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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 the present project, immunofluorescence analysis demonstrated that cell proliferation and satellite cell accumulation is impaired in uPA null mice, and accelerated in mice deficient in the inhibitor of uPA, PAI-1, compared to wild-type mice. Western blot analysis indicated that levels of active hepatocyte growth factors (HGF) are enhanced in PAI-1 null mice compared with wild-type mice, and that phosphorylation of the receptor of HGF, c-met, is similarly enhanced. Taken together, these data indicate that satellite cell activity is regulated by the balance of uPA and PAI-1, perhaps 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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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 [1], 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 is to determine whether the balance of uPA and PAI-1 regulates satellite cell activation and proliferation following muscle injury (Months 1-18 of the project). Wild-type, uPA null and PAI-1 null mice were obtained from Jackson Laboratories, and breeding colonies established. The extensor digitorum longus (EDL) muscles of wild-type, uPA null and PAI-1 null mice were injured using injection of snake toxin (cardiotoxin), and muscles harvested at 1, 3, 5 and 10 days post-injury. Bromodeoxyuridine (BrdU) was injected into mice 1 hour before euthanasia; BrdU is incorporated into newly synthesized DNA and immunofluorescent detection of BrdU in muscle sections was used as a marker for proliferating cells. Satellite cells were detected by immunolabeling of cryosections for the myogenic transcription factor MyoD. Cells positive for MyoD or BrdU were counted on two sections per muscle and averaged across muscles.

Hematoxylin and eosin staining revealed impaired regeneration in uPA null mice and accelerated regeneration in PAI-1 null mice (Figure 1), confirming data from our previous study [1]. Very few BrdU positive cells were observed in control muscle or at 1 day post-injury in each strain of mice. For wild-type and uPA null mice, BrdU positive cells increased at 3 days post-injury, peaked at 5 days post-injury and then decreased again at 10 days post-injury (Figures 1 and 2). Analysis of variance indicated that uPA null mice exhibited significantly fewer BrdU positive cells compared with wild-type mice at each time point ($p < 0.05$). For PAI-1 null mice, BrdU positive cells peaked at 3 days post-injury, with a progress decline thereafter. Analysis of variance indicated that differences between wild-type and PAI-1 null mice were significant at 3 days post-injury ($p < 0.05$). These data indicate significantly impaired cell proliferation in uPA null mice, and accelerated cell proliferation in PAI-1 null mice compared with wild-type mice, consistent with the alterations in healing observed in a previous study [1].

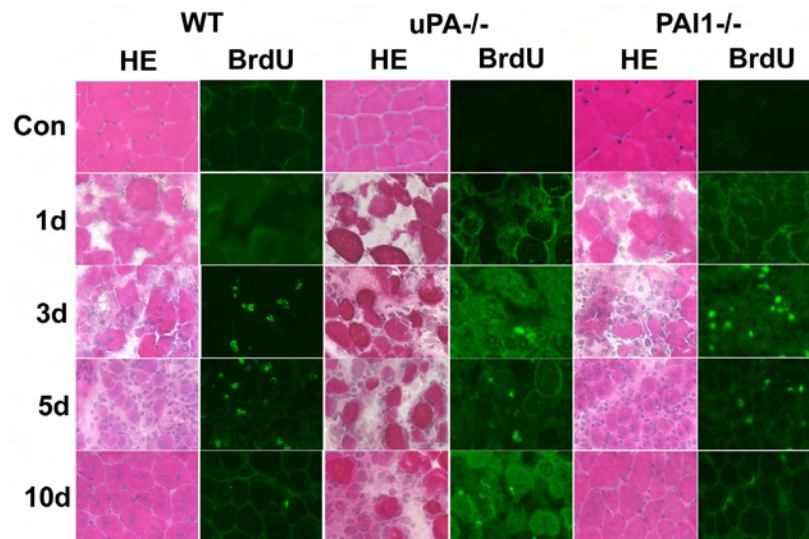


Figure 1. Morphology and cell proliferation in wild-type, uPA null and PAI-1 null mice. EDL muscles were injured with cardiotoxin and collected at 1 to 10 days post-injury. Cryosections were stained with either hematoxylin and eosin or an antibody against BrdU. Note the impaired regeneration and cell proliferation in uPA null mice and accelerated regeneration and cell proliferation in PAI-1 null mice.

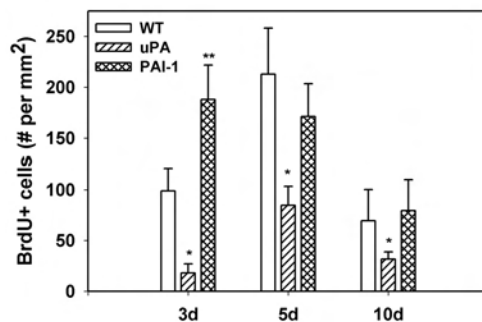


Figure 2. Cell proliferation is impaired in uPA null mice and accelerated PAI-1 null mice. Values shown are means with standard error bars. * value for uPA null mice significantly smaller than that for wild-type (WT) mice. ** value for PAI-1 null mice significantly smaller than that for wild-type mice. N = 8, p < 0.05.

