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Macrophage-Specific Expression of Urokinase-Type Plasminogen Activator Promotes Skeletal Muscle Regeneration

Margaret L. Novak,* Scott C. Bryer,* Ming Cheng,* Mai-Huong Nguyen,* Kevin L. Conley,* Andrew K. Cunningham,† Bing Xue,† Thomas H. Sisson,† Jae-Sung You,‡ Troy A. Hornberger,‡ and Timothy J. Koh*

Macrophages (Mp) and the plasminogen system play important roles in tissue repair following injury. We hypothesized that Mp-specific expression of urokinase-type plasminogen activator (uPA) is sufficient for Mp to migrate into damaged muscle and for efficient muscle regeneration. We generated transgenic mice expressing uPA only in Mp, and we assessed the ability of these mice to repair muscle injury. Mp-only uPA expression was sufficient to induce wild-type levels of Mp accumulation, angiogenesis, and new muscle fiber formation. In mice with wild-type uPA expression, Mp-specific overexpression further increased Mp accumulation and enhanced muscle fiber regeneration. Furthermore, Mp expression of uPA regulated the level of active hepatocyte growth factor, which is required for muscle fiber regeneration, in damaged muscle. In vitro studies demonstrated that uPA promotes Mp migration through proteolytic and nonproteolytic mechanisms, including proteolytic activation of hepatocyte growth factor. In summary, Mp-derived uPA promotes muscle regeneration by inducing Mp migration, angiogenesis, and myogenesis. The Journal of Immunology, 2011, 187: 000–000.

Tissue repair following acute injury involves overlapping phases of inflammation, new tissue formation, and subsequent remodeling. Efficient progression through each phase of healing requires the coordinated response of different cell types and molecular effectors. One such cell type, the macrophage (Mp), is involved in each phase of tissue healing and is thought to play an important role in the repair of a variety of tissues (1–4). First, Mp are thought to help kill pathogens as well as clear damaged tissue and necrotic and apoptotic cells. In addition, Mp can produce a spectrum of chemoattractants, growth factors, and proteinases that promote angiogenesis, tissue repair, and remodeling. However, much remains to be learned about the specific molecular mechanisms that regulate Mp accumulation in damaged tissue as well as the mechanisms by which Mp promote tissue repair.

The hypothesis of the current study is that Mp-specific uPA expression promotes both Mp migration into damaged muscle and subsequent muscle fiber regeneration. We tested this hypothesis by cross-breeding Mp-specific uPA overexpressing mice with uPA-null mice.
null mice to generate mice that express uPA only in Mp. We pos-
tulated that Mp-only expression of uPA would rescue Mp accu-
mulation, angiogenesis, and muscle regeneration following injury in
otherwise uPA-null mice, in part via proteolytic activation of HGF.

Materials and Methods

Mice and breeding

WT and uPA-null mice on a C57BL/6 background were obtained from The
Jackson Laboratory and bred in the animal facility at the University of
Illinois (Chicago, IL). Mp-specific uPA-overexpressing mice were gener-
at with a transgene that used the human scavenger receptor A promoter to
drive expression of the full-length mouse uPA gene (SR-uPA mice, pro-
vided by Dr. D. Dichek, University of Washington, Seattle, WA). These
mice were backcrossed onto a C57BL/6 background for more than eight
generations. For the current studies, mice were also generated that
expressed uPA only in Mp by crossing SR-uPA mice with uPA+/− mice.
The breeding scheme (Fig. 1A) involved first breeding uPA-null males
(uPA−/− SR−/−) with SR-uPA hemizygote females (uPA+/− SR+/−) to
obtain offspring positive for the SR-uPA transgene and heterozygous for
the en-
dogenous uPA gene (uPA+/− SR+/−). Female offspring with this genotype
were then bred with male uPA-null mice to produce Mp-only uPA-
expressing mice (uPA−/− SR+/−), uPA-null mice (uPA−/− SR−/−), Mp-
specific uPA-overexpressing mice (uPA+/− SR−/−), and uPA-heterozygote
(uPA+/− SR+/−) littersmates. All experiments in this study were performed
on mice aged 10–12 wk. The Animal Care Committee at the University of
Illinois approved all experimental procedures.

Genotyping

Mice were genotyped by standard methods using multiplex PCR analysis
of ear punch DNA. Primer sequences for the WT uPA gene were 5′-TCT-
GGAGAGCCCTTATCTG-3′ (forward) and 5′-CTCCCTCAAGATGT-
GGATTG-3′ (reverse), and the mutant uPA gene was amplified by the primer 5′-
CAGCAGACTAGTGAGACGTG-3′ (forward) and 5′-GCTCCTATAATCCTGGAGAG-
GA TGAA-3′ (reverse). To differen-
tiate between uPA+/− SR+/− and uPA−/− SR−/− genotypes, which could not
then be distinguished using only the ear punch DNA, we used PCR analysis of
uPA mRNA expression in the kidney. Kidney mRNA was isolated using
the RNeasy kit (Qiagen) and then reverse transcribed using the Thermoscript
RT-PCR System (Invitrogen). Amplification reactions were performed with
primer sequences 5′-GCTCTTATAATCCTGGAGAGATGAA-3′ (forward) and
5′-ACCTGTCCTTTCGAGCTTCTTCCTC-3′ (reverse).

