/PM patients and controls were stained with anti-IL-22R1, anti-IL-22BP and anti-p-STAT3 antibodies. (A-C) Representative microphotographs showing IL-22R1 immunostainings in (A) healthy controls, (B) PM and (C) DM patients. (E-G) Representative microphotographs showing p-STAT3 immunostainings in (E) healthy controls, (F) PM and (G) DM patients. (I-K) Representative microphotographs showing IL-22BP immunostainings in (I) healthy controls, (J) PM and (K) DM patients. (D, H, L) Representative microphotographs showing staining with isotype antibodies. (A-L) Original magnification 250×. Findings are representative of three independent experiments.
RT-PCR, the phosphorylated form of STAT3 was significantly up-regulated in PM/DM patients and specifically expressed by IL-22R1-expressing cells, providing evidence that an autocrine inflammatory loop is functional in the inflamed muscle of PM/DM patients.

In conclusion, our results indicate that a severe imbalance of IL-22/IL-22BP is characteristic of inflammatory myopathy and is accompanied by a non-conventional expression of IL-22R1 on cells of haematopoietic origin. Although our results may suggest involvement of the IL-22 pathway in the pathogenesis of autoimmune muscle disorders, this study provides no clue as to how or why the inflammation is present. Further studies are clearly required to better clarify the role of IL-22 in the pathogenesis of muscle inflammation and to evaluate whether IL-22 expression may be used as a biomarker of response to treatment or prognosis in patients with inflammatory myopathy.

Rheumatology key messages
- The IL-22 pathway is activated in inflamed muscle of DM/PM patients.
- An imbalance of IL-22/IL-22BP seems to characterize inflammatory myopathy.
- The IL-22 pathway may represent a therapeutic target in DM/PM patients.

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