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Interleukin-17 modulates myoblast cell migration by inhibiting urokinase type plasminogen activator expression through p38 mitogen-activated protein kinase

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ABSTRACT

Interleukin-17 belongs to a family of pro-inflammatory cytokines with pleiotropic effects, which can be associated with several inflammatory diseases of the muscle tissue. Although elevated levels of interleukin-17 have been described in inflammatory myopathies, its role in muscle homeostasis remains to be elucidated. The requirement of the urokinase type plasminogen activator in skeletal myogenesis was recently demonstrated *in vivo* and *in vitro*, suggesting its involvement in the regulation of extracellular matrix remodeling, cell migration and myoblast fusion. Our previous results have demonstrated that interleukin-17 inhibits myogenic differentiation of C2C12 myoblasts *in vitro* concomitantly with the inhibition of cell migration. However, the involvement of urokinase type plasminogen activator in interleukin-17-inhibited myogenesis and migration remained to be analyzed. Therefore, the effect of interleukin-17 on the production of urokinase type plasminogen activator by C2C12 myoblasts was determined in the present study. Our results demonstrated that interleukin-17 strongly inhibits urokinase type plasminogen activator expression during myogenic differentiation. This reduction of urokinase type plasminogen activator production corresponded with the inhibition of cell migration by interleukin-17. Activation of p38 signaling pathway elicited by interleukin-17 mediated the inhibition of both urokinase type plasminogen activator expression and cell migration. Additionally, IL-17 inhibited C2C12 cells migration by causing the cells to reorganize their cytoskeleton and lose polarity. Therefore, our results suggest a novel mechanism by which interleukin-17 regulates myogenic differentiation through the inhibition of urokinase type plasminogen activator expression and cell migration. Accordingly, interleukin-17 may represent a potential clinical target worth investigating for the treatment of inflammatory muscle diseases.

1. Introduction

Interleukin-17A (IL-17) is the first discovered member of the cytokine family, which has been, in addition to its proinflammatory role, associated with human diseases such as rheumatoid arthritis, polymyositis and dermatomyositis (Ivanov and Linden, 2009). IL-17 transduces its signal by binding to a unique cognate type I transmembrane protein receptor. Activated IL-17 receptor (IL-17R), triggers a plethoric range of intracellular signal transduction pathways, such as: protein kinase A; JAK/STAT, NF-kB; and mitogen-activated protein kinases (MAPKs), including ERK1,2 and p38 cascades (Gaffen, 2009; Ivanov and Linden, 2009; Iwakura et al., 2011). Activated signaling pathways induce the expression of proinflammatory cytokines, chemokines, antimicrobial peptides, growth factors and tissue remodeling enzymes (Xu and Cao, 2010). First implications about IL-17's involvement in cell differentiation were based on the findings, which demonstrated its role in hematopoiesis, suggesting that this cytokine is a stromal growth factor, which induces granulopoiesis and erythropoiesis (Schwarzenberger et al., 2000; Huang et al., 2006; Jovcić et al., 2007). Further on, considering the fact that IL-17R is ubiquitously expressed, novel non-immune functions concerning mesenchymal stem cell differentiation have been described showing that IL-17 is able to induce osteogenic (Huang et al., 2009) or to inhibit adipogenic differentiation (Shin et al., 2009) of human mesenchymal stem cells. Furthermore, as we recently reported, IL-17 inhibits myogenic differentiation of mouse myoblast C2C12 cells (Kocić et al., 2012a).

Skeletal muscle regeneration requires resident satellite stem cells able to undergo myogenic differentiation. This regenerative process includes the activation of satellite cells leading them to proliferate, migrate to sites of injury and finally, differentiate into adult muscle cells (Morgan, 2003; Philippou et al., 2008). The urokinase type plasminogen activator (uPA) system has an important contribution in the homeostasis of muscle fibers and muscle regeneration (Philippou et al., 2008). The uPA system consists of uPA,

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uPA receptor (uPAR), plasminogen/plasmin and plasminogen activator inhibitors (PAI) type 1 and 2. Most of these components are expressed in skeletal muscle, especially during the initial regeneration phase following *in vivo* damage (Philippou et al., 2008). uPA seems to be essential for migration, fusion and differentiation of myoblasts *in vitro*, as well as for muscle regeneration *in vivo* (Munoz-Canoves et al., 1997; Lluís et al., 2001). In addition, uPA deficiency exacerbates muscular dystrophy in MDX mice (Suelves et al., 2007).

Taking into consideration that higher expression of IL-17 has been observed in inflammatory myopathies (Tournadre and Miossec, 2012), as well as that our previous work demonstrated the capacity of the cytokine to inhibit myoblast migration and myogenic differentiation (Kocić et al., 2012a), our aim in this study was to investigate whether inhibition of myogenic differentiation elicited by IL-17 involves modulation of uPA expression in C2C12 myoblasts. Obtained results showed that IL-17 strongly inhibits uPA expression in C2C12 cells concomitantly with the inhibition of cell migration, mainly mediated through the activation of p38 signaling pathway.

2. Materials and methods

2.1. Cell culture and reagents

Mesenchymal myoblast C2C12 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum in humidified environment at 37 °C and 5% CO_2. Myogenic differentiation was induced by incubating cells in DMEM supplemented with 2% horse serum, as previously described by Kocić et al. (2012a).

