European Journal of Cell Biology 91 (2012) 139–149

Contents lists available at SciVerse ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.de/ejcb

Inflammatory response in human skeletal muscle cells: CXCL10 as a potential therapeutic target

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ARTICLE INFO

Article history:
Received 9 June 2011
Received in revised form 9 August 2011
Accepted 20 September 2011

This work is dedicated to the memory of Prof. Mario Serio.

Keywords:
Human skeletal muscle cells
Inflammation
CXCL10
Biomolecular target(s)
Immunosuppressants
Inflammatory myopathies
Th1 polarization
Autoimmunity

ABSTRACT

Inflammatory myopathies (IMs) are systemic diseases characterized by a T helper (Th) 1 type inflammatory response and cell infiltrates within skeletal muscles. The mainstay of treatment is drugs aimed at suppressing the immune system – corticosteroids and immunosuppressants. About 25% of patients are non-responders. Skeletal muscle cells seem actively involved in the immune-inflammatory response and not only a target; understanding the molecular bases of IMs might help drug development strategies.

Within muscles the interaction between the chemokine interferon (IFN)γ inducible 10 kDa protein, CXCL10 or IP-10, and its specific receptor CXCR3, present on Th1 type infiltrating cells, likely plays a pivotal role, potentially offering the opportunity for therapeutic intervention.

We aimed to clarify the involvement of human skeletal muscle cells in inflammatory processes in terms of CXCL10 secretion, to elucidate the engaged molecular mechanism(s) and, finally, to evaluate muscular cell responses, if any, to some immunosuppressants routinely used in IM treatment, such as methylprednisolone, methotrexate, cyclosporin A and Infliximab.

We first isolated and characterized human fetal skeletal muscle cells (Hfsmc), which expressed the specific lineage markers and showed the competence to react in the context of an *in vitro* alloresponse.

CXCL10 protein secretion by Hfsmc was similarly induced by the inflammatory cytokines interferon (IFN)γ and tumor necrosis factor (TNF)α, above undetectable control levels, through the activation of Stat1 and NF-kB pathways, respectively; CXCL10 secretion was significantly magnified by cytokine combination, and this synergy was associated to a significant up-regulation of TNFαRII; cytokine-induced CXCL10 secretion was considerably affected only by Infliximab.

Our data suggested that human skeletal muscle cells might actively self-promote muscular inflammation by eliciting CXCL10 secretion, which is known to amplify Th1 cell tissue infiltration *in vivo*. In conclusion, we sustain that pharmacological targeting of CXCL10 within muscular cells might contribute to keep in control pro-Th1 polarization of the immune/inflammatory response.

Introduction

Inflammatory myopathies (IMs) are chronic autoimmune diseases characterized by decreased muscle endurance and symmetrical muscle weakness (Grundtman et al., 2007). On the basis of their different immunopathologies, clinical features and response to therapies, distinct main subgroups have been categorized, such as idiopathic dermatomyositis, polymyositis, inclusion body myositis, necrotizing autoimmune myositis or myositis associated with systemic disorders (Dalakas, 1991, 2011; Mantegazza et al., 1997). Nevertheless, all IM subtypes share some common features such as inflammation, fibrosis and muscle loss together with T helper (Th) 1 immune reaction predominance and the presence of lymphocyte infiltrate into the damaged muscle (De Paepe et al., 2005, 2007, 2009; Grundtman et al., 2007). The mainstay treatment for IMs are immunomodulating agents specifically designed to target immune cells, such as corticosteroids, often administered with second-line immunosuppressants to reduce the corticosteroid dose and related side effects (Tournadre et al., 2010; Wiendl,

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doi:10.1016/j.ejcb.2011.09.011

2008). Approximately 25% of the patients are non-responders to or cannot tolerate these drugs, continue to experience clinical relapses and are left with disability (Dalakas, 2001; Wiendl, 2008).

The need for more specific and safer therapies has been driving the search for a deeper understanding of the pivotal mediators and mechanisms associated with muscular disease also at the molecular level.