MyoD staining was performed in cross-sections of muscle from wild-type, uPA null and PAI-1 null mice harvested at 3 and 5 days post-injury. At 3 days post-injury, analysis of variance indicated that the number of MyoD positive cells was significantly smaller in uPA null mice, and significantly greater in PAI-1 null mice compared with wild-type mice (p < 0.05, Figure 3). All of these MyoD positive cells were small mononuclear cells at this time point, suggesting that they were satellite cells. At 5 days, the number of MyoD positive cells was still significantly smaller in uPA null mice compared with wild-type mice. Many of these MyoD positive cells showed morphology of regenerating muscle fibers, indicating that satellite cells had begun to fuse at this time point. The data strongly indicate that satellite cell proliferation and accumulation is impaired in uPA null mice, and enhanced in PAI-1 null mice compared with wild-type mice.

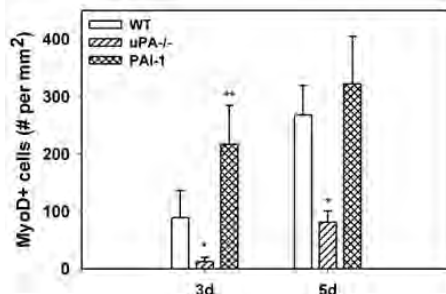


Figure 3. Satellite cell accumulation is impaired in uPA null mice and increased in PAI-1 null mice. Values shown are means with standard error bars. *value for uPA null mice significantly smaller than that for wild-type (WT) mice. **value for PAI-1 null mice significantly larger than that for wild-type mice. N = 8, p < 0.05

The number of satellite cells in uninjured muscle was also counted to determine whether the number of quiescent cells differs between wild-type, uPA null and PAI-1 null mice. Cross sections from uninjured muscles were labeled with a monoclonal antibody against the satellite cell marker Pax7 to identify quiescent satellite cells. The number of Pax7 positive cells was counted in two sections per muscle for 6 mice of each strain. The number of satellite cells in uninjured muscle of wild-type mice (29.1 ± 2.8 cells/mm²), uPA null mice (34.9 ± 4.5 cells/mm²) and PAI-1 null mice (26.9 ± 3.6 cells/mm²) were not significantly different. These data indicate that the differences in the number of satellite cells in uninjured muscle does not account for the differences in cell proliferation, satellite cell accumulation and muscle regeneration between strains.

Task 2. Task 2 is to determine whether the balance of uPA and PAI-1 regulates satellite cell migration and fusion in vitro (Months 13-30 of the project). After optimizing procedures for this Task over the past year, we have established procedures in our laboratory for isolating satellite cells from neonatal mouse muscle. Muscle has been harvested, finely minced, and digested using pronase. After trituration to dissociate cells from tissue debris, the suspension has been filtered, centrifuged and the satellite cells isolated on a Percoll gradient. Cells were then plated in F-10 media supplemented with 20% fetal bovine serum, 2.5 ng/ml basic fibroblast growth factor, and 1% penicillin/streptomycin. Our preliminary experiments with this technique have produced a substantial yield of satellite cells, and we have been able to obtain cultures that are > 90% positive for the myogenic marker MyoD (Figure 4). We are in the process of determining satellite cell migration and fusion in cells isolated from wild-type, uPA null and PAI-1 null mice.

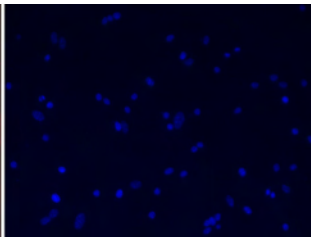
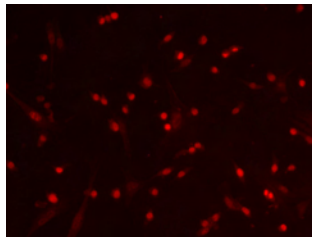


Figure 4. MyoD staining of satellite cell culture. Cells isolated from neonatal wild-type muscle were labeled with antibody against myogenic factor MyoD (left), or stained with DAPI (right). ~90% of the total cell population stained positively for MyoD, indicating that the majority of the cultured cells were satellite cells.

Task 3. Task 3 is to determine whether the balance of uPA and PAI-1 regulates HGF activity during muscle regeneration (Months 25-48 of the project). Since we had problems establishing procedures for Task 2 (problems which have been solved at this time), we started to collect data for Task 3. 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. HGF expression was measured using affinity purification of muscle homogenates with heparin sepharose followed by Western blotting with an antibody against HGF. 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.