Muscle injury

Extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were
injured using cardiotoxin as described previously (5, 11). Briefly, en-
thelial cells were labeled with a CD31 Ab (1:100 dilution; BD Bioso-
ciences), neutrophils were labeled with a Ly6G Ab (1:100 dilution;
BD Biosciences), and macrophages with a F4/80 Ab (1:100 dilution;
Serotec). Following incubation with these primary Abs, sections were in-
cubated with biotinylated mouse adsorbed anti-rat IgG (1:200 dilution;
Vector Laboratories) and avidin D HRP (1:1,000 dilution; Vector Labora-
tories). All sections were then developed using the 3-amin-9-ethylcarbazole
kit (Vector Laboratories) and mounted in Vectashield mounting medium. For
angiogenesis, image analysis software (Scion Image) was used to quantify
the percentage of CD31-stained area relative to the total field area in six ×20
fields from each muscle. For inflammatory cells, the number of positively
stained cells were counted in six ×20 fields for each muscle and normalized
to the volume of muscle sampled (area of field × section thickness).

HGF protein analysis

HGF protein levels were assessed by affinity purification of TA muscle
homogenates, followed by Western blotting as described previously (10).
Briefly, the full volume of muscle homogenate from each sample was
incubated overnight with heparin–agarase beads at 4°C with rotation.
Beads were washed and then boiled in 20 μl SDS sample buffer to elute
bound protein. One-half the total volume of each sample was saved on each
of two SDS-PAGE gels and transferred to nitrocellulose membranes.
After transfer, membranes were blocked in 5% milk for 1 h. Membranes
were incubated with primary Ab that recognizes the α-chain of HGF
(Santa Cruz Biotechnology) and then incubated with secondary Ab conju-
gated to HRP (Pierce). Protein bands were detected using ECL (GE
Healthcare), and band densities were determined by image analysis.

Culture of bone marrow-derived macrophages

Bone marrow-derived Mp were cultured as described previously (11).
Briefly, mouse bone marrow cells were flushed from femurs and tibias and cul-
inated in DMEM supplemented with 10% heat-inactivated FBS, 10% L-
929 cell-conditioned medium (source of M-CSF) 2 mM L-glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich) in a humidified 10% CO2
atmosphere at 37°C. After 4 d in culture, cells were >95% F4/80 positive as
determined by flow cytometry, confirming that the majority of the cultured
cells were mature Mp.

Migration assays

Mp migration was analyzed using a 48-well modified Boyden chamber
(NeuroProbe) with uncoated polycarbonate membranes (5-μm pore size)
separating upper from lower wells. Mp were loaded in the upper well of
the filters (2.5 × 105 cells), and MCAF-1 (10 ng/ml) was placed in the lower
wells to induce migration. Cells and all reagents were diluted in serum-free
DMEM. Recombinant mouse uPA and/or PAI-1, the amino terminal frag-
ment (ATF) of uPA (Molecular Innovations), uPA receptor (uPAR) or HGF-
blocking Abs (R&D Systems), recombinant mouse HGF or fibroblast growth
factor (FGF) (R&D Systems), the PI3K inhibitors LY294002 and wart-
mannin, and the MEK1 inhibitor PD98059 (Calbiochem) were added to the
upper wells as indicated. The recombinant mouse HGF used in these
experiments was in the active form. Migration was allowed to proceed for
90 min at 37°C in 10% CO2. Cells that migrated to the lower surface of the
filters were fixed in methanol and stained with Wright-Giesma (Sigma-
Aldrich). The number of stained cells was counted in five randomly selected
fields per well (×20 magnification). Data were expressed relative to spon-
taneous cell migration (number of cells migrated when lower wells con-
tained media alone). Each condition was replicated in at least eight wells per
experiment in at least three independent experiments.

Western blotting

Bone marrow Mp were pretreated with LY294002 (10 μM), PD98059 (10
μM), or vehicle for 30 min and then stimulated with uPA (10 ng/ml) for
an additional 30 min. Mp were homogenized in buffer containing 40 mM
Tris (pH 7.5), 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 25 mM
β-glycerophosphate, 25 mM NaF, 1 mM Na3VO4, 10 μg/mI leupeptin, and
1 mM PMSF. After homogenates had been centrifuged, the superna-
tant was mixed with concentrated reducing SDS buffer and separated on SDS-
PAGE gels. Proteins were transferred to a polyvinylidene difluoride membrane, and membranes were blocked with 5% powdered milk and then incubated overnight at 4°C with primary Abs against total or phosphorylated protein kinase B (PKB) (473) or ERK1/2 (Upstate Biotechnology). Membranes were washed and then probed with HRP-conjugated secondary Ab. Following another wash, blots were developed using an ECL kit (Amersham Biosciences). Membranes were then stained with Ponceau S to verify equal loading in all lanes. Densitometric measurements were carried out using the public domain National Institutes of Health Image program (ImageJ).

