PEDF-derived peptide promotes skeletal muscle regeneration through its mitogenic effect on muscle progenitor cells

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The inflammatory response activates macrophages to eliminate damaged myofibers (1, 25). Following injury, satellite cells undergo rapid self-renewal and proliferation to form myoblasts, starting from the second day after injury (13). The myoblasts migrate to the site of injury, differentiate, and fuse to form new myofibers (12). In Pax7−/− mice, satellite cells are depleted rapidly during the early postnatal period and skeletal muscle regeneration is severely hampered (29, 34), showing that Pax7 is crucial for satellite cell maintenance and Pax7-positive satellite cells play an essential role in skeletal muscle regeneration (29, 34).

Treatment of rat soleus muscle by local intramuscular injection of bupivacaine, an anesthetic agent, is a reproducible method of inducing extensive muscle necrosis and provides a useful model for studying muscle regeneration. It is well known that muscle regeneration after injury is driven by surviving satellite cells. When activated, these proliferate, differentiate, and fuse to form myotubes, in which the nuclei are positioned at the center of the syncytium (10, 13). The centrally nucleated myofibers may be observed from around 3 days after muscle injury induced by bupivacaine and remain visible up to 14 days (4, 16, 28). Next, the newly formed myofibers grow continuously until they reach the size of mature fibers. During maturation, the central located nuclei move to the periphery of the myofiber. The process of soleus muscle regeneration following bupivacaine injection may be accelerated, such as by the application of heat stress (28). The beneficial effect of heat stress on muscle regeneration is associated with the satellite cells, which are activated in the damaged site (28). The formation of nonmuscle tissues in the repair process could be a consequence of depletion of the pool of satellite cells over time (2, 5). Sufficient numbers of satellite cells seem to be quite important to support complete muscle healing. Pharmacological agents that promote the generation of muscle satellite cells have clinical potential for improving muscle regeneration after injury.

Pigment epithelium-derived factor (PEDF) is a 50-kDa secreted glycoprotein that exhibits multiple biological activities through various motifs. For example, the human PEDF 44-mer motif (amino acid positions Val19-Thr21) determines the neurotrophic activity of PEDF (7). On the other hand, the 34-mer (Asp31-Asn37) is responsible for the antiangiogenic and antifi-
brotic activities of PEDF and does not exhibit neurotrophic effect (18, 40). Previously, we showed that PEDF and the 44-mer can induce the self-renewal of corneal limbal epithelial stem cells (LSCs) (23), indicating that the 44-mer motif has the same mitogenic effect on LSCs as the full-length PEDF protein. Moreover, the 44-mer has been shown to be a potent regenerative agent in repairing the corneal epithelium in mice (23). The expression of PEDF is seen in a variety of human tissues, including skeletal muscle cells and smooth muscle cells (15, 17, 27, 31). Moreover, more PEDF is released from human myotubes upon their contraction in vitro (31). In addition to being a neurotrophic factor, PEDF may also be a muscle trophic factor working in an autocrine or paracrine fashion. In this study, we investigated the effect of PEDF on satellite cell proliferation because these cells play an exclusive role in postnatal muscle maintenance and repair. We also determined whether the process of soleus muscle regeneration can be accelerated by application of the PEDF-derived short peptide (PSP; residues Ser93-Leu112). Specifically, we show that PEDF and the PSP induce the proliferation of satellite cells and C2C12 myoblasts in vitro. We provide evidence that, in rat soleus muscle wounded by the myotoxin bupivacaine, the PSP enhances the regenerative process by increasing the numbers of satellite cells in injured muscle in vivo.

