STAT3 induces muscle stem cell differentiation by interaction with myoD

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A R T I C L E   I N F O

A B S T R A C T

Signal transducers and activators of transcription (STAT) family proteins transduce pivotal biological effects of various cytokines and hormones. STAT3 proteins are known to play a central role in the regulation of growth, differentiation, and survival of many types of cells. However, the function of STAT3 in myogenesis still remains largely unknown. We now provided direct evidence that STAT3 could induce myogenic differentiation and this effect might be mediated by interaction with MyoD—the essential transcription factor during myogenic differentiation. Furthermore, leukemia inhibitory factor (LIF) might be the upstream factor which activated JAK2/STAT3 pathway to stimulate muscle cell differentiation. Taken together, these results provide a molecular basis for further understanding of the muscle regeneration mechanism.

1. Introduction

Muscle stem cells, also known as myoblasts, are normally present as quiescent satellite cells between the basement membrane and the plasma membrane of myofibrils [1]. In response to injury, exercise or in pathogenic states, they become activated, re-enter cell cycle and start to proliferate actively. Eventually, these proliferating satellite cells irreversibly withdraw from cell cycles, differentiate and fuse with existing myofibrils [2]. These sequential events are called muscle regeneration. In this process, the differentiation of myoblasts is controlled by two families of transcription factors, myogenic regulatory factors (MRFs) and myocyte enhancer-binding factor 2(MEF2s) [3], which are regulated by a variety of growth factors such as insulin-like growth factors (IGFs), leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and transforming growth factor-α [4]. In addition, many signaling pathways, such as Ras/MAPK, PI3K/Akt and JAK/STATs are involved in the regulation of myoblasts differentiation. LIF

belongs to the interleukin-6 (IL-6) cytokine family, which is secreted by multiple cell types and mediates a variety of biological effects depending on the target tissue and cell lineage. The intracellular signaling pathways responsible for LIF action involve the activation of Jak and/or Tyk kinases. Activated Jak/Tyk phosphorylates (signal transducers and activators of transcription (STAT1, STAT3 or STAT5) can translocates into the nucleus and transactivate LIF-responsive genes.

STAT proteins transduce pivotal biological effects of various cytokines and hormones [5]. Among seven STAT family members (STAT1-4, 5A, 5B, and 6), STAT3 is activated by several cytokines, such as the IL-6 family (LIF, ciliary neurotrophic factor, oncostatin M, IL-11, and cardiotrophin-1), IGF-I, HGF, EGF, PDGF, and bFGF. Thus, STAT3 plays a central role in the regulation of growth, differentiation, and survival in many types of cells [6]. For example, STAT3 promotes cell growth through the transcriptional regulation of cyclin D1 in fibroblasts [7] and through the transcriptional regulation of c-myc and Pim1 in hematopoietic cell lines [8–9]. In addition, STAT3 maintains embryonic stem cells in an undifferentiated state [10] and inhibits IL-6-induced neuronal differentiation in PC12 cells [11]. These data indicate that STAT3 promotes cell growth and suppresses cell differentiation. However, STAT3 has been reported to mediate cytokine-induced terminal differentiation in other cell types, such as IL-6-induced macrophage differentiation [12], CD40 ligand-induced B-cell differentiation [13], OSM-induced differentiation of an osteosarcoma cell line [14], ciliary neurotrophic factor or OSM-induced astrocyte differentiation [15,16], and LIF-mediated endothelial differentiation in cardiac stem cells [17]. Therefore, the role of STAT3 in cell growth

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and differentiation is divergent according to the cellular background and/or the type of cytokines.

