# Systemic administration of the NF-κB inhibitor curcumin stimulates muscle regeneration after traumatic injury

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**Thaloor, Deepa, Kristy J. Miller, Jonathan Gephart, Patrick O. Mitchell, and Grace K. Pavlath.** Systemic administration of the NF-κB inhibitor curcumin stimulates muscle regeneration after traumatic injury. *Am. J. Physiol.* 277 (*Cell Physiol.* 46): C320–C329, 1999.—Skeletal muscle is often the site of tissue injury due to trauma, disease, developmental defects or surgery. Yet, to date, no effective treatment is available to stimulate the repair of skeletal muscle. We show that the kinetics and extent of muscle regeneration in vivo after trauma are greatly enhanced following systemic administration of curcumin, a pharmacological inhibitor of the transcription factor NF-κB. Biochemical and histological analyses indicate an effect of curcumin after only 4 days of daily intraperitoneal injection compared with controls that require >2 wk to restore normal tissue architecture. Curcumin can act directly on cultured muscle precursor cells to stimulate both cell proliferation and differentiation under appropriate conditions. Other pharmacological and genetic inhibitors of NF-κB also stimulate muscle differentiation in vitro. Inhibition of NF-κB-mediated transcription was confirmed using reporter gene assays. We conclude that NF-κB exerts a role in regulating myogenesis and that modulation of NF-κB activity within muscle tissue is beneficial for muscle repair. The striking effects of curcumin on myogenesis suggest therapeutic applications for treating muscle injuries.

transcription factor; muscle differentiation; dominant-negative; pyrrolidine dithiocarbamate; retrovirus

SKELETAL MUSCLE COMPRISES 80% of the human body and as such is often the site of injury due to trauma, disease, or surgery. Skeletal muscle is one of the few tissues of the body that can regenerate following injury due to a regulated balance of growth, fusion, and differentiation of muscle precursor cells (myoblasts). However, the effectiveness of regeneration can be diminished by genetics (22), age (15, 20, 42, 59), sex (20), loss of structural or supporting structures (25), or muscle type (37).

To date, no effective treatment is available to enhance the repair of injured muscle. Previous attempts to enhance muscle repair after experimentally induced injury used treatment applied directly to the site of injury. Thus infusion or injection of myoblast growth factors, such as basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (10, 32), or heparan-sulfate-like polymers that modulate the stability and/or activity of endogenous growth factors (19, 51) was tried in experimental models of muscle injury with limited success. In another group of studies, myoblast transplantation was used to augment the number of muscle precursor cells present within the damaged site, as a means of enhancing muscle repair after injury. Bischoff and Heintz (11) supplemented their myoblast transplants with soluble muscle extracts derived from crushed muscles and demonstrated an increase in creatine kinase levels in the tissues. The increase in creatine kinase is consistent with an increased formation of myofibers in the degenerating muscle. Myoblast transplants by others (2, 24) demonstrated functional improvement in the muscles after cell transplantation only when the host's own muscle precursor cells were compromised or destroyed, thus indicating that the transplanted myoblasts were outnumbered by the endogenous muscle precursor cells. In contrast, mass and functional capacity of degenerating muscles could be enhanced with myoblast transplantation in immunocompromised animals, although not to normal levels, in a situation where the host's muscle precursor cells were normal (6).

Treatment of muscle injuries would be best achieved through systemic administration of a pharmacological compound. In this manner, diffuse muscle injuries as well as localized ones would be equally accessible for treatment. Here we test the ability of curcumin (diferuloylmethane), a dietary pigment responsible for the yellow color of curry, to enhance repair of multiple muscles after induced trauma. Recently, curcumin has been shown to promote skin wound healing (46), suggesting it might be efficacious in other models of tissue healing.

The anti-inflammatory, anti-carcinogenic, and free-radical-scavenging properties of curcumin have been well documented, along with its low toxicity (3, 17, 43, 48–50, 57). Many of the beneficial effects of curcumin are consistent with its ability to block the activity of the transcription factor, NF-κB. Curcumin has been shown to prevent activation of NF-κB by blocking phosphorylation of the NF-κB inhibitor, IκB (47). Under basal conditions, the inhibitory subunit IκB is bound to the p50/p65 heterodimer of NF-κB in the cytoplasm. A wide array of stimuli activate IκB kinase (18, 28, 29), which phosphorylates IκB, leading to its dissociation from NF-κB. The free NF-κB translocates to the nucleus, binds to a consensus DNA sequence in the promoter elements of numerous genes, and activates transcription (8, 53).

In this report we demonstrate that systemic treatment with curcumin after local muscle injury leads to faster restoration of normal tissue architecture as well as to increased expression of biochemical markers

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associated with muscle regeneration. In vitro studies indicate that curcumin can act directly on myoblasts to increase cell proliferation as well as fusion and differentiation. Curcumin enhances differentiation of muscle cells by inhibiting NF-κB activity, as suggested by in vitro experiments using other pharmacological or genetic inhibitors of NF-κB. The ability of curcumin to increase the rate and extent of muscle regeneration indicates that it may be the first systemically administered drug useful for treating muscle injuries.

## EXPERIMENTAL PROCEDURES

### *Induced Regeneration of Skeletal Muscle and Histological Analyses*

Adult C57BL/6 male mice (4–6 wk old) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed in viral- and pathogen-free conditions. All animals were handled in accordance with the guidelines of the Administrative Panel on Laboratory Animal Care of Emory University.