Statistical analysis

Values are reported as means ± SE. Data were compared across mouse genotypes and/or treatment conditions using ANOVA. The Student-Newman-Keuls test was used for post hoc analysis of ANOVA tests found to be statistically significant. The 0.05 level was taken to indicate statistical significance.

Results

Generation of mice that express uPA only in Mp

To generate mice that express uPA only in Mp, we bred SR-uPA (uPA+/+SR+/0) mice with uPA-null (uPA−/−SR−/−) mice following the breeding scheme in Fig. 1A. SR-uPA mice have Mp-specific uPA overexpression and have been described in detail elsewhere (23, 24). Mice were genotyped using multiplex PCR analysis of ear punch DNA. Our primers could not differentiate the WT uPA genomic DNA from the SR-uPA transgene, and therefore, ear punch DNA was not sufficient to distinguish the uPA−/−SR−/− from the uPA+/−SR+/0 genotype. To differentiate between these genotypes, we used PCR analysis of uPA mRNA expression in the kidney. In this analysis, the uPA+/−SR+/0 genotype exhibited kidney uPA expression, but the uPA−/−SR+/0 genotype did not. Genotyping from a representative litter is shown in Fig. 1A, and over all litters produced, the genotypes followed the expected Mendelian ratios. See Table I for a description of genotypes generated for this study.

As expected, gel zymography demonstrated that uPA activity was absent in both uninjured and injured muscle of uPA−/−SR−/− mice and was absent or barely detectable in uninjured muscle of the other genotypes (data not shown). To determine whether Mp-specific expression of uPA influences the level of uPA activity following muscle injury, uPA activity was assessed in damaged muscle of uPA+/−SR+/0 (WT), uPA+/−SR−/− (uPA heterozygote), uPA+/−SR−/0 (uPA null), and uPA+/−SR−/− littermates. Mice were genotyped using multiplex PCR analysis of ear punch DNA along with PCR analysis of uPA mRNA expression in the kidney. B. TA muscles were collected at 5 d postinjury, and uPA activity was determined by zymography. Top panel, Representative zymogram. Bottom panel, Densitometry results normalized to uPA+/+SR−/0. No detectable uPA activity was seen in uPA-null samples. Bars are means ± SE; n = 4 muscles/group. *p < 0.05, mean value significantly larger than that for uPA+/+SR−/0 mice.

FIGURE 1. Breeding scheme and muscle uPA activity for Mp-only uPA-expressing mice. A, uPA+/−SR−/− males were mated with uPA+/+SR+/0 females to obtain uPA+/−SR+/0 offspring. Female offspring were then bred with uPA−/−SR−/− males to produce uPA+/−SR+/0, uPA−/−SR+/0, uPA+/−SR−/0, and uPA+/−SR−/0 litters. Mice were genotyped using multiplex PCR analysis of ear punch DNA along with PCR analysis of uPA mRNA expression in the kidney. B, TA muscles were collected at 5 d postinjury, and uPA activity was determined by zymography. Top panel, Representative zymogram. Bottom panel, Densitometry results normalized to uPA+/−SR−/0. No detectable uPA activity was seen in uPA-null samples. Bars are means ± SE; n = 4 muscles/group. *p < 0.05, mean value significantly larger than that for uPA+/+SR−/0 mice.
(uPA heterozygote with uPA overexpression in Mp), and uPA−/−SR−/− (Mp-only uPA-expressing) mice. At 5 d postinjury, uPA activity was robust in both uPA+/+SR+/+ and uPA+/−SR−/− muscles and was not different between these genotypes (Fig. 1B). uPA activity was increased in muscles of uPA+/−SR−/− compared with the former strains, indicating that Mp-specific overexpression enhances uPA activity in damaged muscle. Finally, uPA activity in damaged muscle of uPA−/−SR−/− mice was not different from uPA+/+SR+/+, indicating that Mp-only expression was sufficient to restore uPA activity in otherwise uPA-null mice.

**Mp-specific expression of uPA promotes muscle regeneration**

Mice lacking uPA exhibit a profound impairment in muscle regeneration (5, 9). To determine whether Mp-specific expression of uPA is sufficient to promote muscle regeneration, we assessed muscle regeneration in uPA+/+SR−/−, uPA+/−SR−/−, uPA−/−SR−/−, and uPA+/−SR−/− mice. Regeneration was measured as the number of centrally nucleated muscle fibers per square millimeter (Fig. 2B, top panels) and as the percentage of the total muscle area that was regenerating (Fig. 2B, bottom panels). At 5 and 10 d postinjury, muscles from uPA+/+SR−/− mice exhibited robust regeneration, and muscles from uPA−/−SR−/− mice exhibited a complete lack of regeneration (Fig. 2), confirming previous studies (5, 9). Muscles from uPA+/−SR−/− mice showed regeneration that was not different from uPA+/+SR−/− mice (Fig. 2), indicating that a single allele of the uPA gene is adequate to achieve normal regeneration. Muscles from uPA+/−SR−/− mice showed a greater number of regenerating muscle fibers and a larger regenerating area than either uPA+/+SR−/− or uPA+/−SR−/− mice, indicating that increasing uPA production by Mp enhances new muscle fiber formation in otherwise WT mice. The number of regenerating fibers, as well as the percent regenerating area, in muscles from uPA−/−SR−/− mice was not different from uPA+/+SR−/− or uPA+/−SR−/− mice, indicating that Mp-only expression of uPA was sufficient to induce myogenesis in otherwise uPA-null mice. Because serum uPA is elevated in SR-uPA−/− mice compared with their SR-uPA+/+ counterparts (25), we also gave i.v. injections of uPA to uPA+/−SR−/− mice to determine whether an increase in circulating uPA is sufficient to induce muscle fiber regeneration. However, i.v. uPA did not rescue uPA+/−SR−/− muscle regeneration, even at estimated plasma concentrations up to 25-fold greater than those observed in SR-uPA+/+ mice (injection of 5 μg uPA results in expected serum concentration of ~2500 ng/ml uPA versus ~100 ng/ml uPA observed in SR-uPA−/− mice).

