In this study we have investigated the effect and the mechanisms by which tumor necrosis factor-like weak inducer of apoptosis (TWEAK) modulates myogenic differentiation. Treatment of C2C12 myoblasts with TWEAK inhibited their differentiation evidenced by a decrease in the expression of creatine kinase, myosin heavy chain-fast twitch, myogenin, and the formation of multinucleated myotubes. TWEAK also inhibited the differentiation of mouse primary myoblasts. Conversely, the proliferation of C2C12 myoblasts and the expression of a cell-cycle regulator cyclin D1 were increased in response to TWEAK treatment. Inhibition of cellular proliferation using hydroxyurea only partially reversed the inhibitory effect of TWEAK on myogenic differentiation. Treatment of C2C12 myoblasts with TWEAK resulted in the activation of nuclear factor-κB (NF-κB), the (IkappaB) IκB kinase (IKK) complex, and the phosphorylation and degradation of IκBα protein. Inhibition of NF-κB activity by overexpression of a dominant negative mutant of IκBα (IκBαΔN) significantly increased the myogenic differentiation in TWEAK-treated C2C12 cultures. Furthermore, overexpression of a dominant negative mutant of IKKβ (IKKβ44A) but not IKKα (IKKα44M) reversed the inhibitory effect of TWEAK on myogenesis. TWEAK inhibited the expression of myogenic regulatory factors MyoD and myogenin and also induced the degradation of MyoD protein. Finally, inhibition of NF-κB activation through overexpression of IκKBK44A prevented the degradation of MyoD protein. Overall, our data suggest that TWEAK inhibits myogenesis through the activation of NF-κB signaling pathway and degradation of MyoD protein.

Skeletal myogenesis is a highly orchestrated multistep process, which involves the determination of multipotential mesodermal cells to give rise to myoblasts. Exit of myoblasts from the cell cycle is followed by increase in the expression of myogenic transcription factors such as MyoD and myogenin and formation of multinucleated myotubes and the expression of myofibrillar proteins (1, 2).

Muscle injuries are one of the most common traumas occurring in accidents, military combat, and sports. An understanding of the biology of muscle regeneration is critical to the development of effective therapies to repair injured muscles. Myogenesis is required not only for growth but also for maintenance and repair of injured myofibers (3). Myoblasts commitment to differentiate into myotubes is influenced both positively and negatively by several factors. Insulin-like growth factor and low serum conditions promote myotube formation (4, 5). On the other hand, treatment of myoblasts with fetal bovine serum, basic fibroblast growth factor 2, myostatin, or transforming growth factor β1 is known to inhibit their differentiation into myotubes (6–9). Myogenesis is also negatively regulated by oncogenes such as c-fos, Ha-ras, and E1a (10–12).

The TNF2 superfamily (TNFSF), consisting of 19 members, comprises type II transmembrane proteins that are involved in regulation of various cellular responses (13, 14). While regulating normal functions such as immune response, hematopoiesis, and morphogenesis, these ligands have also been implicated in a wide variety of diseases, including tumorigenesis, transplant rejection, septic shock, viral replication, bone resorption, and cachexia (13, 15). Most of the ligands of the TNFSF mediate their effects through the activation of the nuclear transcription factor NF-κB. The members of the NF-κB family consist of p50, p52, p65 (Rel A), c-Rel, and Rel B, which form various homo- and heterodimers (16). The NF-κB dimers in resting cells reside in the cytoplasm in an inactive form bound to an inhibitory protein known as IκBα (IκB). Diverse signaling cascades mediate the stimulation of NF-κB pathways. These signals activate the IκB kinase (IKK) complex by phosphorylation of its IκKα and/or IKKβ subunits by upstream kinases. Active IKK complex then phosphorylates IκB proteins at serine residues to result in their ubiquitization and degradation by proteasome (17–19). This results in the activation of NF-κB, which translocates to the nucleus and binds to the promoter or the enhancer regions of specific genes, thus initiating transcription. Besides NF-κB, TNFSF ligands also activate c-Jun N-terminal kinase and induce proliferation and apoptosis in several cell types (13, 15).

TNF-like weak inducer of apoptosis (TWEAK) or TNFSF12 is a member of the TNFSF that acts on responsive cells via binding to a small cell-surface receptor known as fibroblast growth factor-inducible14 (Fn14) receptor or TWEAK receptor (TWEAKR) (20–22). TWEAK is primarily expressed as a 249-amino acid (aa) type II transmembrane protein. However, like most of the other members of the TNFSF,
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TWEAK is proteolytically processed into a soluble, biologically active 156-aa cytokine (20). Recombinant soluble TWEAK has been shown to induce several biological responses such as cell growth and proliferation (23, 24), angiogenesis (25), and stimulation of apoptosis under some experimental conditions (20, 26). Recently, Maeker et al. (27) through the generation of TWEAK−/− mice showed that, in contrast to TNF-α, TWEAK attenuates the transition from innate to adaptive immunity. Furthermore, TWEAK may also contribute to pro-inflammatory reactions. TWEAK has been shown to activate NF-κB signaling pathway and induce the expression of various NF-κB-regulated pro-inflammatory cytokines and cell adhesion molecules in some cell types (28–31).