MATERIALS AND METHODS

Materials

 Dulbecco’s modified Eagle’s medium (DMEM), horse serum (HS), chicken embryo extract, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Ultrapure alginate (6,000 Da), dimethyl sulfoxide (DMSO), fibronectin, 5-bromo-2′-deoxyuridine (BrdU), and Hoechst 33258 dye were all from Sigma-Aldrich (St. Louis, MO). Collagenase type I and Dispase II were obtained from Roche (Indianapolis, IN). Antibodies used in this study were for active p38 MAPK, active ERK, and active JNK (Promega, Madison, WI), p38 MAPK, ERK1/2 (p44/42 MAPK), JNK, phospho (p)-Akt (Thr308), Akt (pan), p-Erk-1 (Ser204), and p-ATF2 (Thr75) (Cell Signaling Technology, Beverly, MA), Socs3, survivin, BrdU (GTX-42641), and β-actin (GeneTex, San Antonio, TX). Pax7 mAb (MAB1675) was purchased from R&D Systems (Minneapolis, MN). All of the fluorescent dye-conjugated secondary antibodies were purchased from BioLegend (San Diego, CA). PD-98059, SB-203580, SP-600125, LY-294002, and STAT3 inhibitor (no. 573096) were purchased from Calbiochem (La Jolla, CA). Hematoxylin and eosin (H&E) dyes were purchased from Merck (Rahway, NJ). PEDF was purified from human plasma via collagen I-Sepharose resin, as described previously (30), and preserved in a solvent composed of 20 mM sodium phosphate, pH 6.4, 0.2 M sodium chloride, and 1 mM DTT. Short synthetic PEDF peptides were synthesized, modified by acetylation at the NH2-termini and amidation at the COOH-termini for stability, and characterized by mass spectrometry (>95% purity), to order from GenScript (Piscataway, NJ).

Cell Culture

The C2C12 mouse myoblast cell line was from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in DMEM-high-glucose medium supplemented with 10% FBS, 4 mM l-glutamine, 1 mM pyruvate, and 100 U/ml penicillin-100 μg/ml streptomycin at 37°C in the presence of 5% CO2.

Cell Proliferation Assays

C2C12 myoblasts (1×10⁵) were seeded on glass slides and incubated in 10% FBS culture medium for one day. Subsequently, cells were maintained in 1% FBS DMEM for 24 h and then switched to stimulation medium, containing of DMEM supplemented with 1% FBS, 4.5 nM PEDF, and 50 nM PEDF peptide for a further 24 h. C2C12 cell proliferation was evaluated by measuring BrdU incorporation in cells synthesizing DNA. Briefly, BrdU (final concentration, 10 μM) was added to the culture for 6 h. After being fixed with 4% paraformaldehyde (PFA), the cells were exposed to cold methanol for 2 min and then treated with 1 N HCl at room temperature (RT) for 1 h before immunofluorescence.

Myofiber Culture and Fiber-Associated Satellite Cell Proliferation Assay

Single myofibers with associated satellite cells were isolated from the rat extensor digitorum longus (EDL) muscle as described previously (36, 37). Briefly, the hindlimb EDL muscles were digested for 2 h at 37°C in 0.2% (wt/vol) collagenase type I. The collagenase was reconstituted in DMEM (high glucose, with l-glutamine, 110 mg/l sodium pyruvate, and pyridoxine hydrochloride; supplemented with 100 U/ml penicillin-100 μg/ml streptomycin). Following digestion, the muscle was dispersed with a wide-bore pipette to release single myofibers. The myofibers were then suspension-cultured in wells of 24-well plates (n = 4 myofibers/well) with 0.5 ml DMEM containing 20% FBS, 10% HS, and 1% chicken embryo extract, at 37°C in 5% CO2, for up to 24 h. To examine the effect of PEDF peptide on the proliferation of myofiber-associated satellite cells, myofibers were cultured in suspension in DMEM containing 1% FBS, 1% HS, 1% chicken embryo extract, 10 μM BrdU, and 50 nM PEDF peptide for 24 h. Subsequently, myofibers were fixed in 4% PFA for 2 h, exposed to cold methanol for 2 min, and treated with 1 N HCl at RT for 1 h before immunofluorescence.

PEDF Peptide/Alginate Gel Formulation and Bolus Formulation

The PSP 29-mer (Ser93-Thr121) and 20-mer (Ser93-Leu112) were reconstituted in DMSO as stocks (5 mM). Next, ultrapure alginate was mixed with the stock to obtain a 2% wt/vol alginate solution with the PEDF peptide at a final concentration of 10 μM. The alginate solution was then filtered through a membrane filter (pore size, 0.22 μm) and mixed with filtered calcium sulfate (0.21 g CaSO4/ml of dH2O) at a ratio of 25:1 (40 μl of CaSO4/1 ml of the filtered alginate solution). The mixture was left to stand at RT for about 1 h to allow for the cross-linking of the alginate. The resultant sustained-release formulation was then used for the treatment of muscle damage. The 29-mer in phosphate-buffered saline (PBS; 10 μM) was used for bolus delivery.

