Urokinase-type plasminogen activator increases hepatocyte growth factor activity required for skeletal muscle regeneration

Running title: uPA, HGF and muscle regeneration

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ABSTRACT

The plasminogen system plays a crucial role in the repair of a variety of tissues, including skeletal muscle. We hypothesized that uPA promotes muscle regeneration by activating hepatocyte growth factor (HGF), which, in turn, stimulates proliferation of myoblasts required for regeneration. In our studies, levels of active HGF and phosphorylation of the HGF receptor c-met were increased following muscle injury in wild-type mice. Compared to wild-type animals, mice deficient in uPA (uPA-/-) had markedly reduced HGF levels and c-met activation following muscle damage. This reduced HGF activity in uPA-/- animals was associated with decreased cell proliferation, myoblast accumulation and new muscle fiber formation. On the other hand, HGF activity was enhanced at early time points in PAI-1-/- mice compared to wild-type mice and the PAI-1-/- animals exhibited accelerated muscle fiber regeneration. Furthermore, administration of exogenous uPA rescued HGF levels and muscle regeneration in uPA-/- mice, and an HGF blocking antibody reduced HGF activity and muscle regeneration in wild-type mice. We also found that uPA promotes myoblast proliferation in vitro through its proteolytic activity and this process was inhibited by an HGF blocking antibody. Together, our findings demonstrate that uPA promotes muscle regeneration through HGF activation and subsequent myoblast proliferation.

Keywords: skeletal muscle repair, PAI-1, c-met, myoblast
INTRODUCTION

The plasminogen system plays a crucial role in the repair of a variety of tissues. This extracellular serine protease cascade includes the urokinase-type plasminogen activator (uPA) and its primary inhibitor, plasminogen activator inhibitor-1 (PAI-1). The classical function of uPA is to cleave plasminogen to form active plasmin, a broad-spectrum protease with activity towards a variety of extracellular matrix molecules. Genetic manipulations that inhibit plasminogen activation lead to impaired healing of lung, liver, kidney, and skin. Similarly, mice deficient in uPA or plasminogen demonstrate markedly impaired muscle regeneration. In contrast, mice lacking PAI-1 exhibit accelerated muscle regeneration.

Despite its robust effect on skeletal muscle regeneration, the molecular mechanisms by which the plasminogen system regulates this process remain largely undefined. Clearance of the provisional fibrin matrix that is deposited following muscle injury may be one mechanism by which uPA promotes regeneration. However, uPA may also promote tissue repair through other pathways such as the proteolytic activation of growth factors, including hepatocyte growth factor (HGF). HGF is synthesized and secreted as an inactive single chain molecule, and proteolytic cleavage results in formation of the active two-chain form, which has affinity for the c-met receptor. uPA is one of a select group of proteases known to activate HGF, the others being tissue-type plasminogen activator (tPA), factor X, factor XIa, matriptase, and HGF activator. In addition, uPA appears to increase HGF bioactivity in the lung and liver following injury. Like uPA, HGF appears to play important roles in the repair of different tissues, including lung, liver, kidney, and skin, presumably by stimulating the proliferation and migration of cells involved in healing.
HGF has also been implicated in both skeletal muscle development and regeneration following injury. HGF and its receptor, c-met, are required for migration of myogenic cells into the limb buds and the development of limb skeletal muscle \(^{21}\). HGF and c-met are also expressed in non-injured skeletal muscle of adult animals \(^{22,23}\). Although HGF expression is increased at both the mRNA and protein levels after muscle injury \(^{22,24}\), the precise role of this growth factor during muscle regeneration has not been established. Injection of exogenous HGF into non-injured muscle of rats activates quiescent satellite cells \(^{23}\), which are muscle precursor cells required for regeneration. In addition, injection of HGF into damaged muscle of mice has been shown to stimulate myoblast proliferation \(^{25}\). In addition to its in vivo effects, HGF also stimulates both proliferation and migration of myoblasts in vitro \(^{26-28}\), and stretching myoblast cultures has been shown to produce an HGF-dependent increase in cell proliferation \(^{29}\).

The ability of uPA to both activate HGF and promote muscle repair, along with the capacity for HGF to activate myoblasts, led us to hypothesize that uPA promotes skeletal muscle regeneration by activating HGF. To test this hypothesis, we measured HGF and c-met activation along with cell proliferation following muscle injury in wild-type (WT), uPA deficient (uPA-/-) and PAI-1 deficient (PAI-1-/-) mice. We also determined whether administering exogenous uPA would rescue HGF activation and muscle regeneration in uPA-/- mice, and whether an HGF blocking antibody would impair muscle regeneration in WT mice. Furthermore, we tested whether uPA activation of HGF stimulated myoblast proliferation in vitro. The resulting data from these experiments indicate that uPA does indeed promote myoblast proliferation and muscle repair by activating HGF.
MATERIALS AND METHODS

Mice. C57BL/6 (WT), uPA-/- and PAI-1-/- mice were obtained from Jackson Laboratories and bred in our animal facility. The uPA-/- and PAI-1-/- mice had been backcrossed onto the C57BL/6 background for more than 8 generations. Mice were housed in a specific pathogen-free environment at a constant temperature and a 12:12 h light-dark cycle. Experiments were performed on 10-14 week-old mice. All experimental procedures were approved by the Animal Care Committee at the University of Illinois at Chicago.

