The Urokinase-Type Plasminogen Activator Receptor is Not Required for Skeletal Muscle Inflammation or Regeneration

Scott C. Bryer & Timothy J. Koh
Department of Movement Sciences, University of Illinois at Chicago,
1919 W. Taylor St. (m/c 994, Rm 529), Chicago, IL 60612, USA

Running Head: uPAR is Not Required for Regeneration

To whom correspondence should be addressed:
Timothy J. Koh, PhD
Department of Movement Sciences
University of Illinois at Chicago
1919 W. Taylor St. (m/c 994, Rm 529), Chicago, IL 60612, USA
Tel: 312-413-9771, Fax: 312-996-8768
Email: tjkoh@uic.edu
Abstract

The hypothesis of this study was the urokinase-type plasminogen activator receptor (uPAR) is required for accumulation of inflammatory cells in injured skeletal muscle and for efficient muscle regeneration. Expression of uPAR was elevated at 1 and 3 days after cardiotoxin-induced muscle injury in wild-type mice before returning to baseline levels. Neutrophil accumulation peaked 1 day post-injury in muscle from both wild-type and uPAR null mice, while macrophage accumulation peaked between 3 and 5 days post-injury, with no differences between strains. Histological analyses confirmed efficient muscle regeneration in both wild-type and uPAR null mice, with no difference between strains in the formation or growth of regenerating fibers, or recovery of normal morphology. Furthermore, in vitro experiments demonstrated that chemotaxis is not different between WT and uPAR null macrophages. Finally, fusion of cultured satellite cells into multinucleated myotubes was not different between cells isolated from wild-type and uPAR null mice. These results demonstrate that uPAR is not required for the accumulation of inflammatory cells or the regeneration of skeletal muscle following injury, suggesting uPA can act independently of uPAR to regulate events critical for muscle regeneration.

Keywords: muscle repair, macrophage, chemotaxis
Introduction

Skeletal muscle regeneration following injury is a multi-step process that involves the activity of several cell types. Inflammatory and satellite cells, in particular, appear to be required for efficient regeneration (17, 24, 42). The accumulation of inflammatory cells, including neutrophils and macrophages, begins within hours of injury and the classic function of these cells is the clearance of necrotic tissue (33, 45). However, recent evidence suggests an additional role for macrophages in the formation of new muscle tissue. Some potential functions of macrophages in muscle regeneration include production of chemo-attractants for other inflammatory and satellite cells, and production of growth factors which may promote satellite cell activation, proliferation, migration and fusion (8, 24, 27, 29).

The urokinase-type plasminogen activator (uPA) has proven to be a key regulator of skeletal muscle inflammation and regeneration. In uPA null (uPA\(^{-/-}\)) mice, macrophage accumulation is nearly absent in injured muscle, and this is associated with severely impaired muscle regeneration, compared to wild-type (WT) mice (22, 25). In mice lacking the inhibitor of uPA, PAI-1, muscle injury resulted in increased uPA activity, increased macrophage accumulation and accelerated muscle regeneration, compared to WT mice (22). The classic molecular function of uPA is the activation of plasminogen to plasmin to assist in the degradation of extracellular matrix proteins, as occurs during cell migration and matrix remodeling (11, 41). uPA may also regulate the activity of growth factors, either through direct cleavage of pro-peptides (e.g. HGF), or by releasing growth factors from the extracellular matrix (e.g. FGF, HGF) through plasmin and MMP activation (26, 43).
Expression of the receptor for uPA (uPAR) appears to correlate with the migratory potential of different cells, including macrophages and satellite cells. uPAR null (uPAR\(^{-/-}\)) mice demonstrate impaired accumulation of inflammatory cells following peritonitis (28) or pulmonary infection \textit{in vivo} (18, 35, 39). In addition, treatment of monocytes with an anti-uPAR antibody suppressed chemotaxis induced by fMLP \textit{in vitro} (19) and peritoneal macrophages collected from uPAR\(^{-/-}\) mice failed to promote plasminogen activation (7). \textit{In vitro} experiments with muscle satellite cells or myoblasts demonstrated that antibodies against uPA or uPAR impaired cell proliferation, migration and fusion (5, 12, 15, 31, 47). uPAR may contribute to these processes through different mechanisms. uPA binding to uPAR enhances uPA-mediated plasmin activation, and concentrates the proteolytic activity of uPA towards the leading edge of migrating cells (4, 14). uPA binding to uPAR can also initiate intracellular signaling through interactions between uPAR and integrins that may promote cell migration, adhesion, proliferation and differentiation (4, 21, 34, 46). Although the available evidence indicates that uPAR plays an important role in regulating the activity of inflammatory and satellite cells, whether uPAR is required for the activity of these cells during muscle regeneration remains to be established.