Although TWEAK and Fn14 receptor are highly expressed in a variety of tissues, including cardiac and skeletal muscles (20, 21, 32, 33), the physiological roles of TWEAK remain largely unknown. A recent report suggests that the TWEAK/Fn14 pathway induces the proliferation of oval cells and may contribute to liver regeneration and/or pathology (34). TWEAK has also been reported to induce differentiation of monocyte/macrophage cells into functional osteoclasts (35). However, the effect of TWEAK on cells of mesenchymal lineage remains unknown. In this report, using C2C12 myoblasts and mouse primary myoblasts, we have investigated the role and the mechanisms by which TWEAK regulates the differentiation of myoblasts. Our results suggest that TWEAK is a potent inhibitor of skeletal myogenesis. Furthermore, our data indicate that TWEAK inhibits myogenesis through the activation of IKK/NF-κB signaling pathway. Our data also suggest that TWEAK affects the stability of MyoD protein required for the induction of the myogenic program.

EXPERIMENTAL PROCEDURES

Materials—Ham’s F-12 nutrient mixture and Dulbecco’s modified Eagle’s medium (DMEM), were obtained from Invitrogen. Antibody against cyclin D1, IκBα, p50, p65, Bcl-3, JNK1, IKKα, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclohexamide (CHX), hydroxyurea (HU), anti-FLAG antibody, protease inhibitors mixture, horse serum, and fetal bovine serum were from Sigma. Phospho-IκBα and anti-IKKβ antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal antibody against myogenin, MyoD, and IKKγ were obtained from BD Biosciences. Mouse monoclonal MF-20 antibody specific to myosin heavy chain-fast twitch (MyHCf) protein was obtained from the Developmental Studies Hybridoma Bank of the University of Iowa. AlexaFluor 546 goat anti-mouse antibody was purchased from Molecular Probes (Eugene, OR). ImmunoPure Immobilized Protein G beads were obtained from Pierce. Recombinant mouse TWEAK protein and anti-Fn14 antibody were obtained from R&D Systems, Inc. (Minneapolis, MN). NF-κB consensus oligonucleotides and luciferase assay kit were purchased from Promega (Madison, WI). Poly(dI-dC) was obtained from Amersham Biosciences. [γ-32P]ATP (specific activity, 3000 (111 TBq) Ci/mmol) was obtained from Perkin-Elmer Life Sciences. CK activity was measured using a spectroscopy-based kit as described previously (37, 38). Briefly, the cells were washed twice in cold PBS and lysed in lysis buffer A (50 Tris-Cl (pH 8.0), 200 mM NaCl, 50 mM NaF, 1 mM dithiothreitol, 0.3% IPEGAL). Lysates were centrifuged for 4 min at 16,000 × g, the supernatant was collected, and the protein content in the samples was measured using Bio-Rad protein assay reagent. CK activity was measured using a commercially available kit (Stabino Laboratory, Boerne, TX). Specific activity of CK was calculated after correction for total protein and defined as units per milligram of protein (units/mg).

Immunofluorescence—Immunofluorescence was performed as described (37). In brief, C2C12 myoblasts were grown in a 24-well plate and allowed to differentiate into myotubes. The cells were fixed with 3.7% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. After being washed with PBS, the cells were blocked with 1% bovine serum albumin in PBS for 1 h and then incubated with MF-20 antibody (specific to MyHCf protein) at 1:100 dilutions in PBS for 2 h. The cells were washed with PBS, incubated with goat anti-mouse IgG-Alexa 546 (2 µg/ml) for 1 h, and counterstained with 4′,6-diamidino-2-phenylindole for 5 min. Stained cells were analyzed under a fluorescence microscope (Olympus IX 70). Pictures were captured using an Olympus MagnaFire digital camera and software. 4′,6-Diamidino-2-phenylindole and MyHCf staining images were finally merged using MagnaFire.
**Preparation of Cytoplasmic and Nuclear Extracts**—Cytoplasmic and nuclear extracts were prepared as described (40). Briefly, 2 × 10⁶ C2C12 cells were washed with cold PBS, scraped, and suspended in 100 μl of hypotonic lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotenin, 0.5 mg/ml benzamidine) for 10 min. The cells were then lysed with 3.25 μl of 10% IPEGAL, the homogenates were centrifuged, and the supernatants containing the cytoplasmic extracts were stored frozen at −80 °C. The nuclear pellets were resuspended in 25 μl of ice-cold high salt nuclear extraction buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotenin, 0.5 mg/ml benzamidine). After 30 min of intermittent mixing the extracts were centrifuged, and the supernatants containing the nuclear extracts were collected. The protein content was measured using Bio-Rad protein assay reagent. If the extracts were not used immediately, they were stored at −80 °C.