However, the function of STAT3 signal pathway in regulating myogenesis still remains largely unknown. As a transcriptional factor, STAT3 mediates several biological functions including proliferation, differentiation, and survival of myoblasts via activating its target genes (cyclin D1, c-fos, JunB, Bcl-2, and Bcl-xL) [18–29], either by directly binding to regulatory region of target genes or by interacting with other transcriptional factors. It has also been reported that the intrinsic muscle-specific transcriptional factors, such as MyoD, MEF2 and SIX1, play crucial roles in skeletal muscle development in coordination with exogenous growth factors during myogenesis. Thus, it is necessary to demonstrate whether STAT3 can induce myogenic differentiation by interacting with MyoD, MEF2 or SIX1. MyoD is known to regulate myogenic differentiation through its transcriptional activities and protein–protein interactions, leading to cell cycle arrest and myoblast differentiation at the early phase. MEF2 is also capable of directly interacting with members of the MRFs (i.e., MyoD, Myf5, myogenin, and MRF4) to synergistically activate many muscle-specific genes [30,31]. The human SIX1, a member of the SIX classes of homeodomain genes, is such a transcription factor functioning as a master regulatory protein and transactivating the MEF3 site containing reporter genes, such as myogenin [32].

To further elucidate the molecular mechanisms underlying the role of STAT3 in myogenic differentiation, we examined whether the STAT3 protein interacts with these key myogenic regulatory factors. We elucidated the role of STAT3 signal pathway during myogenic differentiation using C2C12 cells, an immortalized myogenic cell line derived from mouse MSCs [37]. Our results confirmed that STAT3 is essential for myogenin differentiation. LIF mediates muscle cell differentiation by JAK2/STAT3 pathway. In addition, STAT3 may also induce myogenin differentiation through biding to another transcription factor, MyoD. These results provided a molecular basis for further understanding of the muscle regeneration mechanism.

2. Methods

2.1. Cell cultures

Mouse MSC-derived C2C12 cells (American Type Culture collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (referred to herein after as growth medium or GM), 100 U/ml penicillin, and 100 μg/ml streptomycin in a 37 °C incubator with 5% CO_2 for proliferation. To induce differentiation, growth medium was substituted by differentiation medium (Dulbecco's modified Eagle's medium containing 2% horse serum, or DM) when cells were near confluent to allow the cells permanently withdraw from cell cycles, differentiate and fuse into myotube.

2.2. Reagents and antibodies

LIF was purchased from Chemicon, AG490 from Biomol, HGF from AMS/Immunoko ntact, and bFGF from Invitrogen. Antibodies used include the mouse monoclonal antibodies to actin (Sigma), JAK1 (Santa Cruz), STAT3, phosphorylated-STAT3 (Cell Signaling Techno logy), MHC (MF20, Developmental studies hybridoma bank), MyoD (Phar Mingen), Flag (m2, Sigma).

2.3. Plasmid construction and transfection

Various expression vectors for Flag-tagged MyD, Flag-tagged SIX1 or Flag-tagged MEF2 were constructed by inserting PCR-generated cDNA fragments into the mammalian expression vectors of pcDNA3 (Invitrogen). All constructed clones were verified by sequencing. In DM 12 h, 5×10^4 cells per well were plated onto 12-well plates to reach 70–80% confluence and were transfected with 200 ng of various plasmids as indicated in the figure legends using LipofectAMINE 2000 (Sigma) in according to the manufacturer's instructions. At 48 h after transfection, cells were extracted for coimmunoprecipitation analysis followed by Western blot analysis.

2.4. siRNA and transfection

All Small interfering RNAs (siRNAs) were synthesized at Dharmacon Inc. Sequences were as follows: for the STAT3 siRNA (human, sense), 5′GAGUCCAAUGUCCU CUAUC; for the unrelated to STAT3-RNAi, 5′AGACUUGACACUUCUCG CU; In GM or in DM 36 h, 5×10^4 cells per well were plated onto 12-well plates to reach 70–80% confluence. Then cells were transfected with various siRNAs of 100 nmol/L as indicated in the figure legends using Lipofect AMINE 2000. At 48 h after transfection, cells in DM 36 h were extracted for Western blot analysis, while cells in GM were treated with LIF (20 ng/ml) for 12 h before Western blot analysis.

2.5. Coimmunoprecipitation

To examine the protein–protein interaction in cultured C2C12 cells, C2C12 cells were lysed in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mg/ml aprotinin and 1 mg/ml leupeptin). Four milligrams of anti-Flag antibody was first incubated with 300 μg of cell extracts in RIPA buffer overnight at 4 °C with rotation; 30 μl of protein A-Sepharose beads was then added, and the mixture was incubated at 4 °C for 1 h with rotation. After extensive washing with the RIPA buffer, bound proteins were eluted out by boiling then subjected to Western blot analysis.