The hypothesis of the current study was that uPAR is required for the accumulation of inflammatory cells in injured muscle and for efficient muscle regeneration. We expected that uPAR\(^{-/-}\) mice would demonstrate reduced accumulation of inflammatory cells following cardiotoxin-induced muscle injury and impaired muscle regeneration.
Materials and Methods

Mice

uPAR null (uPAR\(^{-/-}\)) mice were obtained from Jackson Laboratories on a C57BL/6 background (Bar Harbor, ME). Wild-type (WT; C57BL/6) mice were obtained from Jackson or Harlan (Indianapolis, IN). No differences in muscle inflammation or regeneration were observed between WT mice obtained from different suppliers. All mice were housed in groups of 3-5 at 22-24°C using a 12:12 h light/dark cycle. Food and water were provided ad libitum. All experiments were performed on mice 10-12 weeks old. The Animal Care Committee at the University of Illinois at Chicago approved all experimental procedures.

Muscle Injury and Sample Preparation

Extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were injured via cardiotoxin injection as previously described (22). Briefly, mice were anesthetized with an intraperitoneal injection of either ketamine (100mg/kg) and xylazine (5mg/kg) or tribromoethanol (avertin; 400 mg/kg) and a small incision (1 cm) was made exposing the muscles of the anterior leg. Cardiotoxin (10\(\mu\)M; Calbiochem, San Diego, CA) was administered with two intramuscular injections per muscle to ensure distribution throughout each muscle. The skin incision was closed with 7-0 nylon suture and the procedure was repeated on the contralateral limb. Mice were allowed to recover and muscles were collected at different times following injury. Muscles collected from uninjured mice were used as controls. EDL muscles were mounted in tissue freezing medium and frozen in isopentane chilled with dry ice for histological analysis, and TA muscles were stored in RNA later (Qiagen; Valencia, CA) for RT-PCR analysis.
Administration of uPAR Blocking Antibody

A monoclonal anti-mouse uPAR blocking antibody (R&D Systems, Minneapolis, MN) selected for its ability to block uPA interaction with uPAR was administered via intraperitoneal injection (100µg/injection) 2 hours following cardiotoxin induced muscle injury and 1 – 4 days post-injury in WT mice.

Muscle Morphology

Cryosections were cut from the midbelly of each EDL muscle (10µm thickness) and either stained with hematoxylin and eosin for morphological analysis or processed for inflammatory cell analysis via immunohistochemistry. Morphological analysis was performed using five representative images using a ×40 lens objective for each muscle section (Labphot-2, Nikon; and SPOT software, Diagnostic Instruments, Sterling Heights, MI). For each field, fibers were classified as normal, damaged or regenerating as previously described (22). 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.

Immunohistochemistry

Immunohistochemistry was used to identify inflammatory cell accumulation and uPAR protein expression following injury. Analysis of inflammatory cells was performed using immunohistochemical methods previously described (22). Neutrophils were labeled with a rat anti-mouse Ly6G antibody (1:100 dilution, 2 hour incubation; Pharmingen, San Diego, CA), and macrophages were labeled with a rat anti-mouse F4/80 antibody (1:100 dilution, overnight incubation; Serotec, Oxford, UK), followed by a 1 hour incubation with biotinylated mouse adsorbed anti-rat IgG (1:200 dilution; Vector
Laboratories, Burlingame, CA). uPAR was labeled with an rat anti-mouse uPAR monoclonal antibody (1:100 dilution, 2 hour incubation; R & D Systems, Benicia, CA), followed by 1 hour incubation with biotinylated rabbit anti-goat IgG (1:200 dilution; Vector Laboratories). All sections were then developed using Vector Laboratories AEC kit. Controls included omission of primary antibodies, and incubation of sections with isotype specific control IgG in place of primary antibodies. The number of positive cells were counted in two entire sections for each muscle with the aid of an eyepiece grid and normalized to the volume of muscle sampled (area of section × section thickness), and averaged across sections.