**Electrophoretic Mobility Shift Assay (EMSA)**—NF-κB activation was analyzed by EMSA as previously described (41, 42). In brief, 6 μg of nuclear extracts prepared from TWEAK-treated or untreated cells were incubated with 16 fmol of ³²P-end-labeled NF-κB consensus oligonucleotide 5′-CCGTTGTAGTGTCAGCCGGAA-3′ (underline indicates the binding sites of NF-κB) at 37 °C for 20 min, and the DNA-protein complex was resolved on a 7.5% native polyacrylamide gel. A 5% gel was used for supershift analysis. The radioactive bands from the dried gel were visualized and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuaNT software.

**Western Blot**—Immunoblotting for different proteins was done using a standard protocol as described (41, 43).

**Immunoprecipitation and Kinase Assay**—The IKK complex was precipitated by treating 300–μl protein G-Sepharose beads. After 1 h the beads were washed two times with lysis buffer A and two times with kinase assay buffer and then resuspended in 20 μl of kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM MgCl₂, 2 mM dithiothreitol, 20 μM γ-[³²P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate glutathione S-transferase-IκBα (amino acid residues 1–54). After incubation at 37 °C for 20 min, the reaction was terminated by boiling with 10 μl of 4× Laemmli sample buffer for 3 min. Finally, the protein was resolved on a 10% polyacrylamide gel, the gel was dried, and the radioactive bands were visualized and quantitated by using a PhosphorImager and ImageQuaNT (Molecular Dynamics).

**Quantitative Real-time-PCR**—An RNeasy Mini Kit (Qiagen) was used to extract RNA from cells. Any contaminating DNA was removed using the DNA-free™ kit from Ambion (Austin, TX). Quality and quantity of RNA were analyzed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and NanoDrop instrumentation (NanoDrop Technologies, Wilmington, DE). The quantitation of mRNA expression was carried out according to the manufacturer’s instructions (Stratagene) using the SYBR Green method on ABI Prism 7900 Sequence Detection system (Applied Biosystems, Foster City, CA). Briefly, purified RNA (1 μg) was used to synthesize first strand cDNA by reverse transcription system using Ambion’s oligo(dT) primer and Qiagen’s Omniscript reverse transcriptase according to the manufacturer’s instructions. The first strand cDNA reaction (0.5 μl) was subjected to real-time PCR amplification using gene-specific primers. The primers were designed according to ABI primer express instructions using Vector NTI software and were purchased from Eurogentec (San Diego, CA). The sequences of primers used were as follows: cyclinD1: 5′-CGGATGAGAACAAGCAGACCAT-3′ (forward) and 5′-CTGG- AAAGAATGCGGTGTTGC-3′ (reverse); MyoD: 5′-TGGGATATGGGCTCTATGC-GGCATC-3′ (forward) and 5′-GGTGGATCGAAACA-CGGATCAT-3′ (reverse); myogenin: 5′-CATCCAGTACATGGC-GCTTA-3′ (forward) and 5′-GAGGAAATGATCTCTGGGTT-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase: 5′-ATGAGAAATACGGCTACAGCAA-3′ (forward) and 5′-GCACGCA-ACCTTTATGATGATT-3′ (reverse).

Approximately 25 μl of reaction volume was used for the real-time PCR assay that consisted of 2 X (12.5 μl) Brilliant SYBR Green QPCR Master Mix (Stratagene), 400 nM of primers (0.5 μl each from the stock), 11 μl of water, and 0.5 μl of template. The thermal conditions consisted of an initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, and, for a final step, a melting curve of 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. All reactions were carried out in triplicate to reduce variation. The data were analyzed using SDS software version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using the endogenous control (glyceraldehyde-3-phosphate dehydrogenase), and the normalized values were subjected to a 2⁻ΔΔCt formula to calculate the -fold change between the control and experiment groups. The formula and its derivations were obtained from the ABI Prism 7900 Sequence Detection System user guide.

**Proliferation Assay**—Cellular proliferation was measured using AlamarBlue dye (BioSource International, Camarillo, CA). Cells were seeded into a 96-well plate and incubated in medium containing 2% horse serum in the presence or absence of varying concentrations of TWEAK protein. After 72 h the medium was removed, and 100 μl of 10% AlamarBlue diluted in phenol red-free DMEM was added. The fluorescence was determined 2 h later using a fluorescence plate reader (Fluorolite 1000, Dynex Technologies, Chantilly, VA). AlamarBlue was evaluated using the optimal excitation and emission wavelengths of 546 and 590 nm, respectively. Cellular proliferation was also confirmed by measuring the total protein concentration in each well and by counting the total number of viable cells at the end of the incubation.