Muscle Injury. Extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were injured using cardiotoxin as previously described. Briefly, mice were anesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (5mg/kg) and a small incision (1 cm) was made to expose the muscles of the anterior leg. Cardiotoxin (10μM; Calbiochem) was administered with two intramuscular injections per muscle to ensure distribution throughout the muscle. The skin incision was closed with 7-0 nylon suture and the procedure was repeated on the contralateral limb. At different times following injury, mice were euthanized by cervical dislocation while under anesthesia, and muscles were harvested. EDL muscles were mounted in tissue freezing medium and frozen in isopentane chilled with dry ice for histological analysis. TA muscles were snap frozen in liquid nitrogen for immunoprecipitation or affinity purification and Western blot analysis.

Exogenous uPA and HGF blocking antibody. To restore uPA to the muscles of uPA-/- mice, intramuscular injections of recombinant murine uPA (5 μg/muscle, Molecular Innovations) were administered daily on days 1 through 4 post-injury. To inhibit HGF activity in damaged muscle of WT mice, 200 μg of an HGF blocking antibody (R & D Systems) were administered...
intraperitoneally immediately after injury, and daily thereafter for 5 days. Control mice received an equal amount of nonspecific IgG.

Immunofluorescence. Cell proliferation was assessed in muscle cryosections using incorporation of BrdU into cell nuclei. One hour prior to euthanasia, mice were injected with 30 mg/kg BrdU. Cross sections were cut from the mid-belly of each EDL muscle (10μm thickness), fixed in cold acetone, washed in PBS and incubated in 2N HCl to denature DNA. Sections were neutralized in basic PBS and then incubated in blocking buffer containing 0.2% gelatin and 3% BSA. Proliferating cells were labeled with a BrdU antibody (Roche) for 1 hour, and subsequently incubated with FITC anti-mouse secondary antibody (Jackson ImmunoResearch). Alternatively, sections were stained for the myogenic transcription factor MyoD. These sections were fixed in cold acetone, incubated with blocking buffer and then incubated with primary antibody (Santa Cruz Biotechnology) overnight. Sections were subsequently incubated with TRITC anti-rabbit secondary antibody (Jackson ImmunoResearch) and then mounted in Vectashield mounting medium containing DAPI (Vector Laboratories). The number of DAPI positive nuclei that were also MyoD or BrdU positive was counted in three fields observed using a 40X objective with a Nikon Labphot-2 microscope and SPOT digital camera and software (Diagnostic Instruments) for two sections per muscle and normalized to the volume of muscle sampled.

Morphology. Muscle cross sections were stained with hematoxylin and eosin, and quantitative analysis was performed on five representative images of each muscle section obtained using a 40x objective with a Nikon Labphot-2 microscope and SPOT camera and software. For each field, fibers were classified as normal, damaged or regenerating as described. Regenerating fibers were identified as those containing centrally located nuclei without
evidence of damage. The number and area of each type of fiber were recorded. Damaged area
was then estimated in each muscle section by subtracting the summed area of normal and
regenerating fibers from the total area of each field.

HGF and c-met protein analysis. HGF protein levels were assessed by affinity
purification followed by Western blotting. Muscles were homogenized in buffer A (20 mM Tris, 
ph 7.5, 150 mM NaCl, 0.1% Triton X-100) with protease and phosphatase inhibitors (1 mM 
PMSF, 1 μM leupeptin, 0.3 μM aprotinin, 25 mM β-glycerophosphate, 1 mM Na₃VO₄, 5 mM 
NaF). The full volume of muscle homogenate from each sample was incubated with heparin-
agarose beads at 4C overnight with rotation. Beads were washed and then boiled in 20 μl SDS 
sample buffer to elute bound protein.

C-met protein levels and phosphorylation were assessed by immunoprecipitation
followed by Western blotting. Muscles were homogenized in buffer B (20 mM Tris pH 7.5, 150 
mM NaCl, 0.1% SDS, 1% NP-40) with protease and phosphatase inhibitors. Homogenates (2 
mg) were incubated with anti-c-met antibody (Santa Cruz) at 4C overnight with rotation and then 
protein G sepharose beads for 1 hour. Beads were washed and then boiled in SDS sample buffer.