**Culture of Bone Marrow Derived Macrophages**

Bone marrow-derived macrophages (BMDM) from WT and uPAR<sup>−/−</sup> mice were cultured as described previously (48). Femurs and tibias were collected, cleaned of all tissue and bone marrow was flushed using Bone Marrow Medium (BMM) comprised of DMEM supplemented with 10% heat inactivated FBS, 10% L-929 cell-conditioned medium (source of macrophage colony stimulating factor), 2mM L-glutamine and 1% penicillin/streptomycin (Sigma, St. Louis, MO). Cells were cultured in BMM in a humidified 10% CO<sub>2</sub> atmosphere at 37°C for 4 days. After 4d in culture, greater than 90% of the cells were F4/80 positive as determined by flow cytometry, indicating that the cultures contained predominantly mature macrophages.

**Chemotaxis Assays**

Migration of BMDM from WT and uPAR<sup>−/−</sup> mice were analyzed using a 48-well modified Boyden chemotaxis chamber (Neuro Probe; Gaithersburg, MD) with polycarbonate membranes (5µm pore size) separating upper from lower wells. fMLP
(10^{-7} \text{ M}) diluted in serum free media (DMEM + 1\% BSA + 1\% penicillin/streptomycin) was placed in the lower wells to induce chemotaxis. BMDM were loaded in the upper wells (2.5 \times 10^4 \text{ cells}) and migration was allowed to proceed for 90 minutes at 37\degree C in 10\% CO_2. After non-migrated cells were removed from the upper surface of the filter by scraping, those that migrated to the lower surface of the filters were fixed in methanol and Wright-Giesma stain (Sigma). Results were quantified as the number of stained cells in 5 randomly selected fields per well (\times20 magnification). Data were expressed relative to spontaneous cell migration (number of cell migrated when lower wells contained media alone). Each condition was replicated in 8 wells/experiment in 3 independent experiments.

\textit{RT-PCR}

TA muscles were homogenized and total RNA was extracted using the RNeasy RNA Isolation Kit (Qiagen), following the manufacturers instructions. cDNA was synthesized from 1\mu g of RNA using the Thermoscript RT-PCR System (Invitrogen, Carlsbed, CA). Amplification reactions were performed with 1\mu M primers, 1\mu l cDNA, 3.5mM MgCl_2 and 0.2mM dNTP for a 25\mu l final volume in a GeneAmp 9700 thermal cycler (PE Applied Biosystems, Foster City, CA). Primer sequences are as follows: uPAR; Forward, 5'-GCA GTG TGA GAG TAA CCA GAG CT-3', Reverse, 5'-CCA CAG CCT CGG GTG TAG TCC T-3'; GAPDH; Forward, 5'-ACC ACA GTC CAT GCC ATC AC-3', Reverse, 5'-TCC ACC ACC CTG TTG CTG GTA-3'. cDNA was analyzed by separation on a 1.5\% agarose gel and visualized by ethidium bromide staining.
Satellite Cell Isolation, Culture and Fusion Index

Primary cultures were derived from neonatal hind-limb muscles from WT and uPAR−/− mice essentially as described (3). Briefly, muscle was finely minced, digested in 1% Pronase (Calbiochem, San Diego, CA) and cells were released by trituration. Pooled cells were filtered (100 µm), centrifuged and resuspended before plating on 35mm cell culture dishes. Myoblasts were grown in selective proliferation medium (Ham's F10, 20% fetal bovine serum, 5 ng/ml bFGF, 1% penicillin/streptomycin) (38) on entactin/collagen/laminin (ECL; Upstate Biotechnology, Charlottesville, VA) coated dishes in a humidified 5% CO₂ atmosphere at 37°C until ~75% confluent. Immunofluorescence analysis demonstrated that greater than 90% of myoblasts were positive for MyoD. Myoblasts were trypsinized and replated at a concentration of 2 × 10⁵ cells per well in ECL coated 6-well dishes. After 2 hr, the medium was switched to a low serum, low mitogen differentiation medium (DMEM, 2% horse serum, 1% penicillin/streptomycin) (Gibco, Carlsbed, CA). After 72 hr in differentiation medium, cells were fixed and immunostained with an antibody against MyoD (1:20 dilution, 1 hour incubation; Santa Cruz Biotechnology, Santa Cruz, CA) to label muscle cell nuclei and phalloidin (1:50 dilution, 15 minute incubation; Sigma, St. Louis, MO) to visualize myotubes. The percentage of nuclei in myotubes with ≥ 3 nuclei, were counted as previously described (20). Myotubes from 3 wells were counted (5 fields at ×40 magnification/well) and cell isolations were performed three times for each mouse strain.
Statistics

Values are reported as means ± standard error (SE). Data were compared across different mouse strains and time points using a two-way ANOVA. Holm-Sidak post hoc test was performed at a 0.05 confidence level to indicate statistical significance (Sigma-Stat, Richmond, CA).

