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14. ABSTRACT
Skeletal muscle injuries, caused by intense exercise or trauma, are among the most common injuries in military personnel. Enhancement of muscle repair following injury would minimize time lost and maximize performance during training and combat. We and others have published data demonstrating that the extracellular protease urokinase-type plasminogen activator (uPA) is required for efficient muscle repair, although the underlying mechanisms remain to be elucidated. In this progress report, we present data indicating that satellite cell fusion during muscle regeneration is impaired in uPA null mice, and accelerated in mice deficient in the inhibitor of uPA, PAI-1, compared to wild-type mice. In vitro experiments have demonstrated that uPA causes a dose-dependent increase in the proliferation and migration of wild-type satellite cells. Western blot analysis indicated that phosphorylation of the receptor of HGF, c-met, is impaired in injured muscle of uPA null mice and increased in muscle of PAI-1 null mice. Finally, administration of exogenous uPA has been shown to rescue muscle regeneration in uPA null mice. Taken together, these data support the hypothesis that satellite cell activity is regulated by the balance of uPA and PAI-1, through activation of HGF. Findings from continued work on this project will provide insight into potential manipulation of components of the plasminogen system as a way to enhance muscle repair. Enhancing muscle repair following injury would minimize time lost due to muscle injury both during training and combat, and maximize performance following return from injury.

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Skeletal muscle repair, muscle function, urokinase-type plasminogen activator, satellite cell, hepatocyte growth factor

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Table of Contents

Introduction

Proper skeletal muscle function is essential for nearly all activities required for military training and combat. Injury to skeletal muscle caused by intense exercise or trauma compromises muscle function, and such injuries are among the most common experienced by military personnel. Enhancing muscle repair following injury would minimize time lost due to muscle injury both during training and combat, and maximize performance following return from injury. Our published data indicates that the extracellular protease urokinase-type plasminogen activator (uPA) is required for efficient muscle repair, although the underlying mechanisms remain to be elucidated. One way that uPA could promote repair is by stimulating activity of satellite cells; satellite cells are muscle stem cells required for repair. One mechanism by which uPA could stimulate satellite cell activity is by activating hepatocyte growth factor (HGF); HGF can activate quiescent satellite cells, and stimulate their proliferation and migration. *The guiding hypothesis of this proposal is that the balance of uPA and its endogenous inhibitor, PAI-1, regulates muscle repair.* The purpose of the present project is to determine whether the balance of uPA and PAI-1 regulates activation of HGF and activation and proliferation satellite cells during muscle repair.

Body

Task 1. In the Statement of Work for this project, Task 1 was to determine whether the balance of uPA and PAI-1 regulates satellite cell activation and proliferation following muscle injury. We completed the experiments for Task 1 in the Progress Report submitted for last year. Briefly, our results indicated that there was no difference in the number of quiescent satellite cells in uninjured muscle of wild-type, uPA null and PAI-1 null mice. Following muscle injury, proliferation and accumulation of satellite cells was robust in wild-type mice. However, satellite cell proliferation and accumulation was impaired in uPA null mice and was accelerated in PAI-1 null mice compared to wild-type mice. These data indicate that the balance of uPA and PAI-1 regulates the activation and proliferation of satellite cells during muscle repair.

Task 2. Task 2 of this project is to determine whether the balance of uPA and PAI-1 regulates satellite cell migration and fusion. For in vivo experiments, the extensor digitorum longus (EDL) muscles of wild-type, uPA null and PAI-1 null mice were injured with an injection of snake toxin (cardiotoxin), and bromodeoxyuridine (BrdU; 30 mg/kg) was injected into mice immediately after injury and daily thereafter. EDL muscles were harvested at 5 days post-injury, and satellite cell fusion was assayed in cryosections by immunostaining for BrdU and counting the number of BrdU positive nuclei that had been incorporated into regenerating muscle fibers. In wild-type mice, numerous BrdU positive nuclei were observed within regenerating fibers (Figure 1). In uPA null mice, no BrdU positive nuclei were observed within regenerating fibers and in PAI-1 null mice, increased numbers of BrdU positive nuclei were observed in regenerating fibers compared with wild-type mice. These data indicate that satellite cell fusion was impaired in uPA null mice and enhanced in PAI-1 null mice. In each strain, a few BrdU positive nuclei were observed outside of regenerating fibers, likely indicating unfused satellite cells and/or proliferating fibroblasts. In short, the balance of uPA and PAI-1 appears to regulate the fusion of satellite cells in vivo.