2.6. Western blot analysis

To examine expression levels of myosin heavy chain(MHC), stat3, phosphorylated-STAT3, β-actin, MyoD, Flag-myoD, Flag-MEF2, Flag-SIX1 and JAK1, C2C12 cells lysates in RIPA buffer was centrifuged at 13,000g for 10 min at 4 °C and the supernatant was used for the experiment. An aliquot (50 μg) of protein was separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto 0.2-Am nitrocellulose membrane using Towbin transfer buffer (192 mM glycine, 25 mM Tris, 20% methanol, pH 8.3). The membrane was preincubated with PBS containing 5% milk, and probed with monoclonal anti-MHC (1:1000), monoclonal anti-actin (1:1000), monoclonal anti-STAT3, monoclonal anti-phosphorylated-STAT3 (1:1000), monoclonal anti-MyoD (1:1000), monoclonal anti-Flag, and monoclonal anti-JAK1 (1:1000), in PBS containing 5% milk for 1h at room temperature. The membrane was then washed with PBS containing 0.1% Tween 20 and incubated with an appropriate horseradish peroxidase (HP)-conjugated secondary antibody. After several washes, the blot was developed using an enhanced chemiluminescence reagent (ECL, sigma) according to the manufacturer's instructions.

3. Results

3.1. STAT3 is essential for myogenin differentiation

To identify signal pathway involved in differentiation process of myogenesis, we examined the expression of STAT3 proteins in C2C12 myoblast when the C2C12 myoblast was shifted from GM

to DM for 48 h. As shown in Fig. 1A, the expression of STAT3 and phosphorylated-STAT3 proteins increased upon differentiation, i.e., after the cells were transferred from GM to DM. To further identify the role of STAT3 in myogenin differentiation, we knocked down the endogenous STAT3 with small interference RNA in C2C12 cells cultured in DM for 36 h (Fig. 1B). Compared to the control cells, the myogenic differentiation was markedly blocked in STAT3-siRNA-transfected cells, as demonstrated by Western blot showing the decreased expression of differentiation marker protein—myosin heavy chain (MHC). These results suggest that Stat3 plays a critical role in the process of myogenesis differentiation.

3.2. LIF induces rapid phosphorylation of STAT3 in C2C12 cells

The progresses of muscle regeneration are regulated by a variety of growth factors such as LIF, HGF, and bFGF [33,34]. Therefore, we next examined which cytokines that activate STAT3 during muscle differentiation. The C2C12 myoblast cells grown in the GM were treated with LIF (20 ng/ml), HGF (20 ng/ml), or bFGF (20 ng/ml). Although the expression levels of endogenous JAK1 protein or endogenous stat3 protein were comparable between cytokine-treated and untreated cells (Fig. 2; top and center panels), the levels of phosphorylated-STAT3 proteins were significantly increased in LIF-treated cells compared to those treated with HGF or bFGF (top panel). These results indicate that LIF activates STAT3 by inducing rapid phosphorylation of STAT3.

3.3. Jak2 mediates the effect of LIF-induced STAT3 phosphorylation during myogenic differentiation

To test whether Jak2 is the down-stream protein of LIF to activate STAT3 during normal myogenic differentiation, we assessed the effect of a specific Jak2 inhibitor, AG490, on STAT3 activation by LIF in C2C12 cells that has been induced to differentiate in DM for 36 h. As shown in Fig. 3, in differentiated C1C12 cells, LIF-induced stat3 phosphorylation was repressed by pretreatment with AG490 in a dose-dependent manner. AG490 pretreatment also significantly inhibited myogenic differentiation as indicated by decreased expression of Myosin heavy chain (MHC). These results demonstrate that LIF-induced differentiation of myogenesis through the signal pathway of JAK2/STAT3.