Masseter or tibialis anterior (TA) muscles were subject to a standardized freeze injury as described in Ref. 37. Mice were injected intraperitoneally with 0.15–0.2 ml of either curcumin or vehicle (DMSO) diluted in PBS starting on the day of damage and continuing once daily thereafter. The final concentration of DMSO in the vehicle was 0.1%. Groups of 2–3 animals were killed either 4 or 10 days after damage, and the muscles were removed using standardized dissection methods. For the masseter muscles, the deep belly of the muscle was not included in the dissection. The muscles were processed subsequently either for immunohistochemistry or immunoblot analyses.

For histological analyses, cross sections were collected at 400- to 500-μm intervals along the entire length of the muscle and stained with haematoxylin and eosin. At each interval, four to five serial 14-μm sections were collected for immunohistochemistry. Sections were fixed in 2% formaldehyde and incubated with 10% normal goat serum, 0.1% Triton X-100 in PBS for 30 min. The sections were incubated in succession with F.1652, an antibody against embryonic myosin heavy chain (EMHC) (16) used as an undiluted hybridoma supernatant, a 1:400 dilution of biotin-conjugated $F(ab')_2$ fragment goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA), and a 1:1,000 dilution of Texas-Red-conjugated streptavidin. All incubations were at room temperature. Controls included incubation with biotin-conjugated secondary antibody alone or nonimmune mouse ascites. No staining was observed in these controls. All analyses and photography were performed on an Axiovert microscope (Carl Zeiss, Thornwood, NY) equipped with a video camera (Optronics Engineering, Goleta, CA) and Scion Image software (Scion, Frederick, MD).

### *Immunoblot Analyses for Muscle Regeneration*

Equal amounts of protein (10 μg) (14) were resolved by electrophoresis on 7.5% SDS-PAGE minigels using standard techniques and were electrophoretically transferred to a polyvinylidene difluoride membrane. EMHC was detected using the F1.652 antibody and enhanced chemiluminescence (7). Densitometry of the films was performed using an optical scanner and quantitated using Scion Image software.

### *Analyses of Myoblast Populations After Injury*

The number of myoblasts present in the masseter and TA muscles on different days after injury was determined (37) with the following modifications. Animals were injected intraperitoneally daily with either curcumin or vehicle starting on the day of injury, and groups of three animals were killed 1, 2, or 3 days later. The entire volume of the cell suspension generated from each enzymatically dissociated muscle was suspended in growth medium [GM; Ham's F10, 20% fetal bovine serum (FBS), 5 μg/ml bFGF] and plated in two 35-mm collagen-coated dishes. After 2 days, the cells were processed for immunohistochemistry using an antibody against myoD (Vector Laboratories, Burlingame, CA) at a dilution of 1:20 and biotin-conjugated $F(ab')_2$ fragment goat anti-mouse IgG (Jackson Immunoresearch Laboratories) at a dilution of 1:200. The ABC Elite kit (Vector Laboratories) and diaminobenzidine with nickel enhancement were used to visualize binding of the myoD antibody. All cells in 15 random fields were counted using phase-contrast microscopy. Controls included incubation with biotin-conjugated secondary antibody alone or nonimmune mouse ascites. No staining was observed in controls.

### *Cell Culture*

Myoblasts were derived from the TA muscles of adult mice 2 days after muscle injury and purified to >99% by expansion in GM (40). Differentiation was induced by switching confluent myoblast cultures to serum-containing fusion medium (DMEM, 2% horse serum) for 36–72 h. Primary human myoblasts were derived from a muscle biopsy taken from a 2-yr-old donor and were purified to 99% using flow cytometry (54). The myoblast cell line C2F3 was grown in DMEM containing 5% FBS and 15% iron-supplemented calf serum. All cell culture reagents were from GIBCO BRL (Gaithersburg, MD) unless noted.

### *In Vitro Cell Proliferation Assays*

Cell proliferation was measured as described (37). In brief, primary mouse myoblasts ($2 \times 10^4$) were plated in GM overnight. The media were then replaced with GM containing 10% FBS and various concentrations of curcumin. After 32–72 h, the cells were pulsed with 1 μCi/ml [*methyl*-$^3$H]thymidine (sp act 25 Ci/mmol) for 2 h at 37°C. DNA synthesis was assayed by measuring the amount of radioactivity incorporated into TCA-insoluble counts per minute.

### *In Vitro Fusion and Differentiation Assays*

*Fusion assay.* Confluent cultures of human myoblasts were placed in serum-free fusion medium [DMEM, 1:100 dilution of insulin-transferrin-selenium supplement (GIBCO BRL), 0.1% BSA] containing either curcumin or pyrrolidine dithiocarbamate (PDTC; Sigma Chemical, St. Louis, MO). The media containing drugs was replaced daily. After 48–56 h, the cultures were fixed in 2% formaldehyde and stained with 1 μg/ml ethidium bromide for 5 min. Ethidium bromide brightly stains the nuclei, whereas the cytoplasm stains faintly under ultraviolet illumination. The number of nuclei inside myotubes with three or more nuclei and the total number of nuclei were counted in 20 random fields per dish using fluorescence microscopy. A total of 150–406 nuclei were analyzed for each drug concentration in four independent experiments. Fusion index was calculated for each dish as the ratio of the number of nuclei inside myotubes to the total number of nuclei counted.

*Assay for biochemical differentiation.* Confluent cultures of primary mouse myoblasts were placed in fusion medium containing either curcumin or PDTC. The media containing the drugs were replaced daily. Cells were lysed as described in Ref. 7, and the lysates were processed for immunoblotting

using antibodies to EMHC, sarcomeric actin (s-actin; Sigma), desmin (Sigma), and myogenin (56).