For both HGF and c-met analysis, one-half the total volume of each sample was 
separated on each of two SDS-PAGE gels and transferred to nitrocellulose membranes. After 
transfer, membranes were blocked in 5% milk overnight. Membranes were incubated with 
primary antibody against HGF, c-met or phosphotyrosine (all from Santa Cruz) and then 
incubated with secondary antibody conjugated to horseradish peroxidase (Pierce). Protein bands 
were detected using enhanced chemiluminescence (Amersham) and band densities were 
determined by image analysis.
Cell isolation and real time PCR. Cells were isolated from muscles in WT mice that were harvested 5 days after injury using a protocol modified from the literature. Briefly, muscles were dissected, minced and then digested with 0.1% pronase (Calbiochem). After trituration to dissociate from fiber fragments, the suspension was filtered through 100 µm mesh, the filtrate was centrifuged, and cells were resuspended and counted. The total cell population was separated into macrophage and non-macrophage cells using a Mac-1 antibody conjugated to microbeads and a magnetic column (Miltenyi Biotec). Total RNA was isolated from the Mac-1 positive and negative cells using the RNeasy kit (Qiagen) following manufacturer's instructions and then equal amounts of RNA were used to generate cDNA using the Thermoscript RT-PCR System (Invitrogen). Real-time PCR was performed in a 7500Fast System (Applied Biosystems) using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay primer/probe sets (Applied Biosystems) for mouse uPA, HGF or GAPDH. Relative gene expression was determined using the $2^{\Delta\Delta CT}$ method, with Mac-1 negative cells as baseline and GAPDH as endogenous control gene. Primer efficiency was verified using serial dilutions of sample cDNA.

Myoblast cultures. Primary myoblast cultures were derived from neonatal hind-limb muscles from WT and uPA/- mice using an established method. Briefly, muscle was finely minced, digested in 1% pronase (Calbiochem) and cells were released by trituration. Pooled cells were filtered (100µm), centrifuged and resuspended. A pre-plating technique was used to separate fibroblasts from myoblasts; 1 hour after pre-plating on tissue culture dishes, non-adherent cells (primarily myoblasts) were transferred to another dish coated with entactin/collagen/laminin (ECL; Upstate Biotechnology) and grown in selective proliferation medium (Ham's F10, 20% FBS, 5 ng/ml bFGF, 1% penicillin/streptomycin) in a humidified 5%
CO₂ atmosphere at 37°C. Immunofluorescence analysis demonstrated that greater than 90% of cells cultured in this manner were positive for MyoD.

To test the influence of uPA on myoblast proliferation, WT and uPA-/- myoblasts were plated on ECL coated coverslips, and incubated in low serum medium overnight. Cells were then incubated in low serum (0.5% FBS) or high serum (20% FBS) medium, supplemented with recombinant mouse uPA and/or PAI-1 (Molecular Innovations), HGF or an HGF blocking antibody (R&D Systems), the PI3K inhibitor LY294002 or the MEK1 inhibitor PD98059 (Calbiochem) for 24 hours. Cells were also incubated with 100 μM BrdU and incorporation of BrdU into cell nuclei was detected using a protocol similar to that described for muscle cryosections. The number of positively labeled cells was counted in four fields observed at 20X and normalized to the total number of cells present in each field.

Statistical Analysis. Values are reported as means ± SE. Data were compared across time points, mouse genotypes and/or treatment conditions using two-way analysis of variance (analysis of BrdU and MyoD in muscle cryosections, densitometric analysis of HGF and c-met Western blots, myoblast proliferation experiments), one-way analysis of variance (signaling inhibitor experiments) or t-tests (in vivo HGF blocking antibody experiments). The Student-Newman-Keuls test was used for post-hoc analysis of analysis of variance tests found to be statistically significant. The 0.05 level was taken to indicate statistical significance.
RESULTS

HGF and c-met activation in uPA-/- and PAI-1-/- mice

Because uPA is known to activate HGF, and HGF is thought to play important roles in muscle regeneration, we assessed levels of total and active HGF following muscle injury in WT, uPA-/- and PAI-1-/- mice. In uninjured control muscle, HGF was present in mainly its inactive single chain form (~90 kDa) with a greater amount of HGF isolated from WT and PAI-1-/- mice than uPA-/- mice (Figure 1). After muscle injury in WT mice, total HGF levels were increased by ~2-fold at 3 and 5 days post-injury with ~50% present in the active α-chain form (~60 kDa, Figure 1). In uPA-/- mice, very little total HGF was evident until 5 and 10 days post-injury, and it was present predominantly in the inactive single chain form until 10 days post-injury. In PAI-1-/- mice, total HGF levels were similar to those in WT mice at 1 and 3 days post-injury, but a greater percentage of the total was in the active α-chain form in the PAI-1-/- animals. These data indicate that levels of active HGF are downregulated in the absence of uPA and upregulated when uPA activity is not inhibited by PAI-1.

To determine whether differences in levels of active HGF corresponded to differences in activation of the HGF receptor c-met, we measured phosphorylation of c-met following muscle injury in WT, uPA-/- and PAI-1-/- mice. The β-chain of c-met (~145 kDa) was evident in uninjured control muscle of all mouse strains, with very little, if any, phosphorylation (Figure 2). Following muscle injury in WT and PAI-1-/- mice, total c-met was increased at 3 to 10 days post-injury, with some of the immature c-met present (~190 kDa) (Figure 2). Although total c-met was upregulated in uPA-/- mice, the increase was not as large as observed in the other two genotypes. Following muscle injury in WT mice, there was a progressive increase in c-met phosphorylation with a peak at 5 days post-injury. In contrast, c-met phosphorylation was
significantly blunted in the injured muscles of the uPA-/- mice. Furthermore, the induction of c-met phosphorylation in PAI-1-/- mice was more rapid than that observed in WT animals.