In Vitro Release Test for the PEDF Peptide/Alginate Gel

To determine the release kinetics, 100 μg of FITC-conjugated PEDF peptide were released from 100 mg of alginate gel in 1.5 ml PBS (pH 7.4) in microcentrifuge tubes and placed in an orbital shaking incubator (37°C) over a 6-day period. The tubes were centrifuged at each predetermined time point; 200 μl of supernatant were removed and stored at −80°C for further analysis, and 200 μl of fresh PBS were added to the tubes to replace the supernatant. The concentration of FITC-conjugated PEDF peptide present in the stored supernatants was determined using a fluorimeter in 96-well format. A known, unencapsulated FITC peptide was used to generate a standard curve. Triplicate data were used for analysis.

Muscle Regeneration Model

Experimental procedures were approved by the Mackay Memorial Hospital Review Board and conducted according to national animal welfare regulations. Adult 10-wk-old male Sprague-Dawley rats (total 20 mice) were anesthetized with a mixture of ketamine and xylazine (100 mg/kg ketamine hydrochloride and 20 mg/kg xylazine), and the left hindlimb muscles were surgically exposed. A 5-mm incision was made on the posterior side of the EDL muscle and the muscle was dispersed with a wide-bore pipette to release single myofibers. The myofibers were then suspension-cultured in wells of 24-well plates (n = 4 myofibers/well) with 0.5 ml DMEM containing 20% FBS, 10% HS, and 1% chicken embryo extract, at 37°C in 5% CO2, for up to 24 h. To examine the effect of PEDF peptide on the proliferation of myofiber-associated satellite cells, myofibers were cultured in suspension in DMEM containing 1% FBS, 1% HS, 1% chicken embryo extract, 10 μM BrdU, and 50 nM PEDF peptide for 24 h. Subsequently, myofibers were fixed in 4% PFA for 2 h, exposed to cold methanol for 2 min, and treated with 1 N HCl at RT for 1 h before immunofluorescence.
n = 60; initial body wt = 312 ± 11 g) were anesthetized by an intraperitoneal injection of xylazine (10 mg/kg) and then the soleus muscle was injured by injection with 0.5 ml of bupivacaine (AstraZeneca) unilaterally using a disposable syringe with a 26-gauge needle, as reported previously (28). Briefly, the needle was inserted in the distal portion of the soleus muscle and then withdrawn slowly and longitudinally to the proximal portion with continuous and steady injection of the bupivacaine solution. Thirty minutes after bupivacaine injection, the soleus muscle received 50 µl of blank alginate gel, 50 µl PSP/alginate gel (final concentration 10 µM), or 50 µl PSP bolus (29-mer resolved in 50 µl PBS to a final concentration of 10 µM) injected longitudinally as for the bupivacaine treatment. At 4, 7, and 14 days after bupivacaine injection, the animals were anesthetized by CO2, and soleus muscles were harvested and paraffin-embedded for histological examination.

Histology and Quantification

The soleus muscles were fixed in 4% PFA, dehydrated with a graded ethanol series, and paraffinized. Fixed samples were deparaffinized in xylene and rehydrated in a graded series of ethanol. Tissues were sliced into 5-µm sections. General histology was performed using H&E. Quantification was estimated based on high-quality images (1,208 × 960 pixels buffer) captured using a Nikon Eclipse 80i light microscope.

Quantification of the muscle fiber size distribution. The muscle fiber size was determined on H&E-stained muscle cross sections and quantified using the minimum distance of parallel tangents at opposing particle borders (minimal “Feret’s diameter”). Images were collected by a light microscope, and the minimal Feret’s diameter was measured using the Image-Pro Plus 4.5.1 system. Normalization of the number of fibers in each fiber Feret class was based on the total number of muscle fibers in each image.

Quantification of the centrally nucleated muscle fiber. The number of centrally nucleated muscle fibers was determined on H&E-stained muscle cross sections from at least 100 fibers and then normalized to the total number of muscle fibers in each image. Data represent six rats per group. Approximately six consecutive serial cross sections from a muscle block were obtained for histology and statistical analysis. At last six images were taken from the central degenerative and regenerative regions. The peripheries of the muscle fibers spared from bupivacaine injury were not included in the image analysis.