### *Retroviral Plasmids, Retroviral Production, and Infection*

The NF-κB-responsive plasmid (pDT-06) contains a luciferase gene under the control of a minimal human interleukin-2 (IL-2) promoter with an upstream sextet of a consensus NF-κB response element 5′-ATCAGTTGAGGGGACTTTCC-CAGGC-3′. The control plasmid (pDT-03) contains a triplex of a mutant NF-κB response element 5′-ATCAGTTGAGGCGA-CTTTCCCAGGC-3′. The single nucleotide change from G to C (indicated by underlining) prevents the binding of NF-κB to the oligonucleotide (47). Both plasmids confer neomycin resistance and were created by modifications to the retroviral plasmid pLNCX (13, 30). A mutant p50 subunit of NF-κB (Δsp), which acts as a dominant-negative inhibitor of NF-κB function (27), was a generous gift of Dr. Paul Khavari at Stanford University (45). The vector TJ66 (T. J. Murphy, unpublished results) expresses green fluorescent protein and was used as a control vector.

Retroviruses were prepared by transient transfection of helper-virus-free amphotropic producer cells (38) with the plasmids. Infectious supernatant was collected and used to infect myoblasts (1). Two or three rounds of retroviral infection were performed, which typically resulted in >90% infection efficiency. Myoblasts containing the retroviral reporter plasmids were treated with IL-1β or vehicle. In some cases, the cells were pretreated with 5 μM curcumin or 5 μM PDTC for 1 h in DMEM and 2% FBS, and then fresh media containing IL-1β and curcumin or PDTC were added. After 5 h, the cells were lysed and luciferase assays were performed (1). For experiments with p50 Δsp, luciferase assays or immunoblots were performed 3 days after the last retroviral infection.

## RESULTS

### *Systemic Curcumin Administration Enhances Muscle Regeneration in Multiple Muscles*

Curcumin was tested for its ability to enhance muscle repair after local injury to different muscles in the mouse. We have shown previously that the regenerative capacity of different skeletal muscles after injury can vary greatly (37). In limb muscle, normal architecture is restored 12 days after injury, whereas in masseter muscle much less regeneration occurs during this same time period. At late time points masseter muscles regenerate but exhibit increased fibrous connective tissue in the region of the injury.

Both the masseter and TA muscles were injured, and different doses of curcumin were administered starting on the day of injury. Muscles were collected 4 days after injury. Muscle regeneration was quantitated on immunoblots using EMHC as a measure of the formation of new muscle fibers. A dose-dependent effect on EMHC expression is observed in both masseter and TA muscles with curcumin treatment, with 20 μg/kg being the optimal dose (Fig. 1, *left*). Administration of 20 μg/kg curcumin leads to an eightfold increase in EMHC expression in masseter muscles and a fivefold increase in TA muscles.

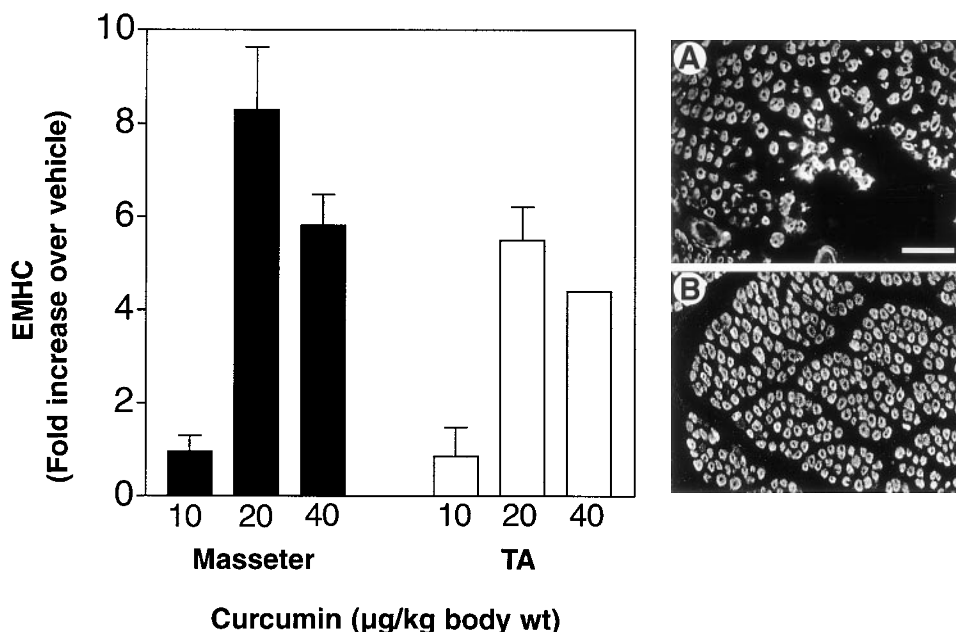


Fig. 1. Curcumin increases muscle regeneration after injury to multiple muscles. *Left*: both masseter and tibialis anterior (TA) muscles in same animals were injured. Immunoblots were performed on electrophoretically separated proteins isolated 4 days after injury from mice treated with vehicle or curcumin. Each bar represents mean ± SD of amount of embryonic myosin heavy chain (EMHC), a marker of regenerated myofibers, in curcumin-treated samples compared with vehicle ($n$ = 2 for TA; $n$ = 3 for masseter). Duplicate samples were pooled for each experiment. *Right*: immunohistochemical evidence of increased regeneration in masseter muscle 4 days after injury. Muscle sections reacted with an antibody against EMHC are shown. *A* (vehicle): regenerated fibers are fewer in number ($n$ = 180) and randomly organized. Areas of the section are devoid of regenerated fibers at this time point. *B* (curcumin 20 μg/kg): centrally nucleated fibers are organized into fascicles characteristic of mature muscle. Number of EMHC-positive fibers = 340. Both sections shown are central to the injury site. Bar = 100 μm.