Proliferation and myoblast accumulation in damaged muscle of uPA-/- and PAI-1-/- mice

Morphological observations from our current study confirmed the persistence of necrotic muscle fibers and lack of formation of regenerating fibers in injured muscles from uPA-/- mice and verified accelerated regeneration in muscles from PAI-1-/- mice (Figure 3). To assess cell proliferation following muscle injury, we counted cells that had incorporated BrdU into newly synthesized DNA. In WT, uPA-/-, and PAI-1-/- mice, there were very few BrdU positive cells in uninjured control muscles or at 1 day post-cardiotoxin administration (Figure 3). In WT mice, BrdU positive cells were evident in damaged muscle at 3 days post-injury, increased to a peak at 5 days and then declined at 10 days. There were significantly fewer BrdU positive cells at all time points after injury in uPA-/- mice compared with WT animals, consistent with the morphological observations of impaired regeneration in these mice. Furthermore, there were significantly more BrdU positive cells in PAI-1-/- mice at 3 days post-injury compared with WT mice, consistent with the accelerated repair in these animals.

To assess myoblast accumulation and muscle fiber formation following muscle injury, we counted MyoD positive nuclei in muscle sections; nuclear staining was confirmed by co-staining with DAPI (cf. Supplementary Figure). At 3 days post-injury in WT mice, MyoD positive nuclei had accumulated at the periphery of damaged muscle fibers and in small cells within spaces between fibers (Figure 4). At 5 days, the number of MyoD positive nuclei increased and many of these were now within small regenerating (central nucleated) muscle fibers. There were significantly fewer MyoD positive nuclei in damaged muscle of uPA-/- mice compared with WT mice.
animals at both 3 and 5 days post-injury, and none of the MyoD positive nuclei appeared to be regenerating fibers. On the other hand, the number of MyoD positive nuclei was significantly increased in PAI-1-/- mice compared with WT animals at 3 days post-injury. Taken together, these data indicate that cell proliferation, myoblast accumulation and muscle fiber formation are regulated by the balance of uPA and PAI-1 present in damaged muscle.

Exogenous uPA administration, HGF levels and muscle regeneration in uPA-/- mice

To determine whether administering exogenous uPA could rescue HGF activity and muscle regeneration in uPA-/- mice, we injected uPA into cardiotoxin-injured muscles of uPA-/- mice. Injured muscles from untreated uPA-/- mice contained relatively low levels of HGF (Figure 5). In contrast, injured muscles from mice treated with exogenous uPA exhibited both increased total and α-chain HGF levels (Figure 5). In addition, muscles from untreated uPA-/- mice were characterized by the persistence of damaged tissue, and the absence of regenerating fibers (Figure 5). In conjunction with the increased levels of HGF, exogenous uPA increased the formation of regenerating fibers, and enhanced the recovery of normal muscle morphology in uPA-/- mice. In contrast, treatment of injured muscles in WT mice with exogenous uPA did not alter the recovery of muscle morphology.

HGF blocking antibody and muscle regeneration

To determine whether HGF activity is required for efficient skeletal muscle regeneration in WT mice, we administered an HGF blocking antibody daily following muscle injury. Treatment with the HGF blocking antibody reduced phosphorylation of c-met compared to control goat IgG (Figure 6). Phosphorylated c-met normalized to total c-met was 37.2 ± 6.5%
for mice treated with control IgG versus 10.2 ± 3.6% for mice treated with HGF blocking antibody, verifying that the blocking antibody reduced HGF activity in damaged muscle. In addition, the HGF blocking antibody significantly reduced the formation of regenerating fibers (Figure 6), indicating that blocking HGF activity following muscle injury impairs muscle regeneration.

Cellular sources of uPA and HGF during muscle regeneration

Since accumulation of macrophages in damaged muscle of uPA-/- mice is dramatically reduced compared to WT mice 7,8,30, and macrophages can produce both uPA and HGF, we determined the level of expression of uPA and HGF in macrophages and non-macrophage cells during muscle regeneration in WT mice. First, we isolated cells from muscle at day 5 post-injury and separated the total population of cells present in the damaged muscle into Mac-1 positive and negative cells; ~50% of these cells were Mac-1 positive. In a previous study, we verified the specificity of this cell sorting method, as Mac-1 mRNA was easily detected in Mac-1 positive cells, but was not detectable in Mac-1 negative cells 34. In the present study, real time PCR demonstrated that both Mac-1 positive and negative cell populations expressed both uPA and HGF following muscle injury, but macrophages expressed 3- and 7-fold higher levels, respectively, than non-macrophage cells (Figure 7). Thus, macrophages appear to be a primary source of these critical mediators of muscle regeneration following injury.