In Vivo Detection of DNA Synthesis by Immunohistochemistry

For the detection of cell expansion, BrdU was reconstituted in DMSO as a stock solution (80 mM). BrdU (150 µl) mixed with 350 µl of PBS was injected intraperitoneally into each rat, 16 h before death. DNA synthesis was assessed by BrdU labeling with anti-BrdU antibodies. Deparaffinized tissue sections were treated with 1 N HCl at RT for 1 h and blocked with 10% goat serum and 5% BSA in PBS containing 0.1% Tween 20 for 1 h at RT. The sections were stained with the following antibodies, including mouse monoclonal antibody specific for BrdU (1:100; GeneTex), rabbit polyclonal antibody to Pax7 (1:100, ab34360; Abcam), and rabbit polyclonal antibody to F4/80 (1:100, sc-25830; Santa Cruz Biotechnology) at 37°C for 2 h. The sections were then incubated with both rhodamine-conjugated donkey anti-mouse IgG antibody and FITC-conjugated donkey anti-rabbit IgG antibody (1:500) for 1 h at RT. Nuclei were monitored by counterstaining with Hoechst 33258 for 7 min. Images were captured using a Zeiss epifluorescence microscope with a CCD camera.

Western Blot Analysis

Cell lysis, SDS-PAGE, and the antibodies used for immunoblotting were as described in our previous study (21). The band intensity was evaluated in immunoblots with a model GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA) and analyzed using Labworks 4.0 software.

Statistics

Results were expressed as means ± SE. One-way ANOVA was used for statistical comparisons. P < 0.05 was considered significant.

RESULTS

PEDF Peptide Enhances the Expansion of Muscle Satellite Cells in Damaged Soleus Muscle

Delivery of therapeutic peptides to muscle tissue may be problematic because agents injected as an intramuscular bolus may leak rapidly into the systemic circulation. A sustained-release design may be required to maintain an adequate, local level of the agent. We determined whether alginate hydrogel can be used as a sustained-release agent for the delivery of PEDF-derived short peptides (PSP 29-mer and PSP 20-mer). The release kinetics of the PSP from the alginic gel were assayed as described in MATERIALS AND METHODS. The assay revealed that the encapsulated peptides were released in a sustained manner over a 6-day period (Fig. 1). There were ~48% of the 29-mer and 35% of the 20-mer peptide remaining in the gel after 24 h. The release of the 29-mer and 20-mer was about 68 and 83% in the first 2 days, followed by a leveling off of the cumulative release curve.

The rat model of a single injection of bupivacaine in the soleus muscle was used to induce myonecrosis and muscle regeneration. We investigated whether the PSP can enhance the proliferation of muscle satellite cells in damaged muscle. The 29-mer/alginate (29-mer), 20-mer/alginate (20-mer), 29-mer solution (bolus), and peptide-free alginate (blank) were
applied in situ immediately after bupivacaine injection. Three days after bupivacaine injection, rats were injected intraperitoneally with BrdU and killed 16 h later. The soleus muscle specimens were double-immunostained for the satellite cell marker (Pax7) and cell proliferating marker (BrdU) (Fig. 2, A and B). The results revealed the increases in the number of BrdU- and Pax7-double positive cells in the 29-mer and 20-mer/alginate gel-treated wounds compared with alginate blank and bolus (68.8 ± 3.9 and 63.2 ± 5.1% vs. 28.7 ± 4.8 and 31.2 ± 3.3%, respectively, Fig. 2C). The BrdU pulse-labeling assay indicates more active Pax7-positive cell proliferation following treatment with the PSP.

It has been established that rat soleus muscle treated with bupivacaine over 3 days shows a higher grade of inflammation, accompanied by macrophage infiltration (13). As depicted in Fig. 2, D and E, immunofluorescence analysis of macrophages, using anti-F4/80 antibody, showed an obvious macrophage accumulation in the injured areas treated with PSP/alginate and alginate blank, respectively. Notably, only a few F4/80-positive macrophages stained positively for BrdU. These observations suggested that treatment with the PSP does not stimulate mitosis of macrophages.

Local Delivery of PEDF-Derived Peptide by a Sustained-Release Device Promotes Muscle Regeneration

We investigated whether the PSP is capable of facilitating the regeneration of damaged muscle. We injected the PSP in soleus muscle that had been injured by bupivacaine. The PSP was injected either as a single bolus solution or formulated into a sustained-release alginate gel to achieve a longer presence in the tissue.