Whereas age-related cardiac fibrosis has been previously reported in SR-uPA mice (24), in the current study, trichrome staining revealed no evidence of fibrosis in uninjured muscles of these transgenic mice (Supplemental Fig. 1). Furthermore, although an increase in collagen-stained area was observed after cardiotoxin injury in muscles of both WT and SR-uPA mice, muscles of SR-uPA mice exhibited significantly less fibrosis than those of WT at 10 d postinjury (Supplemental Fig. 1). These data strongly argue against a profibrotic role for the SR-uPA transgene in skeletal muscle, at least with the 10- to 12-wk-old mice used in this study. Finally, uninjured muscle fiber cross-sectional area was not different between genotypes (uPA+/+SR−/−, 1166 ± 76 μm²; uPA+/−SR−/−, 1278 ± 119 μm²), indicating that muscle fiber development was not altered by the SR-uPA transgene.

**Mp-specific expression of uPA promotes Mp accumulation in damaged muscle**

Previous studies have shown that impaired muscle regeneration in uPA-null mice is associated with a lack of Mp accumulation (5, 9). To determine whether Mp-specific expression of uPA promotes Mp accumulation in damaged muscle, we assayed i.m. Mp numbers by immunohistochemical analysis. At 5 d postinjury, the time of peak Mp accumulation in muscles of WT mice (11, 26), muscles from uPA+/+SR−/− and uPA+/−SR−/− mice contained a large number of Mp that was not different between genotypes (Fig. 3A, Supplemental Fig. 2). In contrast, muscles from uPA+/−SR−/− mice exhibited a nearly complete loss of Mp accumulation. Muscles from uPA+/−SR−/− mice showed a greater number of Mp than either uPA+/+SR−/− or uPA+/−SR−/− mice. Finally, muscles from uPA+/−SR−/− mice exhibited Mp accumulation that was not different from uPA+/+SR−/− or uPA+/−SR−/− mice, indicating that expression of uPA exclusively in Mp is sufficient to restore Mp accumulation in otherwise uPA-null mice. In contrast to Mp accumulation, neutrophil numbers were not different among genotypes at 1 d postinjury (Fig. 3B), the time of peak neutrophil accumulation in WT mice (5, 26). Finally, i.v. uPA treatment did not restore Mp accumulation in uPA+/−SR−/− mice, indicating that increasing serum uPA was not sufficient to rescue Mp accumulation.

**Mp-specific expression of uPA promotes angiogenesis in injured muscle**

Because both uPA and Mp have been shown to promote angiogenesis in ischemic tissues and tumors (12, 22, 27, 28), we used CD31 immunolabeling to determine whether Mp-specific expression of uPA enhances angiogenesis in damaged muscle. In muscles of uPA+/+SR−/− mice, the proportion of CD31-labeled area decreased at 1 d postinjury compared with uninjured muscle, indicating disruption of the vasculature (Fig. 4A). Subsequently, the CD31-labeled area at 3, 5, and 10 d increased above control levels, revealing significant angiogenesis. Maximal CD31 labeling occurred at day 5, and therefore, the 5-d time point was chosen for comparisons among genotypes. CD31 labeling was markedly decreased in muscles from uPA+/−SR−/− mice compared with uPA+/+SR−/−, indicating that angiogenesis was impaired in uPA-null mice (Fig. 4B, Supplemental Fig. 3). uPA+/−SR−/− exhibited CD31 labeling similar to uPA+/+SR−/−, indicating that Mp-derived uPA is sufficient to induce angiogenesis during skeletal muscle regeneration in otherwise uPA-null mice. Finally, abundant angiogenesis was apparent in the muscles of uPA+/−SR−/− mice at 5 d postinjury but was not different from uPA+/+SR−/−.