uPA and myoblast proliferation in vitro

To determine if uPA induces myoblast proliferation in vitro, we compared BrdU incorporation in isolated cells from uPA-/- and WT animals. We found that uPA-/- myoblasts
exhibited a ~65% reduction in proliferation compared to WT cells when cultured in low serum medium, and a lesser but still significant impairment (~25% reduction) when cultured in high serum medium (Figure 8). Subsequent experiments demonstrated that exogenously administered recombinant mouse uPA produced a dose-dependent increase in proliferation of both WT and uPA/- myoblasts (Figure 8). This exogenous uPA had a greater stimulatory effect on uPA/- than WT myoblasts such that there was no difference in proliferation at the highest administered concentration of uPA (100 ng/ml). On the other hand, exogenously administered recombinant PAI-1, the proteolytic inhibitor of uPA, blocked the increase in proliferation induced by uPA, when added at the same concentration as uPA. Finally, the amino terminal fragment (ATF) of uPA, which lacks the proteolytic domain but retains the receptor binding domains, did not stimulate proliferation when added to the cultures of either WT or uPA/- myoblasts (Figure 8). These data indicate that the proteolytic activity of uPA stimulates myoblast proliferation.

To assess whether uPA-induced myoblast proliferation occurred as the result of HGF activation, we cultured myoblasts in the presence of both recombinant uPA and an HGF blocking antibody. Again, uPA increased proliferation of both WT and uPA/- myoblasts (Figure 7). The HGF blocking antibody blocked the uPA-induced increase in proliferation, whereas control IgG had no effect. Furthermore, we cultured myoblasts with recombinant HGF and found a dose-dependent increase in proliferation of both WT and uPA/- cells. Finally, we tested whether inhibiting HGF downstream signaling molecules PI3K or MEK1 reduced uPA-induced myoblast proliferation. Both the PI3K inhibitor LY294002 and the MEK1 inhibitor PD98059 produced a dose-dependent reduction in proliferation (Figure 7). Together, these data indicate that uPA activation of HGF promotes myoblast proliferation likely through PI3K and MEK1.
DISCUSSION

The plasminogen system is required for efficient muscle regeneration, as mice deficient in either uPA or plasminogen exhibit markedly impaired regeneration. Existing evidence suggests that the plasminogen system regulates muscle regeneration in part through facilitation of fibrin removal from injured tissue. Whether other functions of uPA contribute to its profound effect on muscle regeneration remains unknown. Our findings elucidate a critical pathway by which uPA regulates muscle regeneration following injury. First, we demonstrated that uPA upregulates HGF activity following muscle injury. Levels of active HGF and phosphorylation of the HGF receptor c-met were reduced in damaged muscles of uPA-/- mice compared to WT animals. In contrast, HGF and c-met activity were enhanced at early time points in mice deficient in the primary inhibitor of uPA, PAI-1. The effect of uPA on HGF activity was further established by treating injured uPA-/- animals with exogenous uPA protein. The resulting data show that such treatment restored both total and active HGF levels in damaged muscle. Second, our data demonstrate that HGF activity is required for efficient regeneration by stimulating myoblast activity. In each of our experimental models, we observed that increased levels of active HGF were positively associated with cell proliferation, myoblast accumulation and new muscle fiber formation. Furthermore, an HGF blocking antibody reduced formation of new muscle fibers in WT mice. Finally, in vitro experiments showed that uPA promotes myoblast proliferation through its proteolytic activity and that an HGF blocking antibody inhibited this proliferative response.

The correlation of uPA activity with HGF levels following muscle injury was previously explored by Suelves et al. In contrast to our results, the previous study found that levels of active HGF were not different in injured muscles of WT and uPA-/- mice. Reasons for this
discrepancy may have resulted from differences in the time points of analysis, and/or the methods used for processing muscles for Western blots. On the other hand, our findings are consistent with data from a study on liver regeneration following massive hepatic apoptosis\textsuperscript{5}. In this liver study, mice deficient in uPA showed delayed production of active HGF and impaired liver regeneration compared with WT mice. In addition, PAI-1 deficient mice showed accelerated production of active HGF and liver regeneration. Furthermore, in vivo transfection of uPA into the liver of uPA-/− mice rescued production of active HGF and c-met activation and restored liver repair. Our findings are also consistent with in vitro findings demonstrating that uPA promotes HGF activation in fibroblast cultures\textsuperscript{12,36}. In these studies, uPA activated HGF both directly and indirectly, the latter via activation of plasminogen to plasmin.

The total levels of HGF were reduced at early time points in damaged muscle of uPA-/− mice compared with WT mice (Figure 1), which correlates with reduced accumulation of both macrophages\textsuperscript{7,8} and myoblasts (Figure 4) in damaged muscle of uPA-/− mice compared to WT mice. Since both macrophages and myoblasts are known to produce HGF in vitro\textsuperscript{28,37}, and our data show that both macrophages and non-macrophage cells express HGF during muscle regeneration in vivo, with macrophages having 7-fold higher expression (Figure 7), the lower numbers of macrophages and myoblasts likely explains the lower levels of total HGF observed in damaged muscle of uPA-/− mice compared with WT mice. Another possibility is that uPA may directly influence HGF expression in any of these cell types during muscle repair.