At 14 days, necrotic myofibers were replaced by newly formed myofibers in the soleus muscle in all study groups. However, quite a few muscle fibers with centrally located myonuclei remained in muscle treated with blank gel or bolus (Fig. 3, A and B). The presence of these immature muscle fibers indicates a slower maturation of myofibers at day 14, as described previously (13). In contrast, muscle sections from the PSP 29-mer/alginate- and 20-mer/alginate-treated rats had far fewer centrally nucleated fibers than those with blank and bolus treatment (3.9 ± 0.67 and 4.1 ± 0.89% vs. 21.0 ± 3.2 and 21.9 ± 2.8%; Fig. 3B). PSP/alginate-treated muscle seemed to have larger muscle fibers than blank alginate gel-treated muscle (Fig. 3A). To determine the fiber size distribu-

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**Fig. 2.** Immunofluorescence analysis of soleus muscles 4 days after bupivacaine injection and PEDF-derived short peptide (PSP) treatment. A and B: PSP induces muscle satellite cell proliferation in the central area of the damaged soleus muscle. Specimens were assayed by dual-immunofluorescence staining for 5-bromo-2′-deoxyuridine (BrdU, a cell proliferation marker; red) and Pax7 (a muscle satellite cell marker; green). Nuclei were stained by Hoechst 33258 (blue). Pax7 is expressed in the cell nucleus as confirmed by Hoechst 33258 counterstaining. The squares with solid borders show immunostaining for Pax7, BrdU, and nuclear signals. Arrows indicate Pax7/BrdU double-positive cells. Scale bar: 10 μm. C: variation of the percentages of proliferative Pax7-positive cells among the different treatment groups. Results were evaluated from 6 sections/muscle specimen, with 6 rats in each group. The percentage of BrdU- and Pax7-double positive cells (pale pink) per total Pax7-positive cells was calculated. *P < 0.01 vs. blank. D and E: representative images of three independent experiments show dual-immunofluorescence staining of macrophages by F4/80 (green labeling), BrdU (red labeling), and merged. Insets from left to right denote immunostaining for F4/80, BrdU, and the nucleus. Scale bar: 10 μm. *F4/80-positive macrophages.
tion quantitatively, we measured the minimal Feret’s diameter of myofibers from muscle specimens collected at 14 days after injury. The fibers of 29-mer- and 20-mer/alginate gel-treated muscles have a size distribution shifted toward larger fibers compared with blank and bolus treatment (% C). In addition, PSP/alginate-treated fibers had a size distribution similar to uninjured muscle; 56.6% of muscle fibers in PSP/alginate gel-treated and 53.2% of uninjured soleus muscle had a minimal Feret’s diameter between 20 and 25 μm. On the other hand, 59.6 and 56.2% of the fibers in blank and bolus-treated muscle had minimal Feret’s diameters between 10 and 20 μm.

Collectively, our histological analysis revealed that gel delivery of the PSP did not lead to inflammation or fibrosis in the regenerating muscle. PSP/alginate gel significantly increases the regeneration of muscle mass during the recovery period.

**PEDF and the PSP Promote C2C12 Myoblast Proliferation In Vitro**

To determine the influence of PEDF and the PSP 20-mer on the proliferative capacity of C2C12 myoblasts, cells were pulse-labeled with BrdU (6 h; Fig. 4A) and analyzed by immunostaining. It was found that the proliferative ratio of C2C12 cells cultivated in medium containing PEDF or the PSP 20-mer is higher than that of cells cultured in medium containing PEDF solvent or the control peptide 34-mer (13.8 ± 2.6 and 16.0 ±
2.4% vs. 3.8 ± 1.1 and 3.3 ± 1.3%; Fig. 4B). Collectively, the proliferation of C2C12 in culture is enhanced by PEDF and the 20-mer.

The Mitogenic Effect of PEDF on C2C12 Myoblasts is Modulated by Activation of ERK, Akt, and STAT3

To explore the molecular basis of C2C12 proliferation induced by PEDF, C2C12 cells were treated with PEDF for intervals ranging from 5 to 60 min (Fig. 5, A and B). Immunoblots revealed that PEDF caused a rapid and transient increase in p-ERK1/2 and p-Akt levels at 5 min, whereas the peak phosphorylation of p38 MAPK (p-p38) and STAT3 (p-STAT3) occurred between 5 and 10 min after PEDF stimulation. PEDF had no effect on the phosphorylation of JNK.