3.4. STAT3 binded with MyoD in the stage of differentiation

To further elucidate the down-stream molecular mechanism underlying the role of stat3 in myogenic differentiation, we exam-
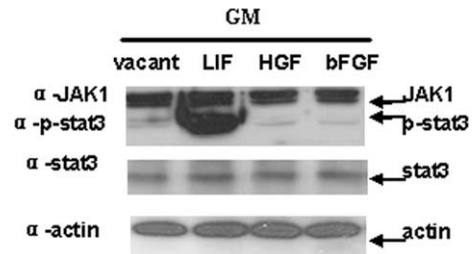

Fig. 2. LIF induced rapid phosphorylation of STAT3 in C2C12 cells. The expression of phosphorylated-stat3 increased when treated with LIF (20 ng/ml) for 24 h compared with HGF (20 ng/ml) or bFGF (20 ng/ml) (the top panel). The expression levels of the endogenous JAK1 protein or endogenous stat3 protein were revealed by Western blot (WB) as indicated (the top and center panel). Vacant: no any disposals as a vacant control.

ined the role of several key transcription factors known to control the expression of muscle-specific gene during myogenic differentiation, such as myoD, MEF2 and SIX1, in LIF-induced SATA3 activation. We transfected C2C12 cells with Flag-six1, Flag-MEF2, or Flag-myoD when the cells were cultured in DM for 12 h. Proteins were prepared from the transfected cells, and analyzed by coimmunoprecipition using a flag-specific antibody and Western blot with an anti-stat3 antibody (Fig. 4; the top panel). As shown in Fig. 4 (the second top panel), stat3 protein was detected in the cells transfected Flag-myoD, but not in those transfected with Flag-MEF2 or Flag-six1. The data indicate that myoD, but not MEF2 or six1, can directly interact with stat3 in myogenic differentiation.

4. Discussion

It is important to understand the molecular mechanisms regulating muscle regeneration, a process involving proliferation, differentiation, and survival of myoblasts. STAT3, a well-known factor for its critical roles in transducing various and important biological effects [5], has been found to be expressed in muscle, but its role during muscle differentiation is poorly defined. By using C2C12 cell-based in vitro muscle differentiation model, we first proved that both the expression level and activation of STAT3 increase upon C2C12 cells differentiation. We also showed that LIF-mediated JAK2/STAT3 signal transduction pathway induces myogenic differentiation. Furthermore, we found that STAT3 could directly interact with myoD, suggesting that myoD might be involved in STAT3-induced myogenic differentiation.
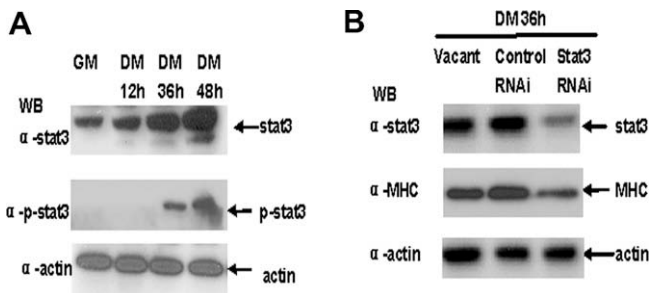

Fig. 1. STAT3 is essential for myogenin differentiation. (A) The expression of STAT3 and phosphorylated-stat3 protein significantly increased upon differentiation in C2C12 from GM to DM 48 h. (B) C2C12 cells were transfected with various RNAi as indicated. Stat3 was knocked down by RNAi and the expression of MHC decreased with stat3-RNAi. Vacant: no RNAi was transfected into cells as a vacant control. Control RNAi: the unrelated to STAT3-RNAi sequence was transfected into cells as a negative control. Stat3-RNAi: the STAT3-RNAi sequence was transfected into cells. WB: Western blot.
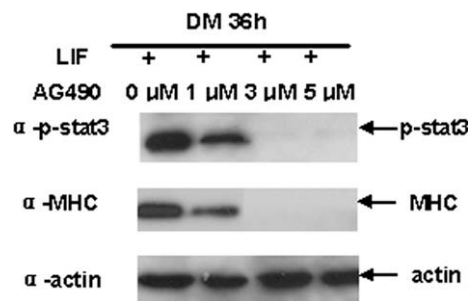