In contrast to our findings that uPA promotes myoblast proliferation, Suelves et al. found that uPA did not stimulate myoblast proliferation in vitro\textsuperscript{35}. One explanation for the differences in results is that Suelves et al. performed their proliferation experiments in high serum medium (20% FBS) whereas most of our experiments were performed in low serum medium (0.5% FBS).
Our data indicate a greater impairment in proliferation of uPA-/- cells in low serum medium than in high serum medium (e.g. Figure 7), and a greater stimulatory effect of exogenous uPA in low serum compared to high serum medium (data not shown). Since HGF activator protein is present in serum, uPA may not be required for proteolytic activation of HGF in high serum medium. Our data are consistent with prior reports in which uPA was shown to stimulate proliferation of human myoblasts in a dose-dependent manner. Furthermore, a blocking antibody against uPA inhibited basic FGF-induced proliferation, indicating that uPA may mediate proliferation induced by FGF. Studies on vascular smooth muscle cells produced similar findings that uPA induces proliferation of these cells and may mediate growth factor-induced proliferation.

Our data demonstrated that (1) intact uPA but not the ATF which lacks the proteolytic domain stimulated myoblast proliferation, (2) the specific proteolytic inhibitor PAI-1 blocked uPA-mediated myoblast proliferation, (3) the HGF blocking antibody reduced uPA stimulation of myoblast proliferation and (4) both the PI3K inhibitor LY and the MEK1 inhibitor PD98059 reduced uPA-mediated myoblast proliferation. Taken together, these data indicate that uPA stimulates myoblast proliferation through proteolytic activation of HGF, and via signaling through PI3K and MEK1. These data are consistent with those of De Petro et al, who reported that uPA promotes proliferation of skin fibroblasts through a mechanism that required proteolytic activity. These findings are also consistent with those of Tanski et al., who showed that proteolytic activity of uPA was required for stimulation of proliferation of smooth muscle cells, and that this occurred through a MEK1-dependent mechanism. HGF has been shown to induce phosphorylation of ERK1/2 in a PI3K-dependent manner in myogenic cells, which is consistent with the idea that uPA activates PI3K and ERK1/2 by activating HGF.
HGF and its receptor, c-met, are required for migration of myogenic cells into the limb buds and the development of limb skeletal muscle \(^{21}\). However, the functions of HGF and c-met in adult muscle have been less clearly defined. HGF was found to be present in uninjured adult skeletal muscle and is a component of crushed muscle extract that activates satellite cells \(^{23}\). In adult muscle as well as lung, liver, kidney and spleen HGF was found primarily in its inactive single chain form, with the level of expression and activation increased following tissue injury \(^{22,45}\). In contrast, other studies have reported that HGF is found primarily in its active \(\alpha\)-chain form in uninjured skeletal muscle \(^{46,47}\). The contrasting findings may be explained by differences in sample preparation. For studies in which single chain HGF was observed, tissue samples were homogenized and HGF levels assayed by Western blotting. In studies reporting primarily \(\alpha\)-chain HGF, muscles were incubated in buffer and HGF released into the buffer was assayed. Thus, freely soluble extracellular HGF may be primarily in the \(\alpha\)-chain form, whereas HGF localized in the extracellular matrix and intracellularly may be primarily in the single chain form. This idea is supported by findings that HGF recovered in lavage fluid from the lung is found only in the \(\alpha\)-chain form \(^{16}\). In our experiments, we assayed HGF in muscle homogenates and found HGF primarily in its single chain form in uninjured muscle. Interestingly, the molecular size of this single chain HGF appeared to be smaller in uninjured control muscle compared with damaged muscle, and showed evidence of returning to this smaller size at 10 days post-injury. The reason for this difference remains to be determined, but we speculate that it may be related to differential post-translational modification of HGF in non-injured and injured muscle. Others have also observed different sizes of the single chain HGF in lung, kidney and spleen tissues \(^{45}\).

We and others have demonstrated that macrophage accumulation following cardiotoxin injury is almost completely abrogated in uPA-/- mice, associated with persistence of necrotic
debris and the lack of muscle fiber regeneration\textsuperscript{7,8}. In addition, we recently showed that transferring WT bone marrow cells to uPA/-/- mice rescues macrophage accumulation and muscle regeneration following injury, suggesting that impaired macrophage function in the absence of uPA plays an important role in the deficient healing\textsuperscript{30}. However, the mechanisms by which macrophages promote muscle fiber regeneration remain to be established. One possible mechanism is the production of uPA and/or HGF by macrophages that have accumulated in damaged muscle. Macrophages are known to express both uPA and HGF\textsuperscript{37,48,49}, both of which would promote satellite cell proliferation and migration required for muscle regeneration. We are currently investigating in further detail the mechanisms by which macrophages promote muscle regeneration and whether their effect on repair may be through the delivery of these key mediators to the site of damage.