Regeneration was also assessed by immunohistochemical staining of muscle sections from vehicle- and curcumin-treated masseter muscles with the use of the antibody to EMHC (Fig. 1, *right*). The newly regenerated fibers in the control samples are fewer in number and randomly organized. In contrast, the newly regenerated muscle fibers in curcumin-treated samples are greater in number and are organized into fascicles characteristic of mature muscle. No expression of EMHC is observed in undamaged muscle fibers of either vehicle- or curcumin-treated animals.

We also analyzed muscle sections from mice 10 days after injury, using standard histological staining, to further characterize the effects of curcumin on regeneration at later time points (Fig. 2). The injured site in masseter muscles from control animals still contains areas devoid of regenerated muscle cells (Fig. 2*A*). In contrast, in curcumin-treated animals the injured site is filled completely with large centrally nucleated myofibers indicative of regenerated muscle fibers (Fig. 2*B*). Together, these data indicate that systemic administration of curcumin enhances skeletal muscle regeneration of multiple muscles in the same animal. The effect of curcumin is dose dependent and occurs at very early times after injury.

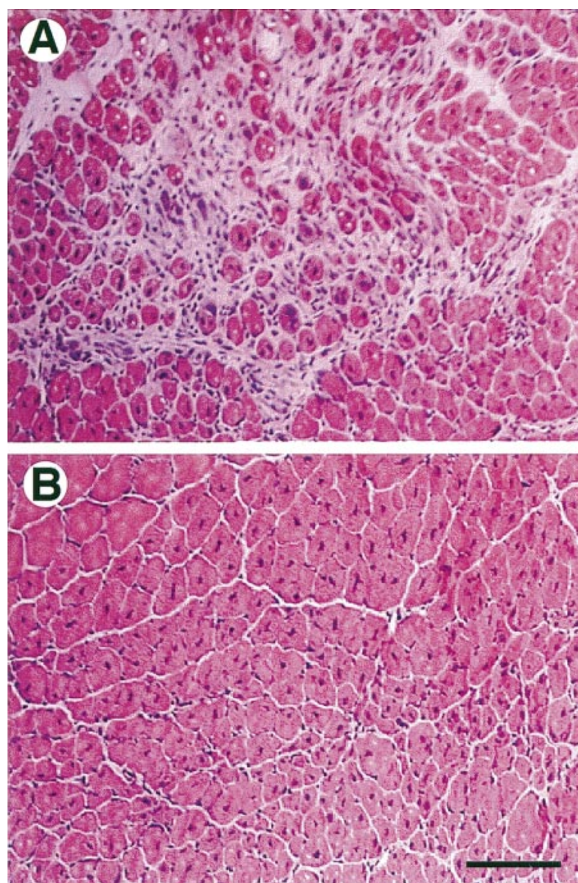


Fig. 2. Histology of masseter muscles 10 days after injury. Mice were treated with vehicle (*A*) or 20 μg/kg ip curcumin (*B*) daily after injury to masseter muscles. In vehicle-treated mice, areas of muscle section are still devoid of regenerating fibers. However, in curcumin-treated mice, normal tissue architecture is restored and is characterized by large centrally nucleated fibers that fill entire region of damage. Both of these sections are central to the injury site. Bar = 100 μm.

### *Cellular Mechanisms of Curcumin Action: Analysis of Myoblast Numbers in Damaged Muscles*

Enhanced formation of newly regenerated myofibers resulting from curcumin treatment may be the result of an increase in the number of muscle precursor cells or an increase in the differentiation of these cells. To elucidate the cellular mechanisms involved in the enhanced regeneration of muscle after injury, we performed immunohistochemical analyses using myoD as a marker of myoblasts isolated from vehicle- and curcumin-treated muscles. These analyses provide an estimate of the number of myoblasts present in the damaged muscles of control and curcumin-treated animals. This type of analysis was not intended to provide a quantitative measure of myoblast number after damage but, rather, served as a relative comparison between untreated and treated muscles. Previous studies indicate correlation between this assay and direct analyses of myoD-positive cells in tissue sections (37).

As shown previously, the greatest number of myoblasts is obtained 2 days after damage with TA muscles, yielding a greater number of myoblasts (Fig. 3*A*) than masseter muscles (Fig. 3*B*), consistent with the greater regenerative capacity of TA muscles (37). However, in general, curcumin treatment does not increase or decrease either the number or percentage (Fig. 3, *C* and *D*) of myoblasts or the total number of cells (Fig. 3, *F* and *G*) obtained in vitro from either TA or masseter muscles after damage. A small but significant decrease in myoblast number with curcumin treatment exists 1 day after damage in masseter muscles only. Taken together, these data indicate that muscle regeneration is significantly enhanced in curcumin-treated muscles without a corresponding large net change in myoblast numbers at these early time points.

### *Effect of Curcumin on Myogenesis In Vitro*

To test whether curcumin has direct effects on myogenic cells, we analyzed primary myoblasts in culture using growth and differentiation assays. Primary mouse myoblasts were treated with different concentrations of curcumin for several days and were pulse labeled with [$^3$H]thymidine to measure population growth. A twofold increase in [$^3$H]thymidine incorporation is observed (Fig. 4). To test the effect of curcumin on myoblast fusion and differentiation, high-density myoblasts were treated with different doses of curcumin in a low-mitogen media. On attaining high density in vitro and by decreasing growth factors, myoblasts fuse with one another to form myotubes. Fusion in vehicle- and drug-treated cultures was assessed by calculating fusion indexes from histological analyses as described in EXPERIMENTAL PROCEDURES. As seen in Table 1, curcumin enhances the fusion of myoblasts twofold compared with vehicle. To determine the effect of curcumin on muscle differentiation, immunoblots of curcumin-treated cells were performed using an antibody against