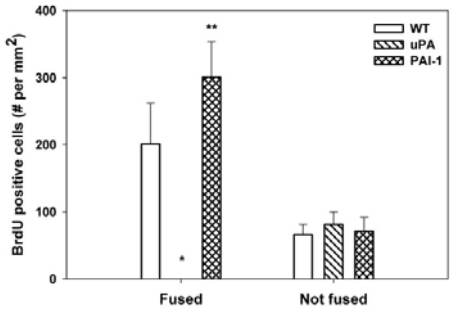

Figure 1. Satellite cell fusion in vivo. Values shown are means with standard error bars. *value for uPA null mice significantly smaller than that for wild-type (WT) mice. **value for PAI-1 null mice significantly greater than that for wild-type mice. N = 8, $p < 0.05$.

For in vitro experiments, satellite cells were isolated from neonatal wild-type mice. Briefly, hindlimb muscles were harvested, minced and digested using 1% pronase. After trituration to dissociate cells from tissue debris, the suspension was filtered, centrifuged and plated in F-10 media supplemented with 20% fetal bovine serum, 2.5 ng/ml basic fibroblast growth factor, and 1% penicillin/ streptomycin. Cells were preplated for 1 hour to allow fibroblasts to adhere to plastic culture dishes, and then the cells remaining in suspension were propagated on tissue culture dishes pre-coated with collagen I. Our preliminary experiments indicated that cells in these cultures are > 90% positive for the myogenic marker MyoD.

We have started to test whether the balance of uPA and PAI-1 regulates the proliferation of muscle satellite cells in vitro. 5×10^4 cells per well were plated in 24 well plates with collagen coated coverslips. Cells were incubated for 24 hours in basal medium to induce growth arrest. Medium was then switched to basal medium containing various supplements and BrdU. Cell proliferation was assayed using immunodetection of incorporation of BrdU into newly synthesized DNA. Low serum medium resulted in a low level of proliferation whereas high serum medium resulted in a high level of proliferation (Figure 2). Active uPA induced a dose-dependent increase in proliferation when placed in low serum medium. The level of uPA-induced proliferation was similar to that of HGF and FGF (100 ng/ml). On the other hand, the amino terminal fragment of uPA, which lacks the proteolytic domain but retains the receptor binding domain, did not stimulate proliferation. These data indicate that uPA stimulates satellite cell proliferation in vitro through its proteolytic activity.

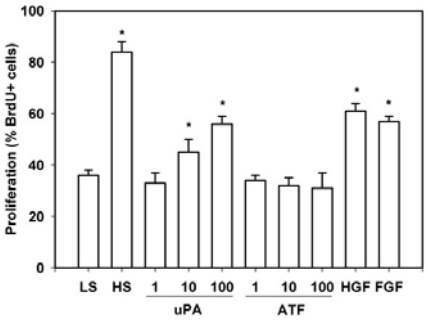

Figure 2. Satellite cell proliferation in vitro. High serum (HS) condition served as a positive control. Units for uPA and ATF are in ng/ml. Values shown are means with standard error bars. *values significantly greater than that for low serum (LS) condition. N = 8-16, $p < 0.05$.

We have also started to test whether the balance of uPA and PAI-1 regulates the migration of muscle satellite cells. 2.5×10^4 cells per well were placed in the upper wells of a Boyden-type migration chamber and 10% extract damaged muscle was placed in the bottom wells to act as chemoattractant. Various supplements were placed in the upper wells with the cells to test whether uPA promotes cell migration. Cells were allowed to migrate through a polycarbonate membrane with 5 μm pores, and the cells

that migrated to the bottom surface were stained and counted. Spontaneous cell migration was measured with media alone in both upper and lower chambers and all other conditions were normalized to this control condition. When muscle extract was placed in the bottom wells, cell migration was increased ~250% above control levels (Figure 3). When uPA was added to the cell suspensions, migration was enhanced in a dose-dependent manner. When HGF was added to cell suspensions, migration was enhanced to a similar level. Interestingly, when an HGF blocking antibody was added to the cell suspensions, migration was reduced to control levels whereas a control antibody had no effect. These data indicate that uPA and HGF promote satellite cell migration and the HGF is required for cell migration in this assay.