Results

uPAR Expression

To confirm that uPAR is expressed in skeletal muscle following injury, RNA was isolated from injured muscle and used for RT-PCR analysis. In WT mice, uPAR mRNA was not detectable in uninjured control muscle, but was elevated at 1 and 3d post-injury (Figure 1A). As expected, injured muscle from uPAR−/− mice showed no expression of uPAR mRNA. Immunohistochemical methods were used to visualize uPAR protein localization within injured muscle from WT mice (Figure 1B). uPAR protein was not detectable in WT uninjured control muscle. Muscle injury was associated with the upregulation of uPAR protein in WT muscle at 1 and 3d post-injury and appeared to be localized between damaged fibers, at sites of inflammatory cell accumulation. As expected, uPAR−/− mice showed no uPAR protein expression within injured muscle (not shown).

Inflammatory Cells

One purpose of this study was to test the hypothesis that uPAR is required for accumulation of inflammatory cells following muscle injury. Immunohistochemical methods were used to quantify inflammatory cell accumulation in injured muscle of WT
and uPAR−/− mice. Uninjured muscles from both WT and uPAR−/− mice contained few neutrophils or macrophages (not shown). Neutrophil accumulation peaked 1d post-injury in both WT and uPAR−/− muscle, while macrophage accumulation peaked between 3 and 5d post-injury, before both cell types returned to uninjured levels by 10d post-injury (Figure 2). The number of neutrophils or macrophages did not differ significantly between strains at any time point, indicating that uPAR is not required for the accumulation of either neutrophils or macrophages in injured skeletal muscle.

Muscle Regeneration

Another purpose of this study was to test the hypothesis that uPA-R is required for efficient regeneration following muscle injury. Cryosections stained with hematoxylin and eosin were used to assess changes in muscle morphology following injury (Figure 3A). Uninjured muscle demonstrated no differences in fiber area between WT (1,340 ± 80 µm²) and uPAR−/− (1,440 ± 110 µm²) mice, suggesting that there were no differences in muscle fiber development between strains. At 3d post-injury, the injury protocol elicited damage to greater than 95% of the area of EDL muscle cross-sections in both WT and uPAR−/− mice and the amount of damage was not different between strains (Figure 3B). At 5d post-injury, both strains demonstrated formation of small central nucleated fibers, which indicate muscle fiber regeneration. The number of regenerating fibers was quantified and was not different between WT and uPAR−/− mice (WT; 792 ± 86/mm², uPAR−/−; 747 ± 87/mm²). Damaged area of muscle sections declined in both WT and uPAR−/− mice from 5 to 40 days post-injury associated with increased regenerating fiber area. There was no difference between strains in either the reduction in damaged area over time, or the increase in regenerating fiber area. This
morphological analysis indicated that uPAR is not required for efficient regeneration of skeletal muscle.

An anti-mouse uPAR blocking antibody was administered to WT mice to verify that uPAR is not required for macrophage accumulation or efficient muscle regeneration. In mice treated with the uPAR antibody, the number of macrophages at 5 days post-injury (39,173 ± 3,442/mm³), number of regenerating fibers (793 ± 90/mm²), and regenerating fiber area (300 ± 100 µm²) was not different from untreated WT and uPAR⁻/⁻ mice. These data further support the hypothesis that uPAR is not required for muscle regeneration.

**Macrophage Migration**

Since macrophage accumulation in injured muscle was impaired in uPA⁻/⁻ mice (22, 25), but not in uPAR⁻/⁻ mice (Figure 2), a modified Boyden chemotaxis chamber was used to further examine the role of uPAR in macrophage chemotaxis in vitro (Figure 4). We first compared spontaneous cell migration (cell movement from upper wells towards media alone in lower wells), and found no significant difference between WT (10.2 ± 1.1 cells/field) and uPAR⁻/⁻ (10.4 ± 0.9 cells/field) macrophages (used as controls for subsequent experiments). We next placed fMLP in the lower wells of the chemotaxis chamber, since fMLP is known to be a potent chemo-attractant for macrophages (40). fMLP increased migration of both WT and uPAR⁻/⁻ macrophages into the lower wells of the chamber approximately 3-fold, with no difference between strains. To confirm that the fMLP-induced increase in cell migration was the result of chemotaxis rather than a general stimulatory effect on cell migration, fMLP was added to cell suspensions in the upper wells as well as to the bottom wells. This treatment reduced the number of
migrated cells of both strains to control levels. The lack of a difference in chemotaxis in vitro between WT and uPAR−/− macrophages is consistent with the in vivo data showing no difference in macrophage accumulation following muscle injury.