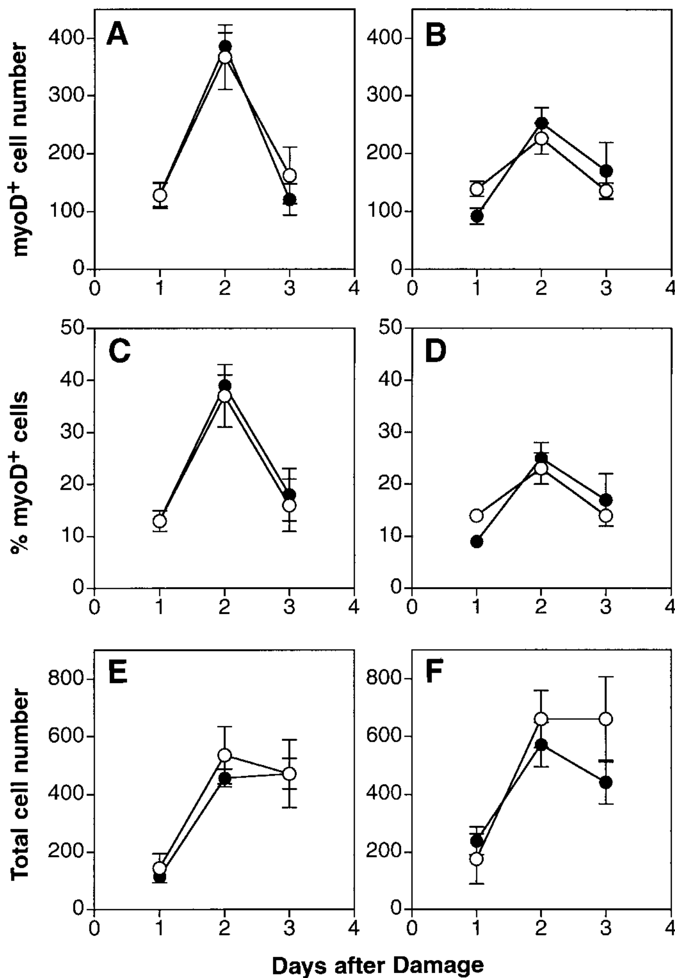


Fig. 3. No net change of myoblast number occurs in regenerating muscles with curcumin treatment. Masseter (*right*) and TA (*left*) muscles were injured and the animals were injected intraperitoneally daily with vehicle (○) or curcumin (●; 20 μg/kg). Muscles were collected on indicated days and enyzmatically dissociated. Liberated cells were plated in vitro and immunostained with an antibody against myoD. Muscle damage increases both number (*A*, *B*) and percentage (*C*, *D*) of myoblasts isolated from these muscles, with peak value occurring 2 days after injury. Curcumin treatment does not alter either peak value or time when peak occurs. Number of myoD-positive cells in different samples was normalized and expressed per 1,000 total cells. Muscle damage also increases total number of cells (*E*, *F*) isolated from these muscles, but no difference is apparent with curcumin treatment. Data are means ± SE ($n$ = 6 for each muscle).

EMHC, a marker of differentiated myotubes (16). Increased levels of EMHC are observed in curcumin-treated cultures (Fig. 5*A*). The increase in expression of EMHC is dose dependent, with a maximal increase of approximately threefold seen at $10^{-6}$ M. Thus curcumin can act directly on muscle cells to stimulate growth as well as both fusion and differentiation.

### *NF-κB Activity and Myogenesis*

Curcumin has been shown to inhibit NF-κB activity in target cells (47). To test whether inhibition of NF-κB can lead to enhanced myoblast fusion and differentiation, assays of myogenesis were performed in high-density myoblast cultures treated with PDTC, another

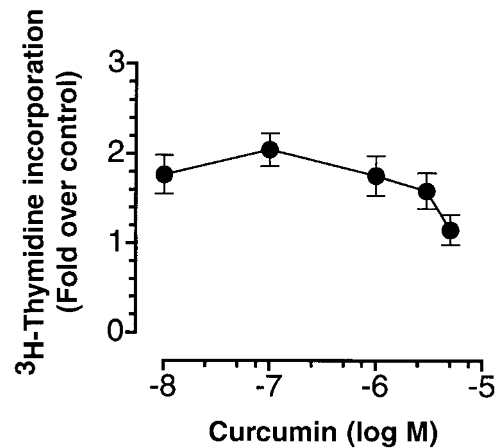


Fig. 4. Curcumin affects myoblast growth in vitro. Primary mouse myoblasts were treated with vehicle or different doses of curcumin for 36–72 h. At end of treatment, cells were pulsed for 2 h with [$^3$H]thymidine, and TCA-precipitable counts per minute were determined. Each point represents mean ± SD of 3 independent experiments each performed in triplicate.

pharmacological inhibitor of NF-κB function (44). Treatment of high-density myoblasts with PDTC should mimic the effects of curcumin on myogenesis if NF-κB is involved in the effects caused by curcumin. In these studies, we focused on fusion and differentiation because these are unique properties of skeletal muscle cells as opposed to cell proliferation. As shown in Table 1, PDTC at $10^{-8}$ M enhances myoblast fusion. In addition, expression of EMHC is increased approximately threefold in PDTC-treated cultures at optimum doses (Fig. 5*B*). Thus two different compounds known to be inhibitors of NF-κB function both stimulate fusion and differentiation of skeletal muscle cells.