**Statistical Analysis**—Results are expressed as mean ± S.D. The Student’s t test or analysis of variance was used to compare quantitative data populations with normal distributions and equal variance. A value of p < 0.05 was considered statistically significant unless otherwise specified.

**RESULTS**

We used C2C12 myoblasts and mouse primary myoblasts to study the role of TWEAK in myogenic differentiation. Before performing differentiation studies, the expression of Fn14 (or TWEAKR) receptors on C2C12 myoblasts and primary myoblasts was confirmed by Western blot and FACS analysis (data not shown).

**TWEAK Inhibits Myogenic Differentiation**—To understand the effects of TWEAK on myogenesis, C2C12 myoblasts were incubated in differentiation medium (DM) in the presence of increasing concentrations of soluble TWEAK protein. Moreover, TWEAK treatment also inhibited the differentiation of mouse primary myoblasts (Fig. 1C). Immunohistochemical analysis of C2C12 and primary myoblasts cul-
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FIGURE 1. Effect of TWEAK on myoblast differentiation. C2C12 myoblasts were incubated in DM for 96 h with increasing concentrations of soluble TWEAK protein, and the differentiation of myoblasts into myotubes was studied. A, data presented here show that TWEAK inhibits CK activity. *, p < 0.01, values significantly different from untreated controls incubated in DM. B, the expression of MyHCf and myogenin was decreased significantly in TWEAK-treated cultures. C2C12 myoblasts and mouse primary myoblasts were incubated in either growth medium GM or differentiation medium DM for 96 and 48 h, respectively, with 0.5 μg/ml TWEAK. The myoblasts were then fixed and analyzed for the expression of MyHCf and formation of multinucleated myotubes by immunofluorescence technique. Representative data presented here show a significant reduction in the number of myotubes in TWEAK-treated cultures. DM, differentiation medium; GM, growth medium.

FIGURE 2. TWEAK Stimulates the Proliferation of C2C12 Myoblasts and Inhibits Their Withdrawal from the Cell Cycle—C2C12 myoblasts were incubated with DM in the presence of increasing concentrations of recombinant TWEAK protein, and the cellular proliferation was measured using AlamarBlue dye. As shown in Fig. 2A, TWEAK treatment significantly increased the proliferation of C2C12 myoblasts in a dose-dependent manner with maximum effect occurring at a concentration of 0.5 μg/ml TWEAK. The effect of TWEAK on cellular proliferation was also confirmed by measuring the total protein content and counting the number of viable cells in each well (data not shown).

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With C2C12 cultures. This could be attributed to the presence of a significantly higher number of myoblasts in primary cultures, which are already committed to differentiation compared with C2C12 myoblasts. This is evident from CK activity in control cultures incubated in GM (Fig. 1, A and C) and from the immunohistochemical data, which show a significantly higher number of MyHCf-expressing cells in primary cultures compared with C2C12 cultures in GM (Fig. 1D). These data thus suggest that TWEAK is a potent inhibitor of myoblast differentiation.

TWEAK Stimulates the Proliferation of C2C12 Myoblasts and Inhibits Their Withdrawal from the Cell Cycle—C2C12 myoblasts were incubated with DM in the presence of increasing concentrations of recombinant TWEAK protein, and the cellular proliferation was measured using AlamarBlue dye. As shown in Fig. 2A, TWEAK treatment significantly increased the proliferation of C2C12 myoblasts in a dose-dependent manner with maximum effect occurring at a concentration of 0.5 μg/ml TWEAK. The effect of TWEAK on cellular proliferation was also confirmed by measuring the total protein content and counting the number of viable cells in each well (data not shown).

Cycin D1 is a major cell-cycle regulator, the expression of which decreases rapidly on withdrawal from the cell cycle and on incubation of myoblasts with DM (45, 46). To understand whether TWEAK affects withdrawal of cells from the cell cycle, we studied the expression of cycin D1 at different time points after induction of differentiation. A sharp decrease in protein levels of cycin D1 was observed on incubation of C2C12 myoblasts in DM (Fig. 2B). In contrast to control myoblasts, cycin D1 levels were significantly higher in TWEAK-treated myoblasts at all the time points studied. We also compared the mRNA levels of cycin D1 in control and TWEAK-treated C2C12 cells after 72 h of incubation in DM. A significantly higher level of cycin D1 transcripts were observed in TWEAK-treated C2C12 cultures compared with untreated controls (Fig. 2C).

