Urokinase-Type Plasminogen Activator Plays Essential Roles in Macrophage Chemotaxis and Skeletal Muscle Regeneration

Scott C. Bryer, Giamila Fantuzzi, Nico Van Rooijen and Timothy J. Koh

*J Immunol* 2008; 180:1179-1188; 
doi: 10.4049/jimmunol.180.2.1179
http://www.jimmunol.org/content/180/2/1179

Why *The JI*?

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References  This article cites 49 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/180/2/1179.full#ref-list-1

Subscription  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Urokinase-Type Plasminogen Activator Plays Essential Roles in Macrophage Chemotaxis and Skeletal Muscle Regeneration

Scott C. Bryer,* Giamila Fantuzzi,† Nico Van Rooijen,‡ and Timothy J. Koh²*

Although macrophages are thought to play important roles in tissue repair, the molecular mechanisms involved remain to be elucidated. Mice deficient in urokinase-type plasminogen activator (uPA⁻/⁻) exhibit decreased accumulation of macrophages following muscle injury and severely impaired muscle regeneration. We tested whether macrophage-derived uPA plays essential roles in macrophage chemotaxis and skeletal muscle regeneration. Macrophage uPA was required for chemotaxis, even when invasion through matrix was not necessary. The mechanism by which macrophage uPA promoted chemotaxis was independent of receptor binding but appeared to depend on proteolytic activity. Exogenous uPA restored chemotaxis to uPA⁻/⁻ macrophages and rescued muscle regeneration in uPA⁻/⁻ mice. Macrophage depletion in wild-type (WT) mice using clodronate liposomes resulted in impaired muscle regeneration, confirming that macrophages are required for efficient healing. Furthermore, transfer of WT bone marrow cells to uPA⁻/⁻ mice restored macrophage accumulation and muscle regeneration. In this rescue, transferred WT cells appeared to contribute to IGF-1 expression but did not fuse to regenerating fibers. These data indicate that WT leukocytes, including macrophages, that express uPA were sufficient to rescue muscle regeneration in uPA⁻/⁻ mice. Overall, the results indicate that uPA plays a fundamental role in macrophage chemotaxis and that macrophage-derived uPA promotes efficient muscle regeneration. The Journal of Immunology, 2008, 180: 1179–1188.

The Journal of Immunology

© 2008 by The American Association of Immunologists, Inc.

*Department of Movement Sciences, and †Department of Human Nutrition, University of Illinois, Chicago, IL 60612; and ‡Department of Molecular Cell Biology, Vrije University, Amsterdam, The Netherlands

Received for publication May 10, 2007. Accepted for publication October 31, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by United States Army Medical Research and Materiel Command Grant W81XWH-05-1-0159 (to T.J.K.). S.C.B. was supported by the National Aeronautics and Space Administration Graduate Student Research Program Grant NNG04GN53H.

2 Address correspondence and reprint requests to Dr. Timothy J. Koh, Department of Movement Sciences, University of Illinois, 1919 West Taylor Street, m/c 994, Room 529, Chicago, IL 60612. E-mail address: tjkoh@uic.edu

Abbreviations used in this paper: uPA, urokinase-type plasminogen activator; WT, wild type; BMDMs, BM-derived macrophages; EDL, extensor digitorum longus; TA, tibialis anterior; BM, bone marrow; MyoD, myogenic regulatory factor; HGF, hepatocyte growth factor; iNOS, inducible NO synthase; uPA⁻/⁻-mx, uPA⁻/⁻ muscle extract.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00
macrophages from uPA−/− mice in vitro, even when matrix invasion is not required. We also expected that uPA would promote accumulation of macrophages in damaged muscle in vivo. Finally, we expected that transfer of WT bone marrow (BM) cells to uPA−/− mice would restore macrophage accumulation in injured muscle and rescue muscle regeneration.

Materials and Methods

Mice

WT, uPA knockout (uPA−/−), uPAR knockout (uPAR−/−), and GFP-transgenic mouse on a C57BL/6 background were obtained from Jackson ImmunoResearch Laboratories and bred in our animal facility. All experiments were performed on mice aged 10–12 wk, except where otherwise noted. The Animal Care Committee at the University of Illinois approved all experimental procedures.