Fig. 3. Jak2 specially mediated the effect of STAT3 during myogenic differentiation. AG490, a specific Jak2 inhibitor, was able to inhibit STAT3 activation induced by LIF. After cells were pretreated with AG490 in a dose-dependent manner as indicated for 30 min and then treated with LIF (20 ng/ml) for 24 h, the level of expression of phosphorylated-stat3 (the top panel) decreased. And the differentiation of the cells was determined by examining the expression of MHC protein as indicated (the center panel).
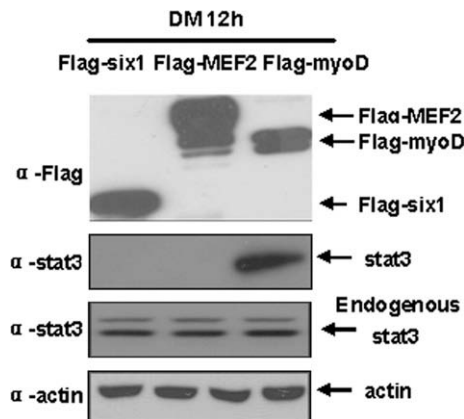
Fig. 4. STAT3 bonded with myoD in the stage of differentiation. C2C12 cell were respectively transfected with Flag-six1, Flag- MEF2 or Flag-myoD. Twelve hours after transfection, cells extracts were subjected to coimmunoprecipitated with antibodies against anti-Flag antibody. First the immunoprecipitated (IP) Flag-six1, Flag-MEF2, Flag-myoD were detected by Western blot (WB) with anti-Flag antibody (the top panel).The transfected plasmids all existed in the cells. Next, the cells lysates were subjected to WB with antibodies against stat3 (the second top panel). Stat3 protein was detected only in the cells transfected Flag-myoD. Furthermore, the cells lysates were subjected to WB with antibodies against endogenous stat3 or endogenous actin. The levels of the endogenous stat3 and endogenous actin were equal revealed by WB (two bottom panels).

The role for STAT3 in myoblast differentiation was further confirmed by STAT3 knockdown experiment using siRNA, in which deliberate knockdown of endogenous STAT3 dramatically blocked myogenic differentiation. The role of STAT3 in cell growth and differentiation is divergent according to the cellular background, the type of cytokines that activates STAT3 and the type of nuclear proteins that interacts with STAT3. It has been reported that STAT3 induces differentiation in some cell lines such as macrophage, B-cell, osteosarcoma cell line, astrocyte and cardiac stem cells [12–17], but suppresses differentiation and promotes cell growth in others [7–11].

We observed that LIF-activated JAK2/STAT3 induces myogenic differentiation. However, a previous report showed that LIF-mediated ERK phosphorylation inhibits myogenic differentiation [35]. In this study, the inhibitory effect of LIF was detected only in the first 12 h after the cells are incubated in the DM, which is STAT3-independnt. Thus, it possible that LIF might play distinct roles in different stages of muscle differentiation. Cytokines may require critical time frame to mediate activation of signal pathway. For example, there is a critical time window (16–20 h) for bFGF to activate ERK and to repress myogenic gene expression and differentiation [36]. These data raise a possibility that LIF may inhibit muscle differentiation by ERK pathway at the early stage (within 12 h), but enhance differentiation through JAK2/STAT3 pathway later.

STAT3, as a key signal transducer and activator of transcription, has been shown to interact with various nuclear proteins and transduce important biological effects in many cell lines. SIX1, MEF2 and MyoD are key myogenic regulatory factors inducing pluripotent precursor cells differentiation. So we tested whether they were involved in stat3 mediated myogenic differentiation. The results showed that stat3 could interact with myoD, but not SIX1 or MEF2 in C2C12 cells in DM for 12 h, suggesting that STAT3-MyoD binding may be responsible for the stimulatory effect of STAT3 on myogenic differentiation. Further studies are needed to understand how STAT3 coordinates with myoD to control myogenic differentiation and how they cross-talk with other signaling pathways. Taken together, our data suggest a possibility to accelerate muscle regeneration by modulating STAT3 pathways.

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