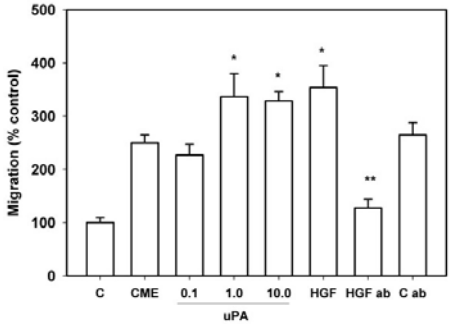

Figure 3. Satellite cell migration in vitro. Values normalized to control (C) with media alone. Migration induced using injured muscle extract (CME). Units for uPA and ATF are in ng/ml. Values shown are means with standard error bars. *values significantly greater than that for low serum condition. **value significantly smaller than that for CME condition. N = 8-16, $p < 0.05$.

We are in the process of completing the in vitro experiments for Task 2. For the wild-type cells, we have planned experiments testing the influence of PAI-1 and a combination of uPA and PAI-1 on satellite cell proliferation and migration. We have had problems breeding uPA mice and thus experiments involving satellite cells from these mice have experienced some delay. However, we have been successful in culturing satellite cells from neonatal uPA and PAI-1 null mice and we are proceeding with experiments similar to those described above for the wild-type mice.

Task 3. Task 3 is to determine whether the balance of uPA and PAI-1 regulates HGF activity during muscle regeneration. In the progress report for last year, we presented data indicating that HGF is upregulated following muscle injury in wild-type mice, and upregulated earlier and to a greater extent in PAI-1 null mice compared with wild-type mice. However, we have had technical difficulties in continuing these experiments. We have identified the problem as an antibody issue, and have obtained new antibody and are in the process of optimizing the procedure for this new antibody. We will obtain new sets of data for wild-type and PAI-1 null mice as well as for uPA null mice using this new antibody.

We have finished analysis of activation of the HGF receptor, c-met. EDL muscles of wild-type and PAI-1 null mice were injured using cardiotoxin, and muscles harvested at 1, 3, 5 and 10 days post-injury. c-met expression and phosphorylation were measured using immunoprecipitation with an antibody against c-met, followed by Western blotting with antibodies against total c-met, or phosphorylated tyrosine. In wild-type mice, c-met phosphorylation was increased in injured muscle of wild-type mice, with peak levels at 3 and 5 days post-injury (Figure 4). c-met phosphorylation was barely evident in uPA null mice and was increased to a greater extent in PAI-1 null compared with wild-type mice. In addition, peak c-met phosphorylation was observed earlier in PAI-1 null mice, at 1 and 3 days post-injury, than in wild-type mice. These

data indicate that the balance of uPA and PAI-1 regulates activation of the HGF receptor following muscle injury.

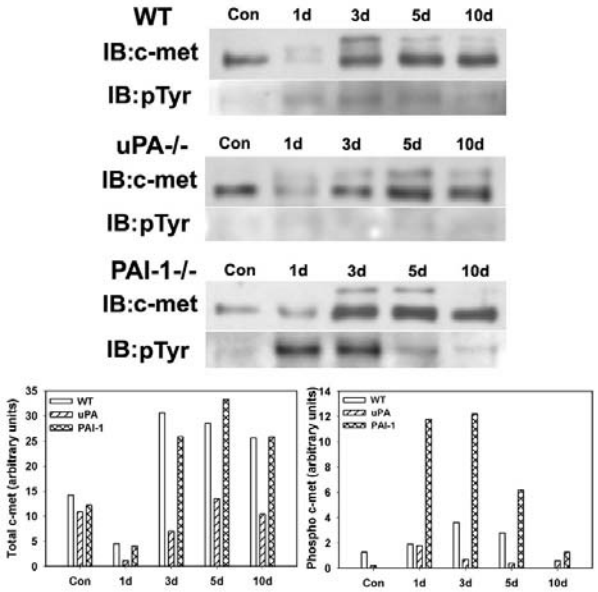

Figure 4. c-met phosphorylation in muscle of wild-type (WT), uPA null and PAI-1 null mice. Note that c-met phosphorylation (pTyr) is elevated in injured muscle of wild-type mice, but not in muscle of uPA null mice, and is elevated earlier and to a greater extent in PAI-1 null mice.