**Mp-specific expression of uPA increases active HGF in injured muscle**

One of the mechanisms by which uPA promotes muscle regeneration is by increasing levels of active HGF in damaged muscle (10). To determine whether Mp-derived uPA regulates HGF activation, we assessed levels of active HGF at 5 d postinjury, the time point at which active HGF levels peak in WT mice (10). In agreement with our previous study, muscles from uPA+/−SR−/− mice contained significantly less active HGF than muscles from uPA+/+SR−/− mice (Fig. 5). Muscles from uPA+/+SR−/− mice contained abundant active HGF that was not different from uPA+/+SR−/−. Finally, levels of active HGF in muscles from uPA+/−SR−/− mice were similar to

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Table 1. Summary of mouse genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Description</th>
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<tbody>
<tr>
<td>uPA+/+SR−/−</td>
<td>WT</td>
</tr>
<tr>
<td>uPA+/−SR−/−</td>
<td>uPA activity similar to WT</td>
</tr>
<tr>
<td>uPA+/−SR−/−</td>
<td>uPA-null</td>
</tr>
<tr>
<td>uPA+/−SR−/−</td>
<td>uPA overexpression in Mp; WT uPA expression in other cell types</td>
</tr>
<tr>
<td>uPA−/−SR−/−</td>
<td>uPA expressed only in Mp</td>
</tr>
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uPA+/+SR0/0, indicating that Mp-derived uPA was sufficient to restore active HGF levels in otherwise uPA-null mice. The double band observed in some lanes may be the result of differential glycosylation or differential proteolysis and has been reported previously (18, 29).

Mechanisms by which uPA stimulates Mp migration in vitro

To explore the mechanisms by which uPA influences Mp migration, we performed experiments with cultured Mp in Boyden chamber-type migration assays. Because preliminary experiments showed no differences in migration between Mp from WT (uPA+/+ SR0/0) and uPA-heterozygote (uPA+/− SR0/0) mice, and between MP from SR-uPA (uPA+/+SR+/0) and SR-uPA/uPA-heterozygote (uPA+/− SR+/0) mice, we confined our experiments to MP from WT, uPA-null, and SR-uPA mice. When MCP-1 was placed in the lower wells of the migration chamber, the number of migrated WT MP was increased ~2.5-fold compared with spontaneous migration control wells (Fig. 6A). uPA-null MP showed

FIGURE 2. Mp-specific expression of uPA promotes muscle regeneration. A, Cryosections of EDL muscles from uPA+/+SR0/0, uPA+/−SR0/0, uPA+/−SR+/0, uPA+/−SR−/−, uPA+/− SR+/0, and i.v. uPA-treated uPA+/−SR0/0 mice collected on day 5 following cardiotoxin injury and stained with H&E for morphological analysis. Original magnification ×40. Scale bar, 50 μm. B, Muscle fiber regeneration was assayed at 5 and 10 d by counting the number of regenerating fibers per square millimeter muscle area and by measuring regenerating area as a percentage of total area. Bars are means ± SE; n = 6–8/time point. *Mean value significantly smaller than that for uPA+/+SR0/0 mice, **mean value significantly larger than that for uPA+/+SR0/0 mice, p < 0.05.
significantly less MCP-1–induced migration, and SR-uPA Mp showed significantly greater migration compared with WT Mp (Fig. 6A). These data indicate that the level of uPA expression in Mp contributes to the ability to migrate in response to chemotactic stimuli.

To further elucidate the mechanisms by which uPA promotes Mp migration, we performed further experiments using recombinant proteins, blocking Abs, and signaling inhibitors. Exogenously administered mouse uPA produced a dose-dependent increase in MCP-1–stimulated migration of both WT and uPA-null Mp (Fig. 6B) and had a greater stimulatory effect on the latter such that there was no difference in migration between the cell types at the highest concentration of uPA (10 ng/ml). Exogenously administered mouse PAI-1 produced a dose-dependent decrease in Mp migration in both WT and SR-uPA Mp (Fig. 6C) and blocked the increase in migration induced by uPA (Fig. 6D), suggesting that the proteolytic domain of uPA is involved in the enhancement of Mp migration, consistent with our previous findings (11). Exogenous PAI-1 also stimulated migration of WT and uPA-null Mp, indicating that at least part of the stimulatory effect of uPA occurs through nonproteolytic mechanisms. A blocking Ab against the uPAR inhibited uPA-stimulated migration of both WT and uPA-null Mp, supporting the role of a receptor binding mechanism.

To determine the role of HGF in promoting Mp migration, we used both recombinant mouse HGF and an HGF-blocking Ab. Exogenously administered active HGF promoted migration of both WT and uPA-null Mp in a dose-dependent manner (Fig. 7A), whereas exogenously administered FGF-2 did not stimulate migration (Fig. 7B). In the presence of control IgG, uPA stimulated migration of both WT and uPA-null Mp, whereas the HGF-blocking Ab prevented this uPA-induced stimulation of migration (Fig. 7C). HGF can induce signaling through both the PI3K and MEK1 pathways (30). In our experiments, the PI3K inhibitors LY294002 and wortmannin blocked uPA-stimulated Mp migration, whereas the MEK1 inhibitor PD98059 did not (Fig. 7D). To ensure the efficacy of the inhibitors and to further investigate the influence of uPA on PI3K and MEK1 signaling in Mp, phosphorylation of the PI3K target PKB and the MEK1 target ERK1/2 was examined. As expected, LY294002 dramatically reduced PKB phosphorylation in both uPA- and vehicle-treated Mp, whereas PD98059 blocked phosphorylation of the MEK1 target ERK1/2 (Supplemental Fig. 4). Importantly, uPA did not increase phosphorylation of either ERK or PKB under these experimental conditions (Supplemental Fig. 4). Thus, although PI3K signaling is necessary for Mp migration, it appears that uPA itself does not activate either PI3K or MEK1 signaling in Mp.
under the conditions used for these migration experiments. In total, the in vitro data indicate that uPA stimulates Mp migration in part through proteolytic activation of HGF and in part by binding to the uPAR.

