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TITLE: Enhancement of Skeletal Muscle Repair By The Urokinase Type Plasminogen Activator System

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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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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 previously completed the experiments for Task 1. Briefly, our results indicated that proliferation and accumulation of satellite cells following muscle injury was robust in wild-type (WT) 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. We completed the in vivo experiments for Task 2 in the Progress Report submitted last year. Briefly, in wild-type mice, numerous BrdU positive nuclei were observed within regenerating fibers. 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.

This year, we have completed experiments investigating the influence of the balance of uPA and PAI-1 on satellite cell proliferation in vitro. To determine if uPA induces myoblast proliferation in vitro, we compared BrdU incorporation in cells isolated from uPA null and WT animals. We found that uPA null 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 1). Subsequent experiments demonstrated that exogenously administered recombinant mouse uPA produced a dose-dependent increase in proliferation of both WT and uPA null myoblasts (Figure 1). This exogenous uPA had a greater stimulatory effect on uPA null 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, exogenous 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 null myoblasts (Figure 1). These data indicate that the proteolytic activity of uPA stimulates myoblast proliferation.

Figure 1. uPA and myoblast proliferation in vitro. Myoblasts were isolated from neonatal WT and uPA null 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 null 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.
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 null myoblasts (Figure 1). 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 null 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 1). Together, these data indicate that uPA activation of HGF promotes myoblast proliferation likely through PI3K and MEK1.

We are in the process of completing the in vitro experiments for Task 2. We plan to determine whether the balance of uPA and PAI-1 regulate myoblast migration and fusion using experimental conditions similar to those used for the proliferation experiments. For assays of migration, we will use the Boyden-type migration chamber described in the Progress Report of last year. For assays of fusion, we will use methods described in the original proposal.

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 described technical difficulties in performing assays of HGF protein levels. We have overcome these difficulties and have measured differences in HGF protein levels between WT, uPA null and PAI-1 null mice (Figure 2).

Figure 2. HGF protein levels following muscle injury in WT, uPA null and PAI-1 null mice. Muscles from WT, uPA null (uPA) and PAI-1 null (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.
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 null mice than uPA null mice (Figure 2). 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 2). In uPA null 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 null 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 null 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 administering exogenous uPA rescues HGF activity and muscle regeneration in uPA null mice, we injected uPA into cardiotoxin-injured muscles of uPA null mice (Figure 3).

Figure 3. Administration of exogenous uPA to uPA null mice. uPA null 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 null mice. Middle: Muscle cryosections stained with hematoxylin and eosin for morphological analysis. Note the restoration of muscle regeneration in uPA null mice after treatment with uPA. 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.
Injured muscles from untreated uPA null mice contained relatively low levels of HGF (Figure 3). In contrast, injured muscles from mice treated with exogenous uPA exhibited both increased total and α-chain HGF levels (Figure 3). In addition, muscles from untreated uPA null mice were characterized by the persistence of damaged tissue, and the absence of regenerating fibers (Figure 3). 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 null mice. In contrast, treatment of injured muscles in WT mice with exogenous uPA did not alter the recovery of muscle morphology.

Key Research Accomplishments

- uPA is required for HGF activity following muscle injury, as uPA null mice had markedly reduced HGF levels following muscle damage. In addition, when uPA activity is not opposed by PAI-1, HGF activity is enhanced at early time points after muscle injury.
- Administration of exogenous uPA rescued HGF levels and muscle regeneration in uPA null mice
- uPA promotes myoblast proliferation \textit{in vitro} through its proteolytic activity and this process was inhibited by an HGF blocking antibody.

Reportable Outcomes

We are in the process of submitting the data generated so far to Blood Journal, which is a prestigious high-impact factor journal.

Conclusion

We have finished experiments for Tasks 1 and 3 and have made good progress on Task 2. Our data so far indicate that the balance of uPA and PAI-1 regulate HGF activity and satellite cell proliferation and fusion in vivo. We also have found that uPA promotes myoblast proliferation \textit{in vitro} likely through its proteolytic activity on HGF. We are currently performing experiments to determine whether uPA and PAI-1 regulate satellite cell migration and fusion \textit{in vitro} by similar mechanisms. In the next year, we will also perform experiments to determine whether increasing uPA expression in muscle following injury through genetic manipulation will enhance HGF activity and/or muscle repair. 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, administering either exogenous uPA or a small molecule inhibitor of PAI-1, which prevents interaction with uPA, could increase uPA activity after muscle injury 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.