HU Partially Reverses the Inhibitory Effect of TWEAK on Myoblast Differentiation—HU, a ribonucleotide reductase inhibitor, inhibits cell growth by delaying entry into the S phase and cell-cycle progression (47, 48). To determine whether the increased proliferation of C2C12 myoblasts in response to TWEAK was responsible for the inhibition of their terminal differentiation, C2C12 myoblasts were treated with HU, and differentiation was induced by incubation in DM in the presence or absence of TWEAK. The proliferation of myoblasts was measured using AlamarBlue dye, and differentiation was monitored by measuring the specific activity of CK. Treatment of C2C12 myoblasts with 100 μM HU completely inhibited the TWEAK-induced proliferation of myoblasts (data not shown). On the other hand, treatment of myoblasts with HU only partially increased CK activity in TWEAK-treated cultures (Fig. 3). These data suggest that TWEAK might also inhibit myogenic differentiation independent of its effect on cellular proliferation.

TWEAK Activates NF-κB Signaling Pathway in C2C12 Myoblasts—To understand the mechanisms by which TWEAK modulates myogenesis, the effect of TWEAK on the activation of NF-κB signaling pathway was studied. Treatment of C2C12 myoblasts with TWEAK increased the DNA-binding activity of NF-κB in a time-dependent manner (Fig. 4A). Different combinations of Rel/NF-κB proteins can constitute an active NF-κB homodimer and/or heterodimer that bind to specific sequences in DNA. To confirm that the retarded band seen in EMSA is indeed NF-κB and to investigate which subunits of NF-κB are activated in response to TWEAK treatment we performed a supershift assay. Preincubation of nuclear extracts from TWEAK-treated C2C12 myoblasts with antibodies against p50 and p65 proteins of NF-κB family shifted the band to higher levels of molecular weight indicating that NF-κB-DNA complex analyzed by EMSA constitutes these proteins. The supershift data also suggest that TWEAK-
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Activated NF-κB complex contained p50/p50 and p50/p65 dimmers (Fig. 4B). Antibodies against other members of NF-κB family such as Bcl3 and irrelevant antibodies against JNK1 did not affect the mobility of the NF-κB DNA complex. Because the activation of NF-κB precedes the phosphorylation and degradation of IκBα, we also studied the effect of TWEAK on the phosphorylated and total levels of IκBα protein in C2C12 myoblasts. As shown in Fig. 4C, treatment of C2C12 myoblasts with TWEAK increased the phosphorylation and degradation (evident at 15 min) of IκBα protein (Fig. 4C). To assay the level of NF-κB-dependent transcriptional activity in C2C12 myoblasts in response to TWEAK, a luciferase reporter assay was used. C2C12 myoblasts were transiently transfected with either pTAL-Luc or pNF-κB-Luc plasmid (contains multiple kappa enhancer elements upstream of the luciferase gene) for 36 h, treated with 0.5 μg/ml TWEAK, and after 24-h luciferase activity in the cells was measured using a luciferase assay kit and a luminometer. Data depicted in Fig. 4D show that TWEAK augments NF-κB transcriptional activity.

**Sustained Activation of NF-κB Is Responsible for the Inhibitory Effect of TWEAK on Myogenesis**—Although TWEAK was found to activate NF-κB activity in C2C12 myoblasts, it remains unclear whether TWEAK-induced activation of NF-κB signaling was transient or prolonged during differentiation. C2C12 myoblasts were incubated in DM with 0.5 μg/ml TWEAK for different time intervals ranging from 0 to 96 h. Activation of NF-κB was monitored by measuring the IKK activity and phosphorylation of IκBα protein. As shown in Fig. 5A, the enzymatic activity of IKK and levels of phosphorylated IκBα protein were significantly higher in TWEAK-treated C2C12 cultures compared with control cultures at all the time points studied.

To understand whether activation of NF-κB in response to TWEAK is responsible for its affect on myogenesis, C2C12 myoblasts were transiently transfected with FLAG-tagged IκBαΔN, a suppressor mutant of IκBα in which 36 aa from the N-terminal (including IKK phosphorylation sites) have been removed (49). The cells were then incubated in DM for 96 h in the presence or absence of TWEAK, and the differentiation of myoblast was assessed by measuring the CK activity. There was no significant difference in CK activity in untreated control and FLAG-IκBαΔN-transfected C2C12 myoblasts. However, overexpression of FLAG-IκBαΔN significantly increased CK activity in the TWEAK-treated C2C12 cultures (Fig. 5B). Similarly, the number of myotubes was significantly increased in TWEAK-treated C2C12 cultures transfected with IκBαΔN (data not shown). These data clearly suggest that TWEAK inhibits myogenesis through the activation of NF-κB transcription factor.