**Discussion**

Mp and uPA have critical and interdependent roles in promoting skeletal muscle regeneration. The present results demonstrate that expression of uPA exclusively in Mp is sufficient to restore Mp accumulation, angiogenesis, and new muscle fiber formation in injured muscles of otherwise uPA-null mice. In addition, over-expression of uPA in Mp further enhances Mp accumulation and muscle fiber regeneration in otherwise WT mice. Whereas levels of active HGF are reduced in injured muscles of uPA-null mice compared with WT, expression of uPA exclusively in Mp is sufficient to restore active HGF to normal levels. In vitro experiments indicate that uPA enhances Mp migration through both proteolytic activation of HGF and receptor binding. These findings improve our understanding of the mechanisms by which uPA and Mp interact to promote healing.

Mp are an important source of uPA during muscle regeneration (10), and conversely, uPA influences Mp function. uPA promotes Mp chemotaxis and is required for invasion of injured muscle (9, 11, 15). The classical function of uPA is proteolytic conversion of plasminogen to active plasmin. This latter enzyme has broad proteolytic activity against a variety of ECM proteins, and the plasmin-mediated degradation of the ECM can influence cell migration, angiogenesis, and tissue repair (12–14). In addition, uPA regulates activity of numerous growth factors, including HGF, either by direct proteolytic activation or by release from sites of sequestration in the ECM (16, 31, 32). In both uninjured and injured skeletal muscle, uPA deficiency is associated with reduced total and active HGF protein, whereas HGF activity after injury is increased in the absence of PAI-1, the primary inhibitor of uPA (10). In the current study, we confirmed that uPA deficiency markedly blunts the increase in active HGF observed after muscle injury (10) and showed thatMp-derived uPA is sufficient to restore active HGF levels in injured muscles of otherwise uPA-null mice. HGF is a well-known myoblast mitogen (33, 34), and we have found that activation of HGF by uPA promotes myoblast proliferation and is necessary for muscle regeneration (10). Thus, restoration of HGF activity may be responsible for the robust regeneration observed in mice that express uPA only in Mp, compared with uPA-null mice.

An important component of muscle regeneration following injury is elaboration of the vascular network to support adequate oxygenation and nutrition for the high level of metabolic activity required. In the current study, uPA-null mice showed impaired angiogenesis, and Mp-derived uPA was sufficient to restore angiogenesis in otherwise uPA-null mice. This suggests that Mp-derived uPA promotes angiogenesis in injured muscle, either directly or by promoting accumulation of Mp, which may then facilitate angiogenesis by other mechanisms. Previous studies have shown that uPA increases capillary density and blood flow after hindlimb ischemia or experimental myocardial infarction (21) and promotes leukocyte invasion and arteriogenesis after femoral artery ligation (22). Similarly, Mp also promote angiogenesis after injury to skin (3) or myocardium (35). Furthermore, HGF promotes angiogenesis after myocardial infarction (36) and during skin wound healing (37, 38). Because Mp produce both uPA and HGF following muscle injury (10), Mp-derived uPA may promote angiogenesis at least in part by enhancing the biological activity of HGF.

Both uPA and HGF are known to promote migration of numerous cell types, including Mp, activated peripheral blood monocytes, endothelial cells, smooth muscle cells, and myoblasts (9, 11, 15, 18, 39–41). Because uPA can proteolytically activate HGF, we hypothesized that HGF activation mediates the uPA-induced increase in Mp chemotaxis. The present data demonstrate that 1) blocking uPA proteolytic activity inhibits Mp chemotaxis in WT and uPA-overexpressing Mp; 2) exogenous active HGF promotes Mp chemotaxis in both WT and uPA-null Mp; and 3) blocking HGF activity prevents the uPA-induced increase in Mp chemotaxis. Because HGF can induce signaling through both PI3K and MEK1 (30), we hypothesized that these pathways may mediate the uPA-induced increase in Mp migration. uPA has previously been shown to induce ERK1/2 phosphorylation in THP-1 Mp in a PI3K- and MEK1-dependent manner (42); however, in the current study, neither PI3K nor MEK1 signaling was induced by uPA. These contrasting results may be due to use of mouse bone marrow-derived Mp versus the human THP-1 cell line or to differing incubation times (30 min of uPA exposure in the current study versus 24 h in Ref. 42). Because uPA did not induce ERK1/2 or PKB phosphorylation under the conditions used for our migration experiments, the reduced Mp migration observed in the presence of PI3K inhibitors seems to indicate that PI3K signaling is permissive for Mp migration but does not directly mediate the uPA effect. Taken together, these data indicated that uPA promotes Mp chemotaxis in part through proteolytic activation of HGF, although the downstream signaling pathways remain to be elucidated.