In summary, we have shown that uPA activation of HGF plays an important role in stimulating the myoblast proliferation required for efficient muscle regeneration. These data extend previous findings of the critical role of the plasminogen system in muscle regeneration, and provide a molecular mechanism by which uPA promotes regeneration. These data also indicate that the plasminogen system may provide an appealing target for interventions to improve muscle regeneration in muscle diseases that involve progressive degeneration (e.g. muscular dystrophy), and other conditions in which muscle regeneration is impaired (e.g. aging).
ACKNOWLEDGMENTS

This research was supported by grants from the Department of Defense (USAMRMC #W81XWH-05-1-0159 to TJK) and the National Institutes of Health (R01 HL078871 to TS).

AUTHORSHIP

T.S. designed the research, analyzed data and wrote the paper. M.-H.N. performed the research, analyzed data and wrote the paper. B.Y. performed the research and analyzed data. M.N. performed the research and analyzed data. R.S. contributed vital tools, analyzed data and wrote the paper. T.K. designed the research, analyzed data and wrote the paper.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest to disclose.
REFERENCES


FIGURE CAPTIONS

Figure 1. HGF protein levels following muscle injury in WT, uPA-/- and PAI-1-/- mice. Muscles from WT, uPA-/- and PAI-1-/- mice collected from uninjured control mice (Con) and on days 1, 3, 5 and 10 following cardiotoxin injury. Top: Muscle homogenates subjected to heparin sulfate affinity purification and then Western blotting for HGF. An equal amount (2.5 ng) of an HGF standard (Std) was loaded onto each gel. SC: single chain, αC: α-chain. Bottom: Densitometric measurements performed for total HGF (single chain + α-chain) and active α-chain HGF. Total HGF normalized to HGF standard on each blot, and α-chain normalized to total HGF in each sample. Bars are means + standard error, n = 4-6 per time point. *mean value significantly smaller than that for WT mice, **mean value significantly larger than that for WT mice, p < 0.05.

Figure 2. c-met protein levels and phosphorylation following muscle injury in WT, uPA-/- and PAI-1-/- mice. Muscles from WT, uPA-/- and PAI-1-/- mice collected from uninjured control mice (Con) and on days 1, 3, 5 and 10 following cardiotoxin injury. Top: Muscle homogenates subjected to c-met immunoprecipitation and then Western blotting for c-met and phosphotyrosine (pTyr). Bottom: Densitometric measurements performed for c-met and pTyr and normalized to c-met level in control sample for each series of samples obtained for each strain. Bars are means + standard error, n = 6 per time point. *mean value significantly smaller than that for WT mice, **mean value significantly larger than that for WT mice, p < 0.05.

Figure 3. Muscle morphology and cell proliferation following muscle injury in WT, uPA-/- and PAI-1-/- mice. Muscles from WT, uPA-/- and PAI-1-/- mice collected from uninjured
control mice (Con) and on days 1, 3, 5 and 10 following cardiotoxin injury. BrdU (30 mg/kg) injected IP into mice 1 hour prior to muscle harvest. Top: Cryosections stained with either hematoxylin and eosin for morphological analysis or a BrdU antibody to detect incorporation of BrdU into newly synthesized DNA. Scale bar = 50 μm. Bottom: Number of nuclei staining positive for BrdU counted in 2 sections for each muscle, averaged, and expressed per mm² muscle area. Bars are means + standard error, n = 4-6 per time point. *mean value significantly smaller than that for WT mice, **mean value significantly larger than that for WT mice, p < 0.05.

**Figure 4. Myoblast accumulation following muscle injury in WT, uPA-/- and PAI-1-/- mice.** Muscles from WT, uPA-/- and PAI-1-/- mice collected on days 3 and 5 following cardiotoxin injury. Top: Cryosections stained with antibody against the myoblast marker MyoD. Scale bar = 50 μm. Bottom: Number of nuclei staining positive for MyoD counted in 2 sections for each muscle, averaged, and expressed per mm² muscle area. Bars are means + standard error, n = 4-6 per time point. *mean value significantly smaller than that for WT mice, **mean value significantly larger than that for WT mice, p < 0.05.

**Figure 5. Administration of exogenous uPA to uPA-/- mice.** uPA-/- mice were subjected to cardiotoxin muscle injury and either left untreated (no treat) or treated with intramuscular injection of exogenous uPA daily from 1 to 4 days post-injury (uPA inject), and muscles collected at 5 days post-injury. Top: Muscle homogenates subjected to heparin sulfate affinity purification and then Western blotting for HGF. Note that treatment with exogenous uPA rescued HGF levels in muscle of uPA-/- mice. Middle: Muscle cryosections stained with
hematoxylin and eosin for morphological analysis. Note the restoration of muscle regeneration in uPA-/- mice after treatment with uPA. Scale bar = 50 μm. Bottom: Quantitative analysis of morphology. WT: wild-type no treatment, WT-I: wild-type injected with uPA, uPA-/-: uPA-/- no treatment, uPA-/-I: uPA-/- injected with uPA. Regenerating fibers identified as central nucleated fibers and counted in 2 sections per muscle and expressed as number per mm² muscle area. Damaged area estimated by subtracting summed area of normal and regenerating fibers from total muscle area. Bars are means + standard error, n = 4-6 per group. *mean value significantly different from that for WT mice.