To prove conclusively that curcumin and PDTC inhibit NF-κB-mediated transcription in skeletal muscle cells, an NF-κB-responsive luciferase reporter in a retroviral vector was introduced into mouse myoblasts in vitro. Curcumin was tested for the ability to block an IL-1β-stimulated increase in NF-κB-mediated transcriptional activity in myoblast cultures containing the NF-κB-responsive reporter. IL-1β has been used in several cell types to induce NF-κB activity (12). As seen in Fig. 6*A*, IL-1β treatment for 5 h results in an ~25-fold increase in luciferase levels. No luciferase activity is detected in cells containing an NF-κB reporter with a mutated consensus binding sequence.

Table 1. *Fusion of myoblasts is increased with either curcumin or PDTC treatment*

| Dose | Fusion Index, fold increase relative to vehicle | |
|---|---|---|
| | Curcumin | PDTC |
| $10^{-8}$ M | ND | 1.9 ± 0.3 |
| $10^{-6}$ M | 2.0 ± 0.4 | 1.3 ± 0.2 |

Values are means ± SD; $n$ = 4. Fusion index was assayed in cultures of human myoblasts treated with the indicated concentrations of drugs in serum-free fusion medium as described in EXPERIMENTAL PROCEDURES. PDTC, pyrrolidine dithiocarbamate.

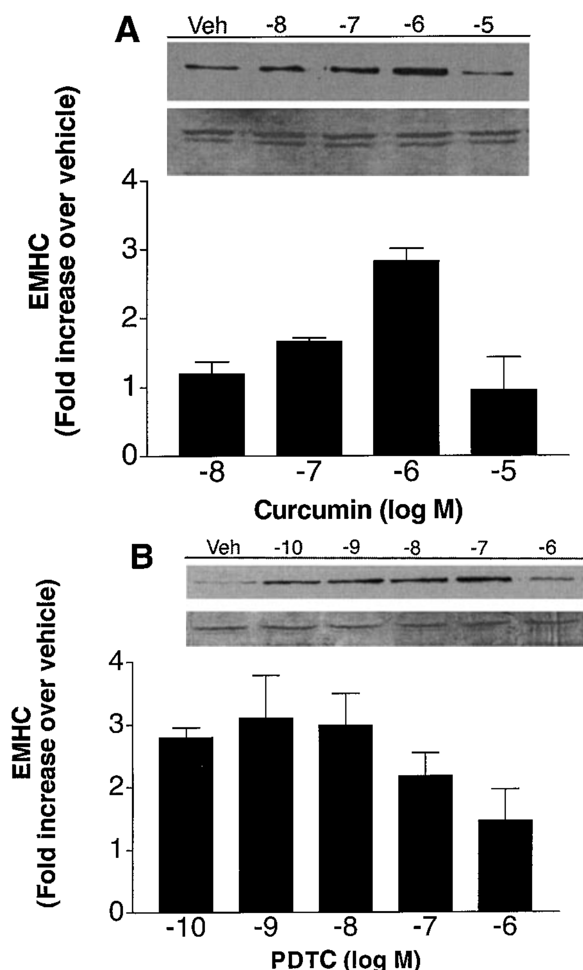


Fig. 5. *A*: curcumin can stimulate muscle differentiation by a direct action on muscle cells. High-density cultures of primary mouse myoblasts were treated with either vehicle (Veh) or different doses of curcumin in serum-containing fusion medium for 36–72 h. Immunoblots of electrophoretically separated proteins were performed using an antibody to EMHC, a marker of differentiated myotubes. Bars represent pooled data showing means ± SD in 2–3 experiments each performed in duplicate. *Inset*: representative immunoblot for EMHC (*top*) and equivalent loading of proteins among lanes as evidenced by Coomassie blue staining of blot (*bottom*). *B*: pharmacological NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), also stimulates muscle differentiation. High-density cultures of primary mouse myoblasts were treated with vehicle or different doses of PDTC for 36–72 h. Immunoblots for EMHC expression were as in *A*. *Inset*: representative immunoblot for EMHC (*top*) and equivalent loading of proteins among lanes as evidenced by Coomassie blue staining of blot (*bottom*).

Pretreatment with curcumin for 1 h followed by IL-1β and curcumin together results in almost complete inhibition of the IL-1β-stimulated response. Pretreatment with PDTC also inhibits the IL-1β-stimulated response. With both drugs, pretreatment was necessary to obtain inhibition of the cytokine-induced response. Because this is a short-term assay, higher doses of curcumin and PDTC were used than those in assays of cell differentiation in Fig. 5. These results demonstrate that both curcumin and PDTC inhibit NF-κB-mediated transcription in myoblasts.

Because these drugs potentially may affect other pathways within muscle cells also, a genetic inhibitor of NF-κB function was used to confirm the contribution of NF-κB to the observed results on myogenesis. The mutant NF-κB subunit p50 Δsp is unable to bind to DNA but is able to form homo- or heterodimers with other members of the c-rel family of proteins, thus acting as a trans-acting dominant-negative inhibitor of NF-κB function (27). To determine that p50 Δsp blocks NF-κB-mediated transcription in muscle cells, primary mouse myoblasts were retrovirally infected with either the NF-κB reporter construct alone or in combination with p50 Δsp. For assessment of the p50 Δsp dominant-negative effects, NF-κB activity was induced with IL-1β as before. As shown in Fig. 6*B*, the mutant effectively blocks the IL-1β-stimulated response.