To determine whether administering exogenous uPA rescues HGF activation and efficient muscle healing in uPA null mice, EDL muscles were injured using cardiotoxin, and uPA was administered via daily intramuscular injections (5 μg). Muscles were harvested at 5 days post-injury and muscle regeneration assessed in hematoxylin and eosin stained cryosections. In wild-type mice, muscle regeneration was robust, with formation of many small regenerating fibers evident at 5 days post-injury (Figure 5). In uPA null mice, regeneration was completely impaired with formation of few, if any, regenerating fibers. When exogenous uPA was administered to uPA null mice, formation of regenerating fibers was restored to wild-type levels. In the coming months, we plan to assess whether administration of uPA rescues HGF activation in uPA null mice. We will also test whether administering exogenous active HGF can rescue muscle regeneration in uPA null mice.

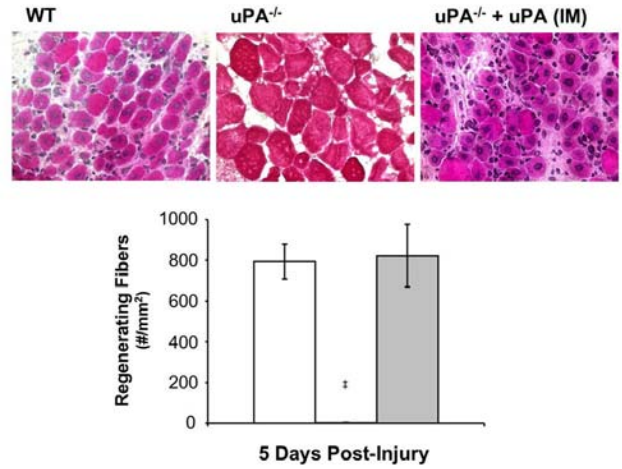

Figure 5. Exogenous uPA restores muscle regeneration in uPA null mice. Top: hematoxylin and eosin stained cryosections from wild-type (WT) uPA null (uPA-/-) and uPA null mice given exogenous uPA (uPA-/- + uPA). Note that recovery of normal morphology is impaired in uPA null compared to wild-type mice, and that exogenous uPA rescues recovery of muscle morphology. Bottom: formation of regenerating fibers in WT (left), uPA null (middle) and uPA null mice given exogenous uPA (right). ‡value significantly smaller than that for wild-type. N = 6, $p < 0.05$.

Key Research Accomplishments

- Satellite cell fusion during muscle repair is impaired in uPA null mice and enhanced in PAI-1 null mice.
- Proliferation of cultured satellite cells is enhanced by uPA in a dose-dependent manner to a similar level as HGF. Proteolytic activity of uPA is required for this stimulation of proliferation.
- Migration of cultured satellite cells is also enhanced by uPA in a dose-dependent manner to a similar level as HGF. Blocking HGF activity using a blocking antibody reduces cell migration.
- Phosphorylation of c-met, the receptor for HGF, is increased following muscle injury in wild-type mice but not uPA null mice, and increased earlier and to a greater extent in PAI-1 null mice compared with wild-type mice.
- Administration of exogenous uPA rescues muscle regeneration in uPA null mice.

Reportable Outcomes

We presented data on rescue of muscle regeneration in uPA null mice at the 2007 Gordon Conference on Tissue Repair and Regeneration in New London, NH.

Conclusion

We have finished experiments for Task 1 and have made good progress on Tasks 2 and 3. We have produced data indicating that uPA and PAI-1 regulate satellite cell proliferation and fusion in vivo and are currently performing experiments to determine whether uPA and PAI-1 regulate satellite activity in vitro and to elucidate the mechanisms involved. We have established the methods required and have promising data from experiments using wild-type cells. We have finished experiments demonstrating that uPA and PAI-1 regulate activation of the HGF receptor and that exogenous uPA rescues muscle regeneration in uPA null mice. However, we have had some problems breeding uPA null mice, and thus data from these mice has been delayed. We have also had some problems with the HGF protein assay, but have resolved these problems and are performing these assays presently. Findings from this work are providing insight into potential manipulation of components of the plasminogen system as a way to enhance muscle repair. For example, a small molecule inhibitor of PAI-1, which prevents interaction with uPA, could increase uPA activity and promote muscle healing. Enhancing muscle repair following injury would minimize time lost due to muscle injury both during training and combat, and maximize performance following return from injury.