In IMs, a cascade of inflammatory mediators, such as cytokines, chemokines and adhesion molecules, modulates biological functions and downstream signaling pathways (Figarella-Branger et al., 2003; Szodoray et al., 2010). Several proinflammatory cytokines, i.e. tumor necrosis factor (TNF)α, interferons (IFNs), interleukines (IL)-1α and β, have been described as being highly present in IM patients, both in muscle tissues and in serum (De Paepe et al., 2009; Lindberg et al., 1995; Lundberg et al., 1995; Nyberg et al., 2000; Schmidt et al., 2008; Szodoray et al., 2010; Wolf and Baethge, 1990). In particular, IFNγ and TNFα, both with strong Th1 association, have been found to be upregulated in IMs (Dalakas, 2001; De Paepe et al., 2009; Lundberg et al., 1995).

During inflammation, muscle cells behave as immunoactive cells, secreting molecules of the monocyte-macrophage lineage (i.e. IL-1α and β, IL-6, TNFα) and chemokines (De Rossi et al., 2000). The latter ones are a class of small chemotactic cytokines which drives leukocyte migration from blood to inflammation sites (Lazzeri and Romagnani, 2005; Zlotnik and Yoshie, 2000) and amplifies inflammatory responses also in IMs (De Paepe et al., 2008; Tournadre and Miossec, 2009). Besides the known involvement of β (or CC-) chemokines, a role for α (or CXC-) chemokines inducible by IFNγ has been recently pointed out in IMs (De Paepe et al., 2007, 2009). In particular, IFNγ inducible 10 kDa protein CXCL10 or IP-10, known to play a predominant role during Th1-mediated responses (Lee et al., 2009; Rotondi et al., 2007), has been recently found raised in IM patient serum (Lee et al., 2009; Szodoray et al., 2010). In IM tissue extracts CXCL10 protein and gene expression have been shown to be significantly increased together with its specific receptor CXCR3, expressed by Th1 infiltrating cells (De Paepe et al., 2005, 2009; Fall et al., 2005; Lee et al., 2009). Indeed, CXCL10-CXCR3 interaction has been hypothesized as a potential target for novel therapeutic interventions (De Paepe et al., 2005, 2007; Lee et al., 2009).

Our *in vitro* investigation aimed to clarify the contribution of human skeletal muscle cells to inflammatory processes in terms of CXCL10 secretion and to elucidate the involved molecular mechanism(s). We also tested human skeletal muscle cell response, if any, to some drugs routinely used in IM treatment – methylprednisolone (MeP), methotrexate (MTX), cyclosporin A (CsA), and the TNFα blocking agent Infliximab.

To perform this study we first established and characterized *in vitro* cultures of human fetal skeletal muscle cells (Hfsmc). In Hfsmc we analyzed the response to the prototypic inflammatory stimuli IFNγ and TNFα, in terms of CXCL10 protein secretion along with some of the involved molecular pathways, and the effect of the above mentioned immunosuppressants; IL-6 and IL-8 were also tested, since both are generic parameters of muscular damage and inflammation (Dieli-Conwright et al., 2009). We also verified Hfscm competence to react in the context of an *in vitro* alloresponse.