**Satellite Cell Fusion**

Previous studies have demonstrated that antibodies against uPA or uPAR impaired satellite cell migration and fusion in vitro (5, 12, 15). In the present study, isolated satellite cells from WT and uPAR−/− neonatal mice were induced to fuse in differentiation medium for 72 hr. The resulting myotubes were stained and the percentage of nuclei in myotubes with ≥3 nuclei were counted (Figure 5). In contrast to previous studies using antibodies against uPAR, cells from uPAR−/− mice demonstrated no impairment in fusion into multinucleated myotubes when compared to WT cells. The ability of uPAR−/− satellite cells to fuse as efficiently as WT cells is consistent with the in vivo morphological data indicating that uPAR is not essential for muscle regeneration.

**Discussion**

Previous studies have indicated that the receptor for uPA (uPAR) plays an important role in inflammatory cell migration (18, 28, 35, 39) and the proliferation, migration and fusion of satellite cells (5, 12, 15), all of which are thought to be required for muscle repair (17, 24, 42). Muscle regeneration in uPA−/− mice is severely impaired, and the impaired healing is associated with a lack of macrophage accumulation following muscle injury (22, 25). In total, these data provided the rationale for the hypothesis of the present study, that uPAR is required for accumulation of inflammatory cells and efficient muscle regeneration. However, we provide evidence that uPAR is not
required for the accumulation of inflammatory cells or for the efficient regeneration of skeletal muscle following cardiotoxin-induced injury. Despite elevated expression of uPAR in injured skeletal muscle from WT mice (Figure 1), accumulation of neutrophils and macrophages in injured muscle of uPAR\(^{-/-}\) mice did not differ from that in WT mice (Figure 2). In addition, muscle regeneration did not differ between uPAR\(^{-/-}\) and WT mice (Figure 3). These results indicate that uPAR is not required for the accumulation of inflammatory cells or for subsequent regeneration of skeletal muscle, and suggest that uPA acts independently of its receptor to mediate events critical for muscle regeneration.

Previous studies using uPAR\(^{-/-}\) mice have indicated that uPAR plays important roles in directed cell migration in several in vivo models of inflammation and infection, including peritonitis, meningitis and pneumonia (18, 28, 35, 39). During thioglycollate-induced peritonitis, leukocyte accumulation was impaired at early time points, but not at later time points (13, 28). The localization of uPAR at the leading edge of migrating inflammatory cells is thought to localize proteolytic activity for directed migration through the extracellular matrix (14, 19). Additionally, several non-proteolytic functions for uPAR during cell migration have been described. uPAR may serve as a chemotactic molecule, as uPAR can be cleaved by plasmin or uPA to form a soluble component (suPAR) that can bind fMLP receptors to initiate directed migration (36). uPAR is linked to the plasma membrane by glycosylphosphatidylinositol (GPI)-anchors, and therefore lacks a direct connection to the cell interior. However, uPAR interactions with transmembrane integrins, fMLP or epidermal growth factor receptors may initiate
intracellular signals important for cell migration, possibly through PI3K/AKT, ERK1/2, Rho, Rac or cdc42 (2, 4, 9, 34, 37).

Based on the evidence supporting an important role for uPAR in cell migration, we expected that uPAR would contribute to cell migration during muscle inflammation and repair. We found that uPAR is upregulated in muscle at 1 and 3 days post-injury, and returned to control levels by 5 days post-injury. The time course of uPAR gene expression correlates with neutrophil accumulation in injured muscle, raising the possibility that elevated uPAR expression in WT muscle following injury may reflect expression by neutrophils. However, uPAR deficiency did not alter accumulation of neutrophils or macrophages in injured muscle, or efficient muscle regeneration. Since the results of our study demonstrate that uPAR is not required for skeletal muscle inflammation or repair, the importance of uPAR may depend on the tissue examined and the model of inflammation utilized. In addition, in the current model of muscle injury, there may have been compensatory mechanisms for the loss of uPAR, allowing cell migration and efficient muscle regeneration to occur. However, no such mechanism has yet been identified.