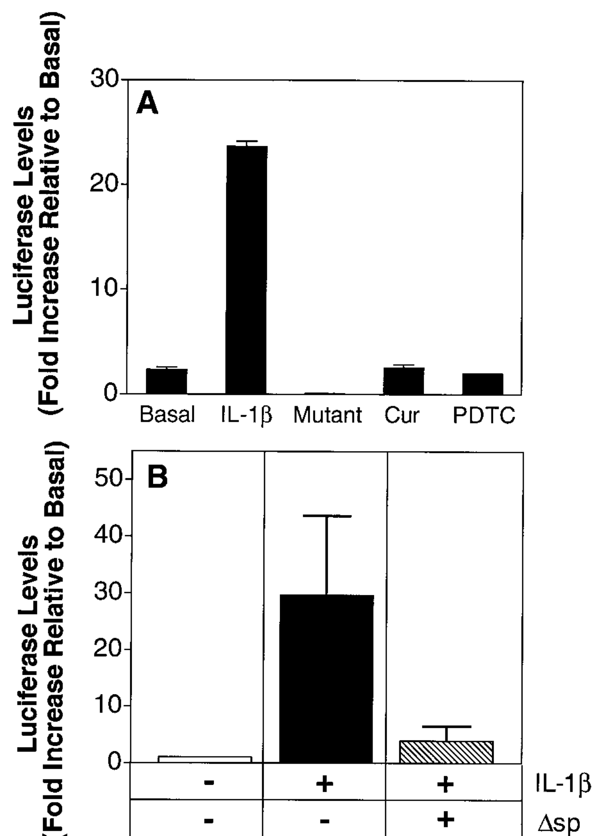


Fig. 6. *A*: curcumin (Cur) and PDTC inhibit NF-κB-mediated transcription in myoblasts. C2F3 mouse myoblasts containing an NF-κB-responsive retroviral reporter plasmid were treated for 5 h with vehicle or 0.5 ng/ml IL-1β to induce NF-κB-mediated transcription. In some cultures, cells were pretreated with either 5 μM curcumin or 5 μM PDTC for 1 h before addition of IL-1β. Luciferase values were subsequently measured. Both curcumin and PDTC inhibit NF-κB-mediated transcription in myoblasts. No NF-κB-mediated transcription in response to IL-1β treatment is observed in myoblasts containing a reporter construct with a mutant NF-κB response element. Each bar represents means ± SD of 2 experiments each performed in duplicate. *B*: retrovirally mediated expression of an inhibitory NF-κB protein also blocks NF-κB-mediated transcription in myoblasts. Primary mouse myoblasts containing an NF-κB-responsive retroviral reporter plasmid only (open and solid bars) or the reporter and the dominant-negative mutant p50 Δsp (hatched bars) were treated for 5 h with vehicle or 0.25 ng/ml IL-1β to induce NF-κB-mediated transcription. Luciferase values were subsequently measured. Each bar represents means ± SD of 3 experiments each performed in duplicate.

To test the effect of p50 Δsp on myogenesis, primary mouse muscle cells were retrovirally infected with either a control vector expressing green fluorescent protein or with one expressing p50 Δsp. Induction of muscle differentiation was assessed using immunoblots and immunohistochemistry in low-density cells in growth medium lacking bFGF, a potent inhibitor of differentiation. A low level of biochemical differentiation is to be expected under these media conditions, but cell fusion does not occur because the cells are not in contact with each other. As observed with the pharmacological inhibitors of NF-κB (Fig. 5), the genetic inhibitor induces expression of the differentiation-associated protein EMHC by 2.4-fold (Fig. 7). Immunohistochemical analyses indicate that only 3.5% of the cells in control cultures express EMHC compared with 37% of the cells in cultures infected with p50 Δsp. Because expression of sarcomeric proteins is an essential part of the myogenic program and serves as a common phenotypic marker for muscle differentiation (5), the expression of other muscle structural proteins such as sarcomeric actin and desmin was also examined on immunoblots. The expression of these two proteins is also enhanced in cells containing the dominant-negative mutant: a twofold induction of s-actin and a 2.8-fold induction of desmin is observed. Because transcription of the myogenic transcription factor myogenin is upregulated on myogenic induction (35) and its activity is crucial for the activation of the entire differentiation program, myogenin expression was also assessed. There is a 1.9-fold induction of myogenin in cultures containing p50 Δsp. Thus inhibition of NF-κB function in myoblasts is sufficient to induce the myogenic differentiation program.

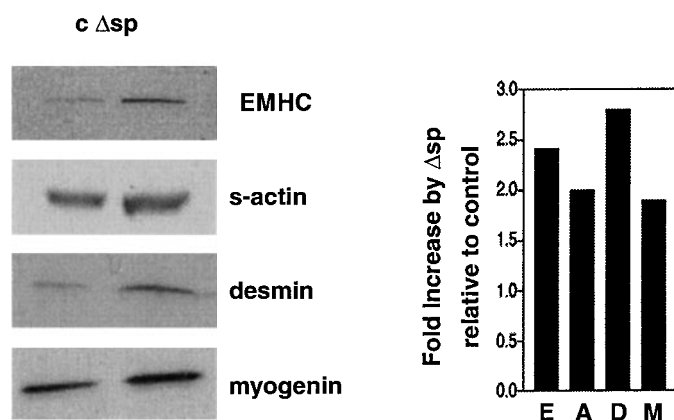


Fig. 7. Expression of dominant-negative mutant p50 Δsp in myoblasts induces expression of differentiation-associated proteins. Primary mouse myoblasts were infected with either a control retroviral vector expressing green fluorescent protein or a vector expressing the mutant p50 Δsp. *Left*: immunoblots of electrophoretically separated proteins isolated from low-density myoblasts in growth medium without basic fibroblast growth factor were performed using antibodies to EMHC, sarcomeric actin (s-actin), desmin, and myogenin. Antibody binding was detected with enhanced chemiluminescence in 2 independent experiments each performed in duplicate. Representative blots are shown. *Right*: immunoblots were subject to densitometric analysis. Increase in expression of differentiation-associated proteins by p50 Δsp relative to control is shown as average of 2 independent experiments each performed in duplicate.