For wild-type mice, HGF protein was not evident in non-injured control muscle, but was increased at 1 to 10 days post-injury (Figure 5). Both inactive single chain (sc) and active α -chain forms were present in injured muscle at all time points, with α -chain levels peaking at 3 and 5 days post-injury. For PAI-1 null mice, HGF protein was also

increased following injury, but peak expression appeared at 1 to 3 days post-injury, earlier than in wild-type mice, and consistent with accelerated satellite cell accumulation and muscle regeneration. In addition, a greater proportion of the HGF was present in the active α -chain form in PAI-1 null compared with wild-type mice, consistent with increased uPA activity in muscle of PAI-1 null mice [1]. uPA is known to activate HGF, and whether HGF can be activated in uPA null mice will be determined in the coming months. An interesting result of these experiments is the increased total levels of HGF in PAI-1 null mice. We will also be working to identify the source of the HGF in the coming months.

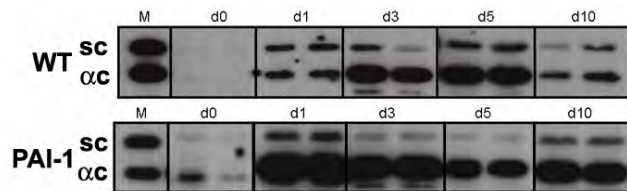


Figure 5. HGF levels in muscle of wild-type (WT) and PAI-1 null mice. Note that HGF levels are higher in PAI-1 null mice, especially at early time points. Note also that the proportion of HGF in the active α -chain form (α c) compared with inactive single chain form (sc) is greater for PAI-1 null mice than wild-type mice.

c-met is the receptor for HGF, and binding of activated HGF to c-met results in its phosphorylation on tyrosine. Such phosphorylation can be used as a read-out of receptor activation, and initiates intracellular signaling for cell proliferation and migration. c-met phosphorylation was increased in injured muscle of wild-type mice, with peak levels at 3 and 5 days post-injury, consistent with the activation of HGF (Figure 6). c-met phosphorylation 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, consistent with the relative timing and magnitude of active HGF levels. In the coming months, we will be performing experiments to determine whether c-met phosphorylation is impaired in uPA null mice compared with wild-type mice.

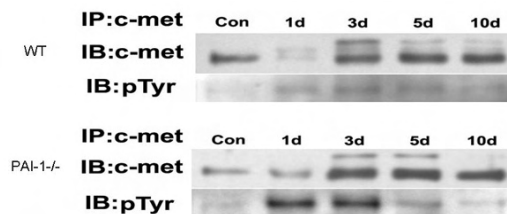


Figure 6. c-met phosphorylation in muscle of wild-type (WT) and PAI-1 null mice. Note that c-met phosphorylation (pTyr) is elevated in injured muscle of wild-type mice, and elevated earlier and to a greater extent in PAI-1 null mice.

Key Research Accomplishments

- Immunofluorescence analysis demonstrated that cell proliferation is impaired in uPA null mice and accelerated in PAI-1 null mice compared to wild-type mice
- Immunofluorescence analysis also demonstrated that satellite cell accumulation is impaired in uPA null mice and increased in PAI-1 null mice compared to wild-type mice
- Immunohistochemical analysis indicates no difference in number of quiescent satellite cells in uninjured muscle of wild-type, uPA null and PAI-1 null mice

- Satellite cell isolation procedure has been optimized and in vitro experiments on cell migration and fusion are underway.
- Affinity purification followed by Western blot analysis demonstrated 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.
- Antibody purification followed by Western blot analysis demonstrated that phosphorylation of c-met, the receptor for HGF, is increased following muscle injury in wild-type mice, and increased earlier and to a greater extent in PAI-1 null mice compared with wild-type mice.

Reportable Outcomes

We presented data on Tasks 1 and 3 at the 2006 Experimental Biology meeting in San Francisco, and at the 2006 DOD Military Health Research Forum in San Juan, Puerto Rico. We also anticipate preparing a manuscript for publication in the middle of 2007.

Conclusion

We have completed experiments for Task 1, and thus we have finished work for Task 1 on schedule. Our experiments to date demonstrate that cell proliferation and satellite cell accumulation are impaired in injured muscle of uPA null mice, and enhanced in PAI-1 null mice, compared to wild-type mice. These findings are consistent with the altered repair and functional recovery we observed in injured muscle of uPA null and PAI-1 null mice compared to wild-type mice [1]. We have also optimized procedures for Task 2, to determine whether the balance of uPA and PAI-1 regulate satellite cell migration and fusion in vitro and in vivo. Establishing procedures for Task 2 took longer than anticipated, and so we have started on Task 3. Our results from the experiments for Task 3 completed so far indicate that HGF is upregulated following muscle injury in wild-type mice, and is upregulated earlier and to a greater extent in PAI-1 null mice. These data indicate that the accelerated muscle regeneration observed in PAI-1 null mice may be a result of enhanced HGF activity. 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.

References

1. **Koh TJ, SC Bryer, AM Pucci, and TH Sisson.** Mice deficient in plasminogen activator inhibitor-1 have improved skeletal muscle regeneration. *Am J Physiol Cell Physiol.* 289:C217-23, 2005.