

Identification of a Role for the Tyrosine Kinase JAK2 in Both Skeletal Muscle Mitogenesis and Myogenesis

A.S. Leaflet R1866

Symantha A. Miller, Graduate Research Assistant, and James M. Reecy, Assistant Professor of Animal Science.

Summary and Implications

This study was conducted to investigate what roles JAK2 might play in skeletal myoblast proliferation and terminal differentiation. These results represent the first characterization of the requirement of JAK2 in skeletal muscle proliferation, and suggest a novel role, potentially independent of kinase activity, for JAK2 in skeletal muscle myogenesis.

Introduction

Janus kinase 2 (JAK2) promotes proliferation in a wide variety of cell types and a number of cancers. Adult skeletal muscle contains a unique population of muscle stem cells called satellite cells that are found between the basal lamina and cell membrane of adult skeletal muscle. During embryonic development and postnatal hypertrophy, skeletal myoblasts and satellite cells proliferate and ultimately differentiate into multinucleated cells. These cells exist in a quiescent state unless activated to proliferate and fuse to existing muscle fibers. This study was conducted to investigate what roles JAK2 might play in skeletal myoblast proliferation and terminal differentiation. We used AG490, a JAK2-specific inhibitor, and dominant negative JAK2 (DN-JAK2) to demonstrate that catalytically active JAK2 is required for proliferation in C2C12 myoblasts. In addition, our research indicates JAK2 also has a novel role in skeletal muscle myogenesis. These results represent the first characterization of the requirement of JAK2 in skeletal muscle proliferation, and suggest a novel role, potentially independent of kinase activity, for JAK2 in skeletal muscle myogenesis.

Materials and Methods

Stable Cell Lines

To create DN-JAK2, WT-JAK2, and pRK5 stable cell lines, the following plasmids were used: pRK5JAK2, pRK5JAK2M, pRK5 and pGKNeo. pGKNeo contained a neomycin cassette, pRK5JAK2 contained wild-type JAK2 cDNA, and pRK5JAK2M contained dominant-negative, mutant JAK2 cDNA. C2C12 cells were co-transfected with Eugene 6, linearized pGKNeo DNA, and linearized pRK5JAK2 (WT), pRK5JAK2M (DN), or pRK5 DNA. To create the TEL-JAK2 (5-12) and pcDNA3 stable cell lines, the plasmids pcDNA3-TEL-JAK2 (5-12) and pcDNA3 were used. Cells were transfected as described for DN-JAK2 stable cells. After 18 hours of incubation in transfection

media, cells were changed to growth media that contained G418. Subclones that expressed the genes of interest were maintained by continual selection with G418. Clones were confirmed by western blot analysis of JAK2 over-expression.

Cell Proliferation

Myoblasts were incubated with 10 μ M BrdU and dCTP for the final 12 hours of the proliferation assay time course. Cells were fixed and incubated with blocking buffer (PBS-T + 1.0% bovine serum albumin) containing anti-mouse BrdU antibody. After incubation, plates were washed with PBS-T. Blocking buffer with secondary anti-mouse IgG, FITC-conjugated antibodies was added to the plates. Cells were washed and incubated with Hoeschst 33258. Total and proliferated nuclei were quantified by visualization of HO33258 and FITC positive nuclei, respectively. To determine the percentage of proliferated cells, FITC-positive nuclei were divided by the total number of nuclei.

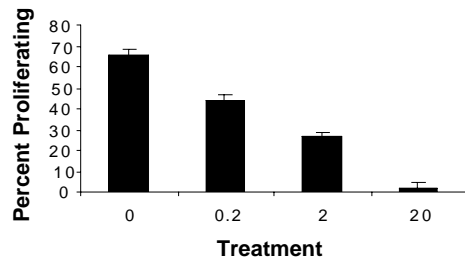
Terminal Differentiation

Cells were photographed throughout experiments. At the time of collection, cells were washed with PBS, and 0.5 M glycylglycine buffer, pH 6.75, was added to the plates. After collection, cells were sonicated and centrifuged to pellet cell debris. The supernatant was collected and creatine kinase (CK) activity was determined as described by the manufacturer's protocol (Sigma). Total protein was quantified with the Bradford protein assay. Creatine kinase units were reported corrected for total protein in nanograms.