**Activation of IKKβ but Not IKKα Is Responsible for the TWEAK-induced Inhibition of C2C12 Differentiation**—Because activation of NF-κB involves the activation of upstream kinases IKKα and/or IKKβ (18), we next investigated the role of IKKα and IKKβ in TWEAK-induced inhibition of myogenic differentiation. C2C12 myoblasts were infected with adenoviruses expressing β-galactosidase (Ad.CMV LacZ), dominant negative IKKα (Ad.IKKα-KM), or dominant negative IKKβ (Ad.IKKβ-KA) gene as described under “Experimental Procedures.” After 24 h the cells were differentiated by incubation in DM for 96 h in the presence or absence of 0.5 μg/ml TWEAK, and the differentiation was studied by measuring CK activity. Interestingly, overexpression of dominant negative IKKβ but not dominant negative IKKα reversed the...
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Inhibitory effect of TWEAK on myogenic differentiation (Fig. 6). No significant difference in CK activity was observed in untreated C2C12 cultures infected with either of the adenoviruses. These results suggest that TWEAK inhibits myogenic differentiation through the activation of IKKβ subunit of IKK complex.

TWEAK Modulates the Expression of Myogenic Regulatory Factors in Differentiating Myoblasts—MRFs play important roles during myogenesis (2). We investigated whether TWEAK can also affect the expression of MRFs in differentiating myoblasts. C2C12 myoblasts were incubated in DM in the presence of TWEAK for 72 h, and the mRNA levels of

FIGURE 4. Effect of TWEAK on activation of NF-κB and phosphorylation and degrada-
tion of IκB protein. C2C12 myoblasts were treated with 0.5 μg/ml TWEAK for the indicated time intervals, and the cytoplasmic and nuclear extracts were prepared. A, a representative EMSA gel presented here shows that treatment of C2C12 myoblasts with TWEAK activates NF-κB. B, supershift analysis of NF-κB-DNA complex from TWEAK-treated samples indicates that it contained p50/p50 and p50/p65 dimmers. C, Western blot analysis of the cytoplasmic extracts using antibodies that detect either phosphorylated IκBα or total IκBα shows that TWEAK induces the phosphorylation and degradation of IκBα protein. The level of an unrelated protein β-actin remained unchanged. D, C2C12 myoblasts were transiently transfected with either pTAL-Luc or pNF-κB-Luc plasmid. After 36 h of transfection cells were incubated without or with 0.5 μg/ml TWEAK for 24 h, cells were lysed, and the luciferase activity in the lysates was measured. The data presented here clearly show that TWEAK increase the transcrip-
tional activity of NF-κB.*, p < 0.01, values significantly different from TWEAK-untreated control transfected with pNF-κB-Luc plasmid. N.S., nonspecific.

FIGURE 5. Role of NF-κB in TWEAK-induced inhibition of myogenesis. A, C2C12 myobla-
sts were incubated in DM for indicated time intervals with or without 0.5 μg/ml TWEAK. The myoblasts were collected, and the activity of IKK was measured as described under “Experimental Procedures.” The level of phosphorylated IκBα protein was measured by Western blot using antibody that detects only phosphorylated IκBα protein. The data pre-

FIGURE 6. Effect of overexpression of either a dominant negative mutant of IKKα or 
IKKβ on the differentiation of myoblasts in response to TWEAK. C2C12 myoblasts were infected with Ad.CMVlacZ, Ad.IKKαKM, or Ad.IKKβKA viruses for 24 h as described under “Experimental Procedures.” The infected cells were then incubated with DM for 96 h with or without 0.5 μg/ml TWEAK. At the end of the incubation period the cells were lysed, expression of FLAG-IκBαΔN protein was confirmed by Western blotting, and CK activ-
ity was measured. The data presented here show that overexpression of FLAG-IκBαΔN reverses the inhibitory effect of TWEAK on myogenesis. *, p < 0.01, compared with TWEAK-treated myoblast transfected with vector alone. KA, kinase assay; WB, Western blot.
MyoD protein showed a significant difference between control and time points after addition of CHX. Quantitative analysis of the levels of MyoD protein was lower in TWEAK-treated C2C12 myoblasts at all the time points measured by Western blotting. Overexpression of IKKβ protein further provided a mechanism for amplification of TWEAK-induced cell death in its target tissues.

**DISCUSSION**

Members of the TNFSF and their receptors are widely expressed in a variety of tissues (13). However, their physiological roles relevant to growth and development remain poorly understood. In this study we demonstrate that TWEAK is a potent inhibitor of muscle differentiation. Incubation of myoblasts with soluble TWEAK inhibits the expression of muscle differentiation markers and myotube formation in vitro. TWEAK also stimulates proliferation and prevents withdrawal of myoblasts from the cell cycle on incubation in DM. Our results demonstrate that TWEAK inhibits myogenesis through the activation of the canonical NF-κB signaling pathway, which involves the activation of IKKβ and the phosphorylation and degradation of IκB protein. Additionally, our data suggest that TWEAK might also suppress the myogenic program by inducing the degradation of MyoD protein. A schematic representation of the biochemical mechanisms that might be involved in the TWEAK-induced inhibition of myogenesis supported by our data, is depicted in Fig. 9.