## DISCUSSION

Previous attempts to increase muscle repair after injury used local infusion or injection of purified growth factors to the injured site with limited success (10, 11, 32). Our studies with curcumin differ substantially from previous work in the area of muscle repair after traumatic injury. First, in our studies, systemic administration was used instead of local delivery to the site of injury. Second, the effect of curcumin treatment is rapid. Only a few days of systemic administration after injury resulted in a pronounced stimulation of muscle regeneration. Third, curcumin is not solely a growth factor but has a dual effect on cultured myoblasts, acting to increase DNA synthesis under growth-permissive conditions and fusion and differentiation under differentiation-promoting conditions. Purified factors delivered locally to sites of muscle injury have all been solely myoblast growth factors (10, 32) and, in the case of bFGF, inhibit muscle differentiation. At first glance it may appear puzzling that we did not observe much change in the number of myoblasts from dissociated muscles after damage. Either curcumin exerts its action on muscle regeneration totally independently of muscle cells or else an increase in the number of myoblasts through activation of quiescent satellite cells and/or myoblast proliferation is balanced by a decrease in number due to fusion of these cells into multinucleated myotubes. Alternatively, curcumin may have changed the ability to isolate myoblasts from muscle tissue, such that any increase in the number of myoblasts was not detectable by our protocols.

Regeneration was assessed both by biochemical and histochemical assays. Immunoblots demonstrated a significant increase in the expression of EMHC, a marker of regenerated muscle fibers, in both the masseter and TA muscles, muscles characterized by markedly different regenerative capacities (37). Increased levels of EMHC in curcumin-treated animals were paralleled by faster restoration of normal tissue architecture as assessed by immunohistochemistry for EMHC and standard histological stainings. Fibrosis was not observed in regenerating muscles of animals treated with curcumin, suggesting that curcumin does not stimulate fibroblasts, which are a major cell type in skeletal muscle compared with muscle precursor cells. Although the effects of curcumin on muscle regeneration are consistent with a myoblast target of action, the beneficial effects of curcumin may arise from effects on cells in addition to muscle cells. Both a functional immune system (20) and angiogenesis (21) have been demonstrated to be necessary for muscle regeneration to occur. The rapid effects of curcumin on muscle regeneration may be due also to its actions as a chemoattractant for immune cells or as an angiogenic agent (Thaloor, unpublished observations).

The use of systemically administered curcumin as an agent to enhance muscle regeneration after trauma bypasses several limitations present in previous experiments that used local delivery of protein growth factors or myoblast transplantation. First, curcumin acts on

the host's own cells to exert its effect and thus abrogates the necessity for large-scale growth of myoblasts for cell therapy and the inherent immune problems associated with myoblast transplantation (36, 40, 41). Second, localized as well as diffuse muscle injuries can be easily treated. Third, surgery is not required to implant a pump to deliver the therapeutic factor. Finally, direct injection into the injured muscle is not required, alleviating further muscle injury from repeated injections.

Curcumin is used as an anti-inflammatory agent in Asia. It inhibits NF-κB, a transcription factor involved in expression of a number of inflammatory cytokines (47). The use of anti-inflammatory agents has mixed results on enhancing muscle regeneration. Naproxen, a nonsteroidal anti-inflammatory drug that inhibits prostaglandin formation via a non-NF-κB-dependent mechanism, has no effect on muscle regeneration after induced injury in normal animals (52). Glucocorticoids, which affect many aspects of the immune system, have a beneficial effect on regeneration in dystrophic mice in some studies (4) but not others (55).

Inhibition of NF-κB-mediated transcription in myoblasts is sufficient to stimulate muscle differentiation, as shown by the studies with the dominant-negative inhibitor of NF-κB function. NF-κB proteins have been extensively studied in lymphoid cells, but little is known of their role in skeletal muscle cells beyond that a prominent decrease occurs in the DNA binding activity of NF-κB early in muscle differentiation (26). In numerous cell types, NF-κB proteins promote cellular proliferation and active cellular stress responses (8, 53). In skeletal muscle cells, an antagonism exists between growth and differentiation. The expression and/or activity of myogenic regulatory factors can be suppressed by factors that promote proliferation (34). Inhibition of NF-κB function by either pharmacological or genetic inhibitors stimulates muscle differentiation, consistent with a growth-promoting activity for NF-κB in skeletal muscle (26). The genes that are regulated by NF-κB in muscle are unknown; however, NF-κB regulates the expression of numerous cytokines in immune cells (9). Several of these cytokines are either mitogens for myoblasts such as IL-6 (7) or inhibit muscle differentiation, such as tumor necrosis factor-α (31). These cytokines are expressed at early times after injury in regenerating muscle (Thaloor and Pavlath, unpublished observations) and as such could be targets for the action of curcumin. Further studies are needed to elucidate NF-κB-mediated signaling pathways in skeletal muscle.

Other targets of action besides NF-κB may contribute to the ability of curcumin to enhance either muscle regeneration in vivo or proliferation, fusion, and differentiation of cultured myoblasts. Curcumin is also known to block the activity of the transcription factor, AP-1 (23). However, inhibition of AP-1 is unlikely to account for our results, because PDTC, which like curcumin stimulates muscle differentiation, increases AP-1 activity (33, 39, 58). Thus the known action that both compounds share is inhibition of NF-κB activity. However, there may exist currently unknown actions that these compounds share.

In summary, curcumin is the first example of a pharmacological agent with a potent effect on stimulating muscle regeneration after trauma. Localized, as well as diffuse, muscle injuries could be treated with curcumin, because it is administered systemically. Enhanced repair of muscle would be beneficial not only in muscle trauma but also in reconstructive surgery and sports-related injuries.

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