The experiments discussed above suggest that the proteolytic activity of uPA is important for promotion ofMp migration in vitro. However, the uPA ATF, which lacks the protease domain, was also able to stimulate Mp chemotaxis, suggesting that uPA may also operate via a nonproteolytic mechanism. In addition, a uPAR-blocking Ab inhibited the uPA-induced increase in Mp chemotaxis, suggesting that receptor binding contributes to the uPA effect. Similarly, previous studies have demonstrated that nonproteolytic and uPAR-binding mechanisms contribute to enhancement of smooth muscle cell migration by uPA (20, 43). Because uPAR lacks an intracellular signaling domain, its...
effects on cell migration are likely exerted through interactions of uPAR with other signaling receptors or through localization of uPA proteolytic activity to the leading edge of the migrating cell (14). In contrast to the data presented in this paper, Mp from uPAR$^{2/-}$ mice exhibit a chemotactic response similar to WT both in vivo and in vitro (26). This may be due to compensatory upregulation of alternate chemotactic mechanisms during uPAR$^{2/-}$ development, which would be avoided in the current study by the use of WT Mp and the uPAR-blocking Ab. Alternatively, the contrasting results may be due to differing chemotactic agents used for the in vitro chemotaxis assays; blocking uPAR decreased Mp chemotaxis toward MCP-1 in the current study, whereas uPAR$^{2/-}$ Mp exhibit normal migration toward fMLP (26).

Because uPA rescues chemotaxis of uPA-null Mp in vitro, i.v. injection of uPA in vivo might have been expected to rescue Mp accumulation and new muscle fiber formation in uPA$^{2/-}$SR0/0 mice. However, no effect of i.v. uPA was observed in the current study, suggesting that Mp production of or access to uPA within the injured muscle is critical to Mp accumulation and muscle fiber regeneration. It is possible that extravasated Mp lose access to circulating uPA and are therefore unable to complete their migration into the injured muscle. In support of this hypothesis, and in contrast to the i.v. treatment reported in this paper, i.m. injection of uPA rescues muscle regeneration and Mp accumulation in uPA$^{2/-}$ mice (11).

Hearts in SR-uPA$^{+/0}$ mice develop fibrosis, which contrasts with our results in skeletal muscle. Moriwaki et al. (24) found that Mp

**FIGURE 6.** uPA promotes Mp migration through both proteolytic and nonproteolytic mechanisms. A, Bone marrow-derived Mp from WT, uPA-null, and SR-uPA mice were cultured for migration experiments in a Boyden chamber-type assay using MCP-1 to induce migration. B, Recombinant mouse uPA enhanced migration of WT and uPA-null Mp. C, PAI-1 reduces migration of WT and SR-uPA Mp. D, PAI-1 inhibited uPA-induced migration of both WT and uPA-null Mp (PAI-1 and uPA added at 10 ng/ml each). E, The ATP of uPA enhanced migration of WT and uPA-null Mp. F, uPAR-blocking Ab inhibited uPA-induced migration of both WT and uPA-null Mp (uPA added at 10 ng/ml, Abs at 25 μg/ml). In all experiments, cells that migrated to the lower surface of the membrane were counted and normalized to spontaneous migration. Bars are means ± SE; n = 12–16/condition. 1, Mean value significantly larger than that for spontaneous migration controls of same strain; 2, mean value significantly smaller for cells of uPA-null mice than that for corresponding experimental condition with WT cells; 3, mean value significantly larger for cells of SR-uPA mice than that for corresponding experimental condition with WT cells; a, mean value significantly larger than that for cells of same strain stimulated only by MCP-1; b, mean value significantly different for cells of indicated strain from that for corresponding experimental condition with WT cells; and c, mean value significantly smaller than that for cells of same strain treated with uPA; p < 0.05.
infiltration and collagen accumulation occurs in hearts of SR-uPA+/0 mice by 15 wk of age. However, in the current study, tri-chrome staining revealed no evidence of fibrosis in injured or uninjured skeletal muscles from SR-uPA+/0 mice. These results are consistent with those of Moriwaki et al. (24) who also found no evidence of fibrosis in non-cardiac organs of SR-uPA+/0 mice. Thus, the ability of uPA to promote fibrosis is likely cardiac specific. Indeed, increased levels of uPA reduce collagen accumulation in murine asthmatic lungs (44), reduce fibrosis in the lung following bleomycin injury (8), and reduce liver fibrosis in rats (45). In skeletal muscle, uPA appears to be antifibrotic as well. Lack of uPA results in fibrin accumulation in injured mouse skeletal muscle, and this fibrin accumulation may contribute to the profound regeneration defect seen in these mice (9). In addition, uPA deficiency increases fibrin deposition and worsens muscle degeneration in the mdx mouse model of Duchenne muscular dystrophy (15). The present study provides further evidence for an antifibrotic role for uPA in skeletal muscle, as collagen-stained area in muscles from SR-uPA mice was decreased relative to WT at 10 d postinjury. The results of the current study confirm and expand upon our previous study in which transfer of WT bone marrow cells to uPA-null mice restored Mp accumulation and muscle regeneration (11). The current study avoided potential adverse effects of radiation and targeted uPA expression specifically to Mp.