**Figure 6. Administration of HGF blocking antibody to WT mice.** WT mice were subjected to cardiotoxin muscle injury and treated with intraperitoneal injection of either HGF blocking antibody or control IgG daily, and muscles collected at 5 days post-injury. Top: Muscle homogenates subjected to c-met immunoprecipitation and then Western blotting for c-met or phosphotyrosine (pTyr). Note that treatment with HGF blocking antibody reduced c-met phosphorylation. Middle: Muscle cryosections stained with hematoxylin and eosin for morphological analysis. Note the impairment of muscle regeneration in WT mice after treatment with HGF blocking antibody. Scale bar = 50 μm. Bottom: Quantitative analysis of morphology. Regenerating fibers identified as central nucleated fibers and counted in 2 sections per muscle and expressed as number per mm² muscle area. Damaged area estimated by subtracting summed area of normal and regenerating fibers from total muscle area. Bars are means + standard error, n = 4-6 per group. *mean value significantly different from that for WT mice.
Figure 7. **uPA and HGF expression in cells isolated from damaged muscle of WT mice.**

Cells were isolated from damaged muscle of WT mice, and sorted into Mac-1 positive and negative cell populations using a magnetic sorting method. Real time PCR indicated that Mac-1 positive cells expressed higher levels of uPA and HGF than Mac-1 negative cells. Bars are means + standard error, n = 3 per group. *mean value significantly different from that for Mac-1 negative cells.

Figure 8. **uPA and myoblast proliferation in vitro.** Myoblasts were isolated from neonatal WT and uPA-/- mice, and cultured for proliferation experiments as described in the Methods section. Cells were incubated in low serum medium without other factors overnight, and then incubated in experimental medium with designated factors added along with 100 μM BrdU for 24 hours for assessment of cell proliferation. The number of BrdU positive cells was counted in 4 fields observed at 20x and normalized to the total number of cells present in each field. Bars are means + standard error, n = 6-12 per condition. a mean value for uPA-/- cells significantly smaller than that for WT cells, b mean value for specific experimental condition significantly larger than that for control condition (low serum or no factors added), c mean value for specific experimental condition significantly smaller than that for uPA treated condition, p < 0.05.
Figure 1

The figure shows a gel electrophoresis analysis of WT, SC, and αC samples at different time points (1d, 3d, 5d, 10d) and controls (Con). The gel is separated into lanes for Std, Con, 1d, 3d, 5d, and 10d, with separate panels for WT, uPA-/-, and PAI-1-/- samples.

The bottom graph illustrates the total HGF (% of standard) and active α-chain HGF (% total) over time for WT, uPA-/-, and PAI-1-/- samples, with significant differences indicated by asterisks (*) and double asterisks (**) for statistical significance.
Figure 2

WT
IB:c-met
IB:pTyr
uPA-/-
PAI-1-/-

![Image of Western Blot](image.png)

![Graph 1](graph1.png)

![Graph 2](graph2.png)
Figure 3

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![Figure 3 Image]

**Graph:**
- WT: Solid line
- uPA-/-: Dashed line
- PAI-1-/-: Dotted line

**Legend:**
- BrdU+ cells (# per mm²)
- 3d, 5d, 10d

**Significance:**
- *: p < 0.05
- **: p < 0.01
Figure 4

WT  uPA-/-  PAI-1-/-

3d

5d

MyoD+ cells (# per mm²)

WT  uPA-/-  PAI-1-/-

3d

5d

* * *
Figure 5

<table>
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<th></th>
<th>Std</th>
<th>No treat</th>
<th>uPA inject</th>
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<tr>
<td>SC</td>
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<tr>
<td>αC</td>
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**WT**

No treat

uPA inject

**uPA-/-**

No treat

uPA inject

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**Graphs**

<table>
<thead>
<tr>
<th>Damaged area (% total)</th>
<th>WT</th>
<th>WT-I</th>
<th>uPA-/-</th>
<th>uPA-/-I</th>
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<table>
<thead>
<tr>
<th>Regenerating fibers (#/mm²)</th>
<th>WT</th>
<th>WT-I</th>
<th>uPA-/-</th>
<th>uPA-/-I</th>
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* indicates statistical significance.
Figure 6

IB: c-met
IB: pTyr

Con ab  HGF ab

Con ab  HGF ab

Damaged area (% total)

Regenerating fibers (#/mm²)

*
Figure 7

The figure shows the mRNA expression (relative to Mac-1 - cells) for uPA and HGF in Mac-1 - and Mac-1 + cells. The expression levels for HGF are significantly higher in Mac-1 + cells compared to Mac-1 - cells, as indicated by the asterisk (*) above the bar for HGF.
Figure 8
Urokinase-type plasminogen activator increases hepatocyte growth factor activity required for skeletal muscle regeneration

Thomas H Sisson, Mai-Huong Nguyen, Bi Yu, Margaret L Novak, Richard H Simon and Timothy J Koh