To confirm that PEDF can activate multiple signaling pathways, we examined the phosphorylation of Elk-1 (a documented nuclear substrate of ERK) and ATF-2 (a documented nuclear substrate of p38 MAPK) after PEDF treatment for 5 min, as well as Socs3 (a target gene of STAT3) and survivin (a target gene of Akt) after PEDF treatment for 24 h. PEDF could enhance the phosphorylation levels of Elk-1 and ATF-2, as well as the expression of Socs3 and survivin, compared with the solvent control (Fig. 5C). Moreover, the involvement of these signaling pathways on its target expression was further evaluated using inhibitors, including PD-98059 (ERK inhibitor), SB-203580 (p38 MAPK inhibitor), STAT3 inhibitor, and LY-294002 (inhibitor of PI3K/Akt signaling). Collectively, these results suggest that PEDF can activate multiple signaling pathways in C2C12 cells.

Chemical inhibitors also were used to examine the involvement of ERK, p38 MAPK, Akt, and STAT3 in C2C12 cell proliferation induced by PEDF. BrdU pulse-labeling assays revealed that pretreatment with ERK, STAT3, or Akt inhibitor suppressed the PEDF-induced cell proliferation from 13.8 ± 2.6% to 2.8 ± 0.6, 2.0 ± 0.8, and 1.5 ± 0.7%, respectively (Fig. 6A). Inhibition of p38 MAPK by SB-203580 slightly

Fig. 5. PEDF induces phosphorylation of ERK1/2, p38 MAPK, STAT3, and Akt in C2C12 myoblasts in a time-dependent fashion. A and B: cells were exposed to PEDF for the time periods indicated. Immunoblotting was performed to detect the active phosphorylated forms (top) and the unphosphorylated forms (bottom) by reincubation with the antibodies indicated. Representative blots and densitometric analysis are shown from three independent experiments. *P < 0.05 vs. untreated cells (time 0). C: PEDF induces the activation of multiple signaling in C2C12 myoblasts. Cells were pretreated with PD-98059 (10 μM; ERK inhibitor), SB-203580 (10 μM; p38 MAPK inhibitor), 50 μM STAT3 inhibitor or LY-294002 (10 μM; Akt inhibitor) for 1 h and then treated with PEDF for 5 min to detect phosphor (p)-Elk and p-ATF-2 or treated with PEDF for 24 h to detect Socs3 and survivin by Western blotting with antibodies as indicated. The intensities of p-Elk, p-ATF-2, and Socs3/survivin on the immunoblots were determined by densitometry and normalized to Elk-1, ATF-2, and β-actin, respectively. The experiment was repeated three times. *P < 0.001 vs. solvent-treated cells. **P < 0.005 vs. PEDF-treated cells.
increased the mitogenic effect of PEDF, suggesting that p38 MAPK may play a role in inhibiting PEDF mitogenic action. Meanwhile, exposure of C2C12 to PEDF for 24 h resulted in a 3.0 ± 0.3-fold induction of the cyclin D1 protein, a proliferative marker, compared with solvent-treated cells. The induction was significantly blocked by pretreatment with ERK, STAT3, or Akt inhibitor (Fig. 6B). The PSP 20-mer was as effective as the full-length PEDF in inducing C2C12 proliferation (Fig. 4), and the mitogenic effect was also abolished by pretreatment with inhibitors of ERK, Akt, and STAT3 (our unpublished data). These data suggest that simultaneous activation of ERK, STAT3, and Akt is required for PEDF and the 20-mer to stimulate C2C12 cell proliferation.

**PEDF and the PSP Induce the Proliferation of Muscle Satellite Cells In Vitro**

To explore the mitogenic effect of the 20-mer on satellite cells, individual myofibers were isolated from the EDL muscles of rats and cultured in suspension with the 20-mer and BrdU for a further 24 h before staining the myofibers for BrdU and Pax7. As shown in Fig. 7, the BrdU-positive satellite cells were visualized using immunofluorescence microscopy. The percentage of BrdU-positive cells was calculated.