Materials and methods

Chemicals

Dulbecco Modified Eagle Medium (DMEM)/Ham's F-12 medium (1:1) with and without phenol red, RPMI 1640, phosphate buffered saline Ca^{2+}/Mg^{2+}-free (PBS), bovine serum albumin (BSA) fraction V, antibiotics, NaOH, absolute ethanol, EDTA–trypsin solution, Ficoll-Hypaque, Bradford reagent and all reagents for Western blot, methotrexate (MTX), methylprednisolone (MeP), cyclosporin A (CsA) were from Sigma–Aldrich Corp. (St. Louis, MO, USA). The chimeric monoclonal antibody Infliximab (Remicade®) was from Centocor B.V. (Leiden, The Netherlands). Fetal bovine serum (FBS) and fetal calf serum (FCS) were purchased from Hyclone (Logan, UT, USA). L-Glutamine, nonessential amino acids, pyruvate, and 2-mercaptoethanol were from Gibco Laboratories (Grand Island, NY). The Coomassie Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Collagenase type IV was from Worthington (Lakewood, NJ, USA). IFNγ, TNFα and ELISA kits for CXCL10, IFNγ, TNFα, IL-6 and IL-8 measurement were from R&D Systems (Minneapolis, MN, USA). For flow cytometry analysis, PE-conjugated anti-CD119 monoclonal antibody (mAb) (GIR-208, mouse IgG1) was from BD Biosciences (Mountain View, CA, USA); PE-conjugated anti-TNFRII mAb (22235.311, mouse IgG2a) was from R&D Systems; conjugated isotype-matched control Abs (mouse IgG1: clone 15H6, mouse IgG2a: clone HOPC-1) were from Southern Biotechnology Associated Inc. (Birmingham, AL, USA). For RNA extraction, the RNeasy Mini reagent kit was purchased from Quiagen Italy (Milan, Italy). The TaqMan Reverse Transcription Reagents kit, all primer/probe mixes (Taqman Gene® Expression Assays), CXCL10 (ID number Hs00171042-ml), IFNγR (ID number Hs00166223-m1), TNFαRII (ID number Hs00153550-m1), Pax-3 (ID number Hs00240950-m1), Pax-7 (ID number Hs00242962-m1), MyoD (ID number Hs00323851-m1), myogenin (ID number Hs01072232-m1) and 1× Universal Master Mix were from Applied Biosystems (Forster City, CA, USA). Quantitative PCR human reference total RNA was purchased from Stratagene (La Jolla, CA, USA). The polyvinylidene difluoride membranes (Hybond-P) were from Amersham Bioscience (Little Chalfont, UK).

For Western blot, immunocytochemical and/or immunohistochemical analysis, primary Abs: goat polyclonal Ab (pAb) anti-tropomyosin, mouse anti-β actin (β actin) mAb, mouse anti-myogenin mAb, rabbit anti-myosin heavy chain (MYH) pAb, rabbit anti-NF-kB p65 (NF-kB, C-20) pAb were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-phospho Tyr701 Stat1 (pStat1) pAb was from Cell Signaling (Danvers, MA, USA); mouse anti-sarcomeric actin mAb (Clone Alpha-Sr-1) was from DakoCytomation (Glostrup, Denmark); rabbit anti-myosin 1β pAb was from Sigma–Aldrich; rabbit anti-myostatin pAb was from Chemicon International Inc. (Temecula, CA, USA). Mouse anti-CD3 mAb (clone HIT3a) was from BD Biosciences.

Alexa Fluor 488 conjugate goat anti-rabbit and goat anti-mouse Abs were from Molecular Probes (Eugene, OR, USA); FITC conjugate rabbit anti-goat and peroxidase-secondary Abs were from Sigma–Aldrich. The ultravision large volume detection system anti-polyvalent was from Lab-Vision (Fremont, CA, USA). 3′,3′-Diaminobenzidine tetrahydrochloride was from Sigma–Aldrich.

All reagents for Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) were from GE Healthcare. Trypan blue was from Euroclone (Pavia, Italy). Plasticware for cell cultures and disposable filtration units for growth media preparation were purchased from Corning (Milan, Italy).

Cell cultures and tissues

Hfsmc were isolated from 11 fetal skeletal male muscles (four upper and seven lower limbs) obtained after voluntary abortion (10–12 weeks of gestation). Legal abortions were performed in authorized hospitals, and written certificates of consent were obtained. The use of human fetal tissue for research purposes was approved by the Committee for investigation in humans of the Azienda Ospedaliero-Universitaria Careggi, Florence, Italy (protocol n° 6783-04). All samples have been handled in the same way and maintained in ice-cold PBS until processed for culture preparation,