Results and Discussion

Inhibition of JAK2 blocks myoblast proliferation in C2C12 cells

To determine if JAK2 signaling was required for C2C12 myoblast proliferation, myoblasts were incubated with 0.2, 2, or 20 μ M AG490 (Figure 1A). C2C12 cells incubated with AG490 had a dose-dependent decrease in myoblast proliferation ($p \leq 0.0001$; Figure 1). Cells treated with 20 μ M of AG490 had almost no BrdU incorporation (2.3%) vs. control cells (66%). These results indicate that JAK2 activity may be required for C2C12 myoblast proliferation. As a second way to examine the effect of JAK2 on proliferation, the rate of proliferation was quantified in stable C2C12 lines that expressed dominant-negative (DN) and wild-type (WT) JAK2. Cells that expressed dominant-negative JAK2 had decreased levels of proliferation as compared to cells that expressed wild-type JAK2 or control cells ($p \leq 0.0001$). These results indicate that expression of a dominant-negative JAK2 attenuates proliferation in C2C12 myoblasts.

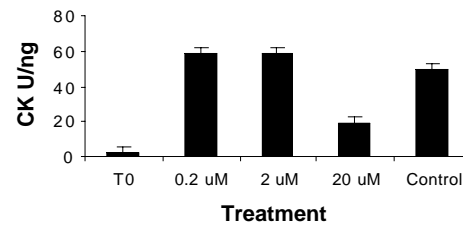
Figure 1. AG490 inhibits myoblast proliferation.

Constitutively active JAK2 increases proliferation in C2C12 myoblasts

To determine if JAK2 could increase proliferation, constitutively active JAK2 was expressed in C2C12 cells. The constitutively active JAK2 construct consisted of a fusion protein between the *ets* transcription factor TEL and JAK2. TEL-JAK2 myoblasts had a significantly higher percentage of myoblasts that proliferated than control cells ($p \leq 0.01$). These results demonstrate that expression of a constitutively active form of JAK2 was sufficient to increase proliferation in C2C12 cells.

Inhibition of JAK2 with AG490 blocks terminal differentiation in C2C12 cells

To determine if JAK2 signaling was required for terminal differentiation in C2C12 myoblasts, cells were cultured for 72 hours in differentiation media (DM) that contained 0.2, 2 or 20 μ M AG490. Creatine kinase activity, a marker of terminal differentiation in skeletal muscle, was significantly inhibited by 2 μ M ($p \leq 0.05$) and 20 μ M ($p \leq 0.0001$) AG490 (Figure 2). The results indicate that, like proliferation, terminal differentiation is inhibited by AG490. However, only high concentrations of AG490 inhibited myogenesis, whereas low concentrations were sufficient to block myoblast proliferation.

Figure 2. AG490 inhibits terminal differentiation in myoblasts.

C2C12 cells stably transfected with DN-JAK2, WT-JAK2 and TEL-JAK2 have enhanced terminal differentiation

To confirm the AG490 results, we quantified the terminal differentiation of stable C2C12 lines that express DN-JAK2, WT-JAK2 or control cells. Interestingly, cells that over-express JAK2 (DN- and WT-JAK2 C2C12 cells) had significantly higher levels of CK activity than control cells ($p \leq 0.05$). Strikingly, DN-JAK2 C2C12 cells had the greatest increase in CK activity ($p \leq 0.0001$; Figure 5A). This is in contrast to our previous experiments in which AG490 was used to block JAK2 activity. In addition, cells that over-express a constitutively active JAK2 (TEL-JAK2) had significantly higher levels of CK activity over control cells ($p \leq 0.001$; Figure 5B). These results suggest a role for JAK2 protein in the promotion of terminal differentiation in C2C12 cells that is independent of kinase activity.

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