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**Fig. 6.** ERK, STAT3, and Akt inhibitor prevent PEDF-induced C2C12 cell proliferation. Inhibitor treatments are described in the legend of Fig. 5C and were followed by PEDF treatment for a further 24 h. A: cell proliferation was determined by BrdU labeling for 6 h as described in the legend of Fig. 4. **P < 0.005 vs. PEDF-treated cells. B: Western blot analysis of the expression of cyclin D1 in C2C12 cells treated as described above. Representative blots and densitometric analysis with the SD from three independent experiments are shown. *P < 0.01 vs. solvent-treated cells. **P < 0.05 vs. PEDF-treated cells.

**Fig. 7.** PSP induces proliferation of Pax7-positive satellite cells in vitro. Myofibers and associated satellite cells were isolated from rat EDL muscle and incubated for 24 h with low-serum medium supplemented with 50 nM 20-mer and 10 μM BrdU and 1 μM of the inhibitors indicated, as described in MATERIALS AND METHODS. Satellite cells (Pax7) and BrdU were detected by immunofluorescence microscopy. A: representative image from three independent experiments is shown. B: 20 randomly selected fields in each treatment were photographed, and the percentage of BrdU- and Pax7-double positive cells/total Pax7-positive cells was calculated. *P < 0.001 vs. untreated cells. **P < 0.005 vs. 20-mer-treated cells.
cells in 20-mer-treated myofibers were greater in number than in the solvent control (26.8 ± 4.4 vs. 2.8 ± 0.9%; Fig. 7B). In addition, the mitogenic activity of the 20-mer on cultured satellite cells was significantly suppressed by ERK, Akt, and STAT3 inhibitor but not by p38 MAPK inhibitor. A BrdU-labeling assay also revealed that PEDF was capable of inducing satellite cell proliferation, compared with the solvent control, in the myofiber culture (19.2 ± 1.1 vs. 2.8 ± 0.9%; unpublished observations). Collectively, PEDF and the PSP 20-mer mitotically activate muscle satellite cells in vitro. The mitogenic activity may be achieved through the ERK, Akt, and STAT3 signaling pathways.

DISCUSSION

Although muscle has the ability to repair damage spontaneously, sufficient numbers of satellite cells that are expanded at an early stage after muscle trauma are crucial for complete muscle regeneration. This study is the first to show that PEDF promotes the growth of satellite cells, a finding confirmed directly using myofibers in culture. A short PEDF peptide (PSP), 20 amino acids in length and with the mitogenic activity of the full-length PEDF, has been identified. We provide evidence that a single injection of PSP hydrogel into damaged muscle can enhance the regeneration of the soleus muscle. This finding is supported by the higher number of proliferating satellite cells during the early regenerating stage (day 4 post-bupivacaine injection) in PSP-treated rats compared with blank-treated rats. The expansion of satellite cells induced by the PSP in injured muscle is closely associated with the changes in the number of centrally nucleated muscle fibers (day 7) and the size of the regenerating fibers (day 14). Our study has demonstrated, in vitro and in vivo, that PEDF is a newly identified mitogen for myogenic progenitor cells.

In this study, an injectable, biodegradable alginate gel was used to release the PSP in the damaged muscle, leading to improved muscle repair. A single injection of a solution of the PSP (bolus) in the injured muscle did not improve muscle regeneration significantly. Most of the bolus drug delivery strategies tested thus far have yielded limited success. Likely reasons include rapidly depleted local concentrations, inappropriate gradients, and rapid degradation in the inflammatory environment of the damaged tissue. Our data reveal that this alginate hydrogel delivery system is useful for PSP delivery and improves soleus muscle regeneration in a rat myonecrosis model.

Our study revealed that PEDF has a promitotic effect on muscle satellite cells. On the other hand, PEDF exerts a proapoptotic effect on endothelial cells and hepatic stellate cells (20, 22). These seemingly contradictory effects can be explained by the different domains of PEDF, 34-mer and 44-mer. The 34-mer becomes associated with the laminin receptor to induce endothelial cell apoptosis (6). In hepatic stellate cells, the 34-mer has been shown to bind to the Wnt coreceptor LRP6 to block the fibrogenic response (40). However, the 34-mer peptide had no effect on muscle satellite cell proliferation in the present study. PSP 29-mer and 20-mer are small peptides derived from the 44-mer that mimic PEDF to induce satellite cell proliferation. Recently, cumulative evidence indicates that the patatin-like phospholipase domain-containing protein 2 (PNPLA2) receptor is essential for mediating the promitotic signaling of PEDF on human embryonic stem cell and neural stem cell (11, 19). Further studies are required to determine the expression of PNPLA2 in muscle satellite cells and the involvement of PNPLA2 in mediating PEDF/PSP promitotic signaling.