Previous in vitro studies have indicated that uPAR may be required for various physiological functions of satellite cells. Antibodies against uPA and uPAR impaired satellite cell migration and fusion into multi-nucleated myotubes (5, 12, 15). In addition, a non-catalytic fragment of uPA that retains the capability to bind uPAR also impaired satellite cell migration and fusion (5, 12, 47). The authors interpreted these data as supporting a role for uPAR bound uPA during cell migration. In the present study, we demonstrate that the fusion of cultured satellite cells into multinucleated myotubes was
not different between cells isolated from WT and uPAR\textsuperscript{−−} mice (Figure 5), and that formation of regenerating fibers is not different in injured muscle of WT and uPAR\textsuperscript{−−} mice, both of which indicate that uPAR is not required for fusion. Similarly contrasting results between studies using antibodies against PAI-1 in vitro and those using a gene knockout model of PAI-1 in vivo have been reported for satellite cell fusion. While others have found that antibodies against PAI-1 resulted in impaired satellite cell migration and fusion \textit{in vitro} (12), we found accelerated formation of regenerating fibers in PAI-1\textsuperscript{−−} mice compared with WT mice \textit{in vivo} (22). Potential explanations for the difference between previous and current results include unintended effects of molecular treatments designed to block uPAR, including possible steric inhibition of satellite cell migration and fusion by bound molecules, or triggering of uPAR mediated events by the binding of these molecules. In addition, the difference between studies could be explained by the availability of compensatory mechanisms induced in uPAR\textsuperscript{−−} cells, allowing satellite cell fusion despite the absence of putative uPAR-mediated mechanisms that may be involved in fusion of WT cells.

Similar to skeletal muscle, healing of other tissues does not appear to require uPAR. Combined deficiency of the uPAR gene and the tissue-type plasminogen activator (tPA) gene did not result in impaired wound healing in skin, indicating that neither uPAR nor tPA are required for skin healing (6). In contrast, combined deficiency of uPA and tPA resulted in a profound impairment in skin healing, consistent with our previous findings that uPA is required for muscle healing (6, 22). In addition, healing of arterial vessel injuries in uPAR\textsuperscript{−−} mice was not impaired, nor was the migration of smooth muscle cells during this process, indicating that uPAR is not required for vessel
healing (10). Despite evidence supporting a role for uPAR in the migration of various cells during tissue injury, uPAR does not appear to be required for the efficient repair of skin, blood vessels or skeletal muscle.

Our previous and current studies indicate that uPA promotes macrophage migration in injured muscle and efficient muscle repair independently of uPAR, possibly as a soluble factor. Soluble uPA may fulfill all the functions required of uPA during muscle repair. Soluble uPA can activate plasmin, which in turn can activate a subset of matrix metalloproteases, to cleave ECM molecules and "clear a path" for migrating cells (10, 23). Soluble uPA can also directly activate HGF through cleavage of the inactive single-chain to an active two-chain form \textit{in vitro} (26, 32). HGF is thought to play an important role in stimulating satellite cell proliferation and migration, and may promote macrophage migration as well (1, 16, 30). Finally, soluble uPA activation of plasmin can result in release of growth factors from their storage sites in the ECM (43). uPA may promote activity of both HGF and FGF in this manner, which in turn, may play key roles in the regulation of satellite cell activity and inflammatory cell recruitment (44, 49). We are currently investigating the precise mechanisms by which uPA promotes muscle regeneration and the cellular sources of uPA (e.g. satellite cells, neutrophils, macrophages, fibroblasts) during this process.

In summary, previous studies provided evidence that uPAR plays an important role in the migration of inflammatory cells, and the proliferation, migration and fusion of satellite cells, all of which are thought to be required for muscle repair. The major findings of the present study were that genetic deficiency of uPAR does not influence the accumulation of neutrophils and macrophages following muscle injury, or
subsequent muscle regeneration. In addition, cultured satellite cells from uPAR−/− mice demonstrated efficient fusion. Previous studies on uPA and muscle regeneration demonstrated that uPA is required for macrophage accumulation in injured muscle and for formation of regenerating muscle fibers (22, 25). Thus, uPA appears to regulate macrophage accumulation in injured muscle and muscle regeneration independent of its receptor.