The TWEAK and TWEAKR/Fn14 transcripts have been found to be co-expressed in a variety of cell types and tissues, suggesting that TWEAK released from these cells may function in an autocrine manner to influence cell behavior (22). An alteration in the expression of TWEAKR/Fn14, reported in response to several stimuli such as cytokines and tissue injury, further provides a mechanism for amplification of TWEAK-induced cell death in its target tissues.

**FIGURE 7.** Effect of TWEAK on the mRNA and protein levels of myogenin and MyoD. C2C12 myoblasts were incubated in DM for 72 h with or without 0.5 μg/ml TWEAK. The mRNA levels of myogenin and MyoD were measured using QRT-PCR and the protein levels were measured by Western blotting. A, a data presented here show that treatment of myoblasts with TWEAK decreases the mRNA levels of myogenin and MyoD with decrease in myogenin mRNA level being more drastic than MyoD mRNA level. *, p < 0.05, values significantly different from corresponding untreated controls. B, representative immunoblots presented here show that TWEAK treatment drastically inhibits the protein levels of MyoD and myogenin without affecting the levels of an unrelated protein, β-actin.

**FIGURE 8.** Effect of TWEAK on the stability of MyoD protein. A, C2C12 myoblasts were plated in a 6-well plate and treated with 0.5 μg/ml TWEAK. After 3 h of treatment with TWEAK, 50 μM CHX was added to each well, and the level of MyoD protein was measured at different time points by Western blot. A representative immunoblot presented here indicates that TWEAK induces the degradation of MyoD protein in C2C12 myoblasts. B, quantitative estimation from four independent experiments shows that TWEAK-treatment rapidly decreases the levels of MyoD protein in C2C12 myoblasts. *, p < 0.05, values significantly different from corresponding TWEAK-untreated controls at 2, 3, and 6 h. C, C2C12 myoblasts were infected with either Ad.CMVlacZ or Ad.IKKβKA adenovirus as described under “Experimental Procedures.” The infected myoblasts were then incubated in DM for 72 h with or without 0.5 μg/ml TWEAK, and the levels of MyoD were measured by Western blot. The data show that overexpression of IKKβKA increases MyoD protein levels in TWEAK-treated C2C12 cultures.
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FIGURE 9. A putative mechanism of action of TWEAK in myogenesis. Binding of TWEAK to its receptor initiates a signaling cascade leading to the activation of IKKβ. Activated IKKβ activates NF-κB, which induces the expression of growth stimulatory molecules such as cyclin D1 leading to the proliferation of myoblasts. Activation of NF-κB also decreases the cellular levels of MyoD protein. Increased proliferation and/or degradation of MyoD are antagonist to myogenic differentiation.

lar effects in certain physiological and pathological conditions (32, 33, 50, 51). Although the TWEAK-induced signal transduction pathway has not been completely understood yet, it appears that TWEAK signals via Fn14 receptor in a manner similar to that described for other members of TNFSF, which involves the recruitment of TNF-receptor associated factors (TRAFs) to the cytoplasmic domains of their receptors. Indeed, two independent groups have recently reported the recruitment of TRAF1, -2, -3, and -5 to the cytoplasmic tail of Fn14 receptor in response to TWEAK stimulation (52, 53).

Our study provides initial evidence that the TWEAK/Fn14 signaling system can regulate the myogenic program by modulating both proliferation and differentiation of myoblasts (Figs. 1 and 2). Our data showing that TWEAK stimulates the proliferation of myoblasts are consistent with published reports (23, 34, 54) that suggest that TWEAK can induce the proliferation of several other cell types in vitro. Although increased cellular proliferation is generally antagonist to their differentiation, TWEAK-mediated inhibition of differentiation might not be attributed only to its effect on myoblast proliferation. Inhibition of TWEAK-induced cellular proliferation using HU only partially reversed the inhibitory effect of TWEAK on myoblast differentiation (Fig. 3). These results thus suggest that TWEAK can independently modulate the proliferation and differentiation of myoblasts. It is also possible that TWEAK modulates proliferation and differentiation of myoblasts through the activation of divergent signal transduction pathways. For example, insulin-like growth factor induces myoblast proliferation through the activation of mitogen-activated protein kinase signaling pathway and differentiation by activation of Akt pathway (55–57).

NF-κB transcription factor has been shown to play an important role in both proliferation and differentiation of myogenic cells (45, 58). Multiple NF-κB binding sites have been reported in the promoter region of cyclin D1, an important cell-cycle regulator that, upon association with cdk4 and cdk6, promotes the progression of cells from G0/G1 to S phase of cell cycle (45). Indeed, we observed higher transcript and protein levels of cyclin D1 in TWEAK-treated C2C12 myoblasts (Fig. 2, B and C). Activation of NF-κB has also been shown to inhibit myogenic differentiation independent of cell-cycle regulation (59, 60). To understand the mechanisms by which TWEAK inhibits myogenesis we investigated the effect of TWEAK on activation of NF-κB. Treatment of myoblasts with TWEAK rapidly increased the DNA-binding activity of NF-κB and induced the expression of reporter gene (Fig. 4). In contrast to other members of the TNFSF, it seems that the effect of TWEAK on NF-κB activation is sustained. Significantly higher IKK activity and the levels of phosphorylated IκBα were observed in TWEAK-treated C2C12 cultures during differentiation (Fig. 5A). These data are consistent with a published report also suggesting that TWEAK causes constitutive long lasting NF-κB activation (31).