Recombinant mouse IL-17 and TGF-β1 were provided by R&D Systems (Minneapolis, MN, USA). Epsilon aminocaproic acid (EACA) was from Sigma–Aldrich (St. Louis, MO, USA). PAI-1, p38 inhibitor SB203580, MEK1,2 inhibitor PD98059 and anisomycin were obtained from Calbiochem (Darmstadt, Germany) and used at concentrations 5 μg/ml, 10 μM, 25 μM and 10 ng/ml, respectively. Anti-uPA mouse monoclonal antibody, SAM-3, was kindly provided by Dr. F. Castellino (University of Notre Dame, IN, USA).

2.2. Zymography assay

The activity of secreted uPA was assayed by zymography, as described by Villar et al. (2010). Briefly, 5×10^4 cells/well were seeded in 24-well plates and cultured overnight, then the cells were washed three times with PBS and 0.35 ml of serum-free culture medium was added per well. Cells were cultivated for additional 24 h with appropriate treatments described in Section 3. Protein-normalized conditioned media were subjected to electrophoresis under non-reducing conditions. The acrylamide gels were then placed on top of 1% agarose gels containing 0.5% casein and 2 μg/ml plasminogen and incubated at 37 °C for 24 h. uPA-dependent proteolysis of agarose gels was detected as a clear band. The intensity of the bands was quantified using NIH-Image J software.

The activity of secreted matrix metalloproteinase 2 (MMP-2) was assayed as described by Santibáñez et al. (2002). Serum-free conditioned media obtained after the same cell cultivation protocol as described above, for uPA analysis, were subjected to SDS-PAGE under non-reducing conditions in 8% polyacrylamide gels containing 0.1% gelatin. After being washed twice with 2.5% Triton X-100, gels were incubated for 24 h in 100 mM Tris–HCl, pH 8.5 with 10 mM $CaCl_2$. After the gels were stained with Coomassie blue, the MMP-2 activity was detected as a transparent band inside the gel. The intensity of the bands was quantified using NIH-Image J software.

2.3. Semi-quantitative RT-PCR assay

Cells were seeded in 6-well plates (2×10^5 cells/well) and after corresponding treatments described in Section 3, total RNA was isolated using TRIzol (Applichem, Darmstadt, Germany) and reverse transcribed by SuperScript First Strand Synthesis System for RT-PCR from Invitrogen (Carlsbad, CA, USA), using oligo (dT) as a primer.

PCRs were performed using 200 ng of cDNA. The reaction conditions were: a first step of denaturation at 94 °C for 5 min, followed by cycling reactions at 94 °C for 45 s, annealing for 30 s, and extension at 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. Primer sets, annealing temperatures and number of cycles used for each primer set are listed in Supplementary Table 1. GAPDH was amplified as a control for the amount of cDNA in each sample. The intensity of the bands was quantified using NIH-Image J software.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2012.11.010.

2.4. Plasmids, transient transfections and reporter assays

To monitor the activation of p38, PathDetect Gal4-CHOP Trans-Reporting System for p38 kinase pathway (Stratagene, La Jolla, CA) was used. pFC-MEK3 plasmid (constitutively active MEK3 Stratagene) was used as a positive control. The p-4.8 uPA–Luc reporter plasmid (−4.8 kb of murine uPA promoter) was provided by Dr. Munoz-Canoves (CRG, Spain). The promoterless pGL2-basic reporter was from Promega (Madison, USA). PCMV-β-galactosidase expression vector was kindly provided by Dr. C. Bernabeu (CIB, Spain) and was used as an internal control for transfection efficiency. ERK1,2 signaling was determined using pSRE-Luc (kindly provided by Dr. A Corbi, CIB, Madrid Spain). Dominant negative and constitutively active p38α constructs were kindly provided by Dr. R. Kishore (Feinberg Cardiovascular Research Institute, Chicago, USA). The pcDNA3.1 empty vector was obtained from Invitrogen.

C2C12 myoblasts seeded in 24-well plates (2×10^5 cells/well) were transfected with different plasmids as described in Kocić et al. (2012a) using Superfect transfection reagent (Qiagen, Hilden, Germany). Transfected cells were then treated for 24 h, as indicated in specific experiments, and cell extracts were prepared. Luciferase assays were done using the Luciferase Assay System (Promega, Madison, WI, USA) according to manufacturer's instructions. Luciferase activities were normalized with respect to parallel β-galactosidase activities. β-galactosidase assays were performed using the Galacto-Light Plus System from Tropix (Bedford, MA, USA) according to manufacturer's instructions.

2.5. Wound healing assay

Cells were grown until confluence in 24-well plates, when a scratch was performed in the cell monolayer using a pipette tip. Cells were cultivated for additional 24 h under treatment specified in Section 3. After this migration period, cells were fixed and stained with 0.1% crystal violet in methanol. Cell migration into the scratch area was photographed using inverted light microscope and quantified by TScratch software (Computational Science and Engineering Laboratory, Swiss Federal Institute of Technology, ETH Zurich, Zurich, Switzerland). The C2C12 conditioned medium, which was used as an exogenous source of uPA, was prepared by