Acknowledgements

The authors would like to thank William Billich for technical assistance.

Grants

S. C. Bryer was supported by the National Aeronautics and Space Administration: Graduate Student Research Program (# NNG04GN53H).
Figure 1

uPAR Expression Elevated in WT Muscle Following Injury

(A) RT-PCR analysis demonstrated that uPAR mRNA levels were elevated in WT TA muscles at 1 and 3 days post-injury before returning to uninjured levels by 5 days. (B) Immunohistochemical analysis demonstrated that uPAR protein levels were elevated in WT EDL muscle at 1 and 3 days post-injury and appear to be localized to sites of inflammation before declining at 5 days. Images are representative of n = 3 muscles examined per strain for each condition.

Figure 2

Inflammatory Cell Accumulation Not Impaired In Muscle From uPAR<sup>−/−</sup> Mice

Inflammatory cell accumulation following cardiotoxin-induced injury was quantified using immunohistochemical analysis with antibodies against the Ly6G (neutrophil) and F4/80 (macrophage) antigens. No difference in inflammatory cell accumulation in injured EDL muscles between WT and uPAR<sup>−/−</sup> mice was seen at any time point following cardiotoxin-induced injury. Data are means ± SE; n=8, 8, 8, and 6 (WT); n=7, 8, 8 and 6 (uPAR<sup>−/−</sup>) at 1, 3, 5 and 10d post-injury.
Figure 3

Regeneration Not Impaired in Muscle From uPAR<sup>−/−</sup> Mice

(A) Hematoxylin and eosin-stained EDL muscle cryosections from uninjured control (Con) WT and uPAR<sup>−/−</sup> mice and following injury (3-40 days). Note the appearance of centronucleated fibers by 5 days post-injury in muscle from both WT and uPAR<sup>−/−</sup> mice, indicative of regenerating fibers. (B) Damaged area, estimated in each muscle section by subtracting the summed area of normal and regenerating fibers from the total area of each field, was not different between muscle from WT and uPAR<sup>−/−</sup> mice from 3 to 40 days post-injury. (C) Regenerating fiber area is not different between muscle from WT and uPAR<sup>−/−</sup> mice from 5 to 40 days post-injury. Data are means ± SE. (*, p ≤ 0.05 compared to uninjured (within strain). n = 8, 8, 8, 6, 7 and 4 (WT); n = 6, 7, 8, 6, 7 and 4 (uPAR<sup>−/−</sup>) for Control and 3, 5, 10, 20 and 40 days post-injury.

Figure 4

Chemotaxis Is Not Different in uPAR<sup>−/−</sup> Macrophages Compared to WT

fMLP induced chemotaxis was not different between WT and uPAR<sup>−/−</sup> macrophages as determined using a Boyden-type chemotaxis chamber. Data are means ± SE (n = 24 wells per condition).
Figure 5

Satellite Cells From uPAR<sup>−/−</sup> Mice Demonstrate Efficient Fusion

Isolated satellite cells cultured in differentiation media for 72 hours were stained for f-actin (green) and MyoD (red) to visualize myotubes and muscle nuclei, respectively. Fusion was quantified as the percentage of nuclei in myotubes with ≥ 3 nuclei. No difference in fusion was found between cells from WT and uPAR<sup>−/−</sup> mice. Data are means ± SE (n = 3 fusion experiments performed for each strain).
References


15. Fibbi G, Barletta E, Dini G, Del Rosso A, Pucci M, Cerletti M, and Del Rosso M. Cell invasion is affected by differential expression of the urokinase plasminogen


Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>uPAR</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uPAR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uPAR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Uninjured  1d  3d  5d

B

WT

H & E  uPAR Ab

1d  3d  5d
Figure 2

[Bar chart showing neutrophil and macrophage counts over days post-injury for WT and uPAR+ conditions.]

OPTIONS

- Neutrophil #/mm³
- Macrophage #/mm³

Days Post-Injury:

1d, 3d, 5d, 10d
Figure 4

[Graph showing comparison of chemotaxis with different conditions]
Figure 5

![Image of fluorescence microscopy results showing WT and uPAR knockout cells with quantitative fusion index graph.](Image137x215 to 475x665)

- **WT**
- **uPAR**

Fusion Index (%)

0 50 100

≥ 3 Nuclei