To study the involvement of NF-κB in TWEAK-induced inhibition of myogenesis, we used dominant negative inhibitors of IκBa and IKK. Interestingly, overexpression of IκBaΔN (a degradation resistant mutant of IκBa) significantly increased muscle differentiation in TWEAK-treated C2C12 cultures (Fig. 5B), suggesting that activation of NF-κB contributes to the inhibition of myogenesis in response to TWEAK. The activation of different subunits of NF-κB is governed by an upstream kinase, IKK, which consists of two catalytic subunits IκKα and IκKβ and a regulatory subunit IKKγ. Despite the structural similarity between IκKα and IκKβ, the targeted disruption of IκKα and IκKβ in mice revealed that their functions are not interchangeable (reviewed in Ref. 61). IκKα plays a crucial role in lymphoid organ development, adaptive immunity, and keratinocyte differentiation, but it is not required for the cytokine-induced activation of NF-κB. In contrast, IKKβ is essential for activation of canonical NF-κB pathways, which is based on IκBβ degradation and is activated in response to many pro-inflammatory stimuli, including TNF-α and interleukin-1β (16, 17). A recent study showed insulin-like growth factor II induces the differentiation of L6 myoblasts through the activation of IκKα but not IκKβ (62). Using adenoviral vectors, we observed that overexpression of a dominant negative mutant of IκKβ but not IκKα increased differentiation in TWEAK-treated myoblast cultures (Fig. 6). Altogether, our data suggest that TWEAK inhibits myogenic differentiation through the activation of canonical NF-κB signaling pathway that involves activation of IκKβ and degradation of IκB protein.

MyoD is a major MRF (others include Myf-5, myogenin, and MRF4) that has been shown to play specific roles in muscle cell activation and transition from proliferation to differentiation that occurs upstream of terminal differentiation (63, 64). MyoD induces myogenesis by withdrawal from the cell cycle (65–67) and through activation of muscle specific genes (68–70). The activity of MyoD is regulated both at transcriptional and post-transcriptional levels (64, 71, 72). Furthermore, activation of NF-κB has been shown to decrease the expression of MyoD mRNA at the post-transcriptional level (58, 73). Activated NF-κB also promotes the degradation of MyoD protein by the ubiquitin-proteasome pathway (60).

Interestingly, our results suggest that TWEAK reduces the levels of MyoD protein by both reducing the expression of MyoD mRNA and destabilization of MyoD protein (Fig. 8). A recent report by Langen et al. (60) also suggests that TNF-α, an important member of TNFSF, also inhibits myogenic differentiation by destabilization of MyoD protein. Although the exact mechanisms by which TWEAK affects MyoD protein stability is yet to be investigated, our data indicate that TWEAK-mediated degradation of MyoD protein is downstream to the activation of NF-κB (Fig. 8C). Collectively, the data presented in this report suggest that TWEAK inhibits myogenic differentiation through the activation of NF-κB signaling pathway. Activated NF-κB can inhibit myogenesis by augmenting the expression of growth stimulatory molecules such as cyclin D1, which may increase myoblast proliferation and/or increase the expression of molecules, which decrease MyoD levels (Fig. 9).

This study is the first one to demonstrate the effect of TWEAK on skel-
etal muscle cells. Certainly, more investigations are required to understand the regulation of TWEAK/Fn14 signaling system in muscle physiology and pathophysiology. TWEAK/Fn14 pathway may promote the proliferation of satellite cells in response to muscle injury. On the other hand, like many other pro-inflammatory cytokines, TWEAK might induce muscle atrophy by preventing the formation of new muscles. It is important to recognize that, in mice, TWEAK and its receptor Fn14 are located on chromosomes 11 and 17, respectively. We have previously shown that chromosomes 11 and 17 contain the quantitative trait loci regions, which regulate skeletal muscle size (74). Preliminary studies by our group suggest that the expression of TWEAK is increased during wound healing further suggesting that components of TWEAK signaling pathway may play an important role in tissue repair and/or regeneration.

Acknowledgments—We thank Dr. John F. Engelhardt of the University of Iowa for providing dominant negative IKKα and IKKβ adenoviral vectors. We also thank Dr. Ramon Langen for his useful suggestions and Nia Wedhas for her excellent technical support.

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