It was observed that, although the regenerated myofibers at day 7 postinjury varied in size and shape in all groups, the fiber sizes of PEDF peptide-treated muscle were generally larger than those of the untreated fibers. In this present study, the increased myofiber size in PSP/alginate gel-treated muscle was extended to day 14 postinjury. The results show that PEDF stimulated the growth of regenerating myofibers and accelerated the process, as confirmed by the larger size of PEDF-treated fibers at 7 and 14 days after injury compared with the untreated muscle. A similar effect was reported for heat stress and leukemia inhibitory factor (LIF), which promote recovery of fiber size in damaged muscle through the stimulation of satellite cell expansion (3, 28). The present study also shows that PSP can have a significant impact on muscle fiber size and satellite cell expansion in damaged sites.

Mammalian skeletal muscles are able to regenerate following injury, and the regeneration process is generally through three sequential but overlapping stages that include 1) the initial inflammatory response, 2) the activation and differentiation of satellite cells, and 3) the growth and remodeling of the regenerated muscle tissue (12, 13). Several types of cells are involved in the process of skeletal muscle regeneration. For example, in the initial stage, proinflammatory M1 macrophages are important for the phagocytosis of debris caused by injury (1, 25), whereas the anti-inflammatory M2 macrophages that promote myogenic growth and differentiation are also critical for supporting the regeneration of skeletal muscles (1, 32, 41). In this regard, the switch of muscle macrophages from an M1 to M2 phenotype in injured muscle is mainly induced by interleukin (IL)-10 (41). PEDF induces IL-10 expression in macrophages (42). It will be interesting to investigate whether the PSP has the same function as PEDF in inducing IL-10 expression in macrophages and the potential effect of the PSP on the conversion of macrophage phenotypes in injured muscle in vivo.

Several signaling pathways have been implicated in the proliferative responses of myoblasts to various growth factors, including STAT3 (38), Akt (14), and ERK1/2 (26). PEDF/PSP activates the STAT3, Akt, and ERK1/2 signaling pathways in a time-dependent manner, and our pharmacological evidence suggests that these various signaling pathways are involved in the induction of C2C12 myoblast proliferation by PEDF or PSP. This multiple signaling is reminiscent of other mitogens known to cause proliferation of C2C12 myoblasts. For example, induction of the STAT3, Akt, and ERK1/2 signaling pathways has been implicated in LIF-induced C2C12 myoblast proliferation (38). On the other hand, it has been found that PEDF impairs skeletal muscle glucose uptake, induced by insulin in vitro, by a mechanism involving JNK signaling (15). However, in our study, we did not find that JNK phosphorylation was induced by PEDF/PSP in C2C12 myoblasts.

Another key finding of this study is that the PSP can induce satellite cell proliferation in vitro and in injured muscle in vivo. We reported recently that the PSP regulates the self-renewal of limbal stem cells by activating STAT3 signaling (23). Indeed, STAT3 signaling plays a pivotal role in controlling muscle growth and regeneration.
satellite cell expansion (38, 39). STAT3 signaling regulates several targets closely associated with cell cycle progression, including cyclin D1 and SOCS3 (9, 35). Our study revealed that pharmacological blockade of STAT3 inhibited the PSP-induced increase of muscle satellite cell proliferation. In addition, a previous study indicated that a basal/permissive level of PI3K/Akt activity is necessary for LIF to induce rat satellite cell proliferation (38). The PI3K inhibitor LY-294002 prevents PSP-induced rat satellite cell proliferation, suggesting that a basal PI3K/Akt activity is also crucial for the mitogenic activity of PSP.

In summary, this study is the first to show that exogenous PEDF and its derived short peptide can induce the proliferation of satellite cells and C2C12 myoblasts in vitro. The overall effects of the PSP that promote a quicker recovery, particularly in the regulation of the inflammatory response during muscle regeneration, await further examination. The mitogenic effect of the PEDF peptide on myogenic progenitors may provide a therapeutic strategy for the treatment of muscle injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


Barnard W, Bower J, Brown MA, Murphy M, Austin L.

Bernard A, Gao-Li J, Franco CA, Bouceba T, Huet A, Li Z.

Beitzel F, Sillence MN, Lynch GS.


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