In SR-uPA+/0 mice, the human scavenger receptor promoter is used to drive uPA expression. Thus, the transgene is expected to be expressed exclusively in mature Mp (23, 46). However, low-level expression of the SR-uPA transgene was also detected in non-cardiac organs of SR-uPA+/0 mice. Thus, the ability of uPA to promote fibrosis is likely cardiac specific. Indeed, increased levels of uPA reduce collagen accumulation in murine asthmatic lungs (44), reduce fibrosis in the lung following bleomycin injury (8), and reduce liver fibrosis in rats (45). In skeletal muscle, uPA appears to be antifibrotic as well. Lack of uPA results in fibrin accumulation in injured mouse skeletal muscle, and this fibrin accumulation may contribute to the profound regeneration defect seen in these mice (9). In addition, uPA deficiency increases fibrin deposition and worsens muscle degeneration in the mdx mouse model of Duchenne muscular dystrophy (15). The present study provides further evidence for an antifibrotic role for uPA in skeletal muscle, as collagen-stained area in muscles from SR-uPA mice was decreased relative to WT at 10 d postinjury.

The results of the current study confirm and expand upon our previous study in which transfer of WT bone marrow cells to uPA-null mice restored Mp accumulation and muscle regeneration (11). The present study overcomes at least two limitations of the bone marrow transplant model used in the previous study. First, the current study avoided irradiation of the mice, which could influence the function of other cell populations in addition to bone marrow cells, including muscle satellite cells. Second, the bone marrow transfer approach did not restrict uPA expression to Mp because other bone marrow-derived cells could have contributed to production of uPA. By using transgenic SR-uPA mice, the current study avoided potential adverse effects of radiation and targeted uPA expression specifically to Mp.

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FIGURE 7. uPA promotes Mp migration through an HGF-dependent mechanism. Bone marrow-derived Mp from WT and uPA-null mice were cultured for migration experiments in a Boyden chamber-type assay using MCP-1 to induce migration. A, HGF enhanced migration of WT and uPA-null Mp. B, FGF did not alter migration of WT or uPA-null Mp. C, HGF-blocking Ab inhibited uPA-induced migration of WT and uPA-null Mp (uPA added at 10 ng/ml, Abs at 25 μg/ml). D, Effects of the PI3K inhibitor LY294002 (10 μM), the PI3K inhibitor wortmannin (1 μM), or the MEK1 inhibitor PD98059 (10 μM) on uPA-induced (10 ng/ml) migration of WT and uPA-null Mp. In all experiments, cells that migrated to the lower surface of the membrane were counted and normalized to spontaneous migration. Bars are means ± SE; n = 12–16/condition. a, Mean value significantly larger than that for cells of same strain stimulated only by MCP-1; b, mean value significantly different for cells of indicated strain from that for corresponding experimental condition with WT cells; and c, mean value significantly smaller than that for cells of same strain treated with uPA; p < 0.05.

Disclosures
The authors have no financial conflicts of interest.
References


Supplemental Figure 1. SRuPA decreases fibrosis of injured skeletal muscle. Cryosections of uninjured (Con) EDL muscles and EDLs at 5 and 10 days post-injury were stained by Masson’s trichrome method. A: Representative 20x images of WT and SRuPA EDLs at 5 and 10 days post-injury. Scale bar = 100μm. B: Collagen accumulation was quantified as percentage blue-stained area. Bars are mean +/- standard error, n=3-5 per time point. * mean value significantly smaller than WT at same time point, P<0.05.
Supplemental Figure 2. Mp-specific expression of uPA promotes Mp accumulation in damaged muscle. Mp accumulation was assessed at 5 days post-injury by immunostaining EDL cryosections for the Mp-specific F4/80 antigen. Representative 20x images from each genotype, and from uPA-/SR0/0 mice given daily i.v. uPA treatment, are shown. Scale bar = 100μm.
Supplemental Figure 3. Mp-specific uPA expression promotes angiogenesis after muscle injury. Angiogenesis was assayed at 5 days post-injury by immunostaining EDL cryosections for CD31 antigen to label endothelial cells. Representative 20x images from each genotype are shown. Scale bar = 100μm.
Supplemental Figure 4. uPA does not alter phosphorylation of PKB or ERK1/2. Bone marrow Mp were treated with recombinant mouse uPA (10 μg/mL), the PI3K inhibitor LY294002 (10 μM), and the MEK1 inhibitor PD98059 (10 μM). Phosphorylation of the PI3K target PKB (Panel A) and the MEK1 target ERK1/2 (Panel B) was assessed by Western blotting with phosphospecific antibodies. Blots are representative of n = 4 independent experiments of 2-3 replicates each.