Culture of BM-derived macrophages (BMDMs)

BMDMs from WT, uPA−/−, and uPAR−/− mice were cultured as described previously (31). Briefly, BM was flushed from femurs and tibias using DMEM supplemented with 10% heat-inactivated FBS, 10% l-2929 cell-conditioned medium (source of macrophage CSF), 2 mM l-glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were cultured in a humidified 10% CO2 atmosphere at 37°C. After 4 days in culture, cells were harvested, and viability was confirmed by trypan blue exclusion. For experiments, cells were resuspended in serum-free medium (DMEM plus 1% BSA plus 1% penicillin/streptomycin) and cultured in 96-well plates.

Mesenchymal stem cells (MSCs)

Bone marrow stromal cells were isolated from femurs and tibias of 10–12-wk-old WT and uPA−/− mice by flushing with PBS, and cell suspensions were centrifuged and plated at a density of 5 × 10^4 cells/well in 24-well plates. The plates were seeded with 100 μl of low-density MSCs (two i.m. injections per muscle) and 2 ml of growth medium (5 mg/kg) and cardiotoxin (10 μM; Calbiochem) was administered with two i.m. injections per muscle. In some mice, exogenous uPA (Molecular Innovations) was administered to EDL and TA muscles from uPA−/− mice via i.m. injections (5 μg/mouse) at 1–4 days post-injury. Mice were euthanized and muscles collected at 0–20 days post-injury. One hour before muscle collection, BrdU (30 mg/kg) was administered via i.p. injection.

Macroscopic depletion with clodronate liposomes

Clodronate liposomes were used extensively to specifically deplete macrophages in vivo by inducing apoptosis in these cells without affecting other cell types (32). Clodronate was a gift from Roche and clodronate liposomes were prepared as described previously (32). Clodronate liposomes were administered to WT mice via i.p. injection (200 μl; 6 mg/ml) immediately after, and 2 and 5 days following cardiotoxin-induced muscle injury.

Lethal irradiation and BM transfer

BM recipient mice (5–7-wk-old WT and uPA−/− mice) were subjected to lethal irradiation by opposed lateral beams, each field contributing 50% of the total dose (50 Gy at 1.02 Gy/min). BM was collected from the femurs and tibias of donor WT and uPA−/− mice, RBCs were lyed, the remaining cells were resuspended in HBSS (Mediatech), and injected retro-orbitally (20 × 10^6 cells per mouse in 200 μl) into recipient mice at 1 day after lethal irradiation. Mice were allowed to recover for 30 days and then EDL and TA muscles were injured via cardiotoxin injection.

Muscle morphology

Cryosections from the midbelly of each EDL muscle (10-μm thickness) were either stained with H&E for morphological analysis or processed for immunohistochemistry. Morphological analysis was performed using five representative images (×40 magnification) for each muscle section (Labphot-2, Nikon; and SPOT software, Diagnostic Instruments). For each field, fibers were classified as normal, damaged, or regenerating as described (19). The number and area of each type of fiber were recorded. Damaged area was estimated in each muscle section by subtracting the summed area of normal and regenerating fibers from the total area of each field.

Immunohistochemistry

Muscle injury and sample preparation

Extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were injured via cardiotoxin injection as previously described (19). Briefly, mice were anesthetized with an i.p. injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) and cardiotoxin (10 μM; Calbiochem) was administered with two i.m. injections per muscle. In some mice, exogenous uPA (Molecular Innovations) was administered to EDL and TA muscles from uPA−/− mice via i.m. injections (5 μg/mouse) at 1–4 days post-injury. Mice were euthanized and muscles collected at 0–20 days post-injury. One hour before muscle collection, BrdU (30 mg/kg) was administered via i.p. injection. Following euthanasia, muscles were either mounted in tissue freezing medium and frozen in isopentane chilled with dry ice for histological analysis or stored in RNAlater (Qiagen) for RNA isolation.

RT-PCR

TA muscles or BMDMs were collected in RNAlater (Qiagen), homogenized, and total RNA was extracted using the RNeasy RNA Isolation Kit (Qiagen), following the manufacturers instructions. cDNA was synthesized from 1 μg of RNA using the Thermoscript RT-PCR system (Invitrogen Life Technologies). Amplification reactions were performed with 1 μM primers, 1 μl cDNA, 3.5 mM MgCl2, and 0.2 mM dNTPs for a 25 μl final volume in a GeneAmp 9700 thermal cycler (PE Applied Biosystems). Primer sequences are listed in Table 1. cDNA was analyzed by separation on a 1.5% agarose gel and visualized by ethidium bromide staining.

Data analysis

Values reported in the text and figures are given as means ± SE. Data were compared across different mouse strains and time points using a two-way ANOVA. Holm-Sidak post hoc test was performed with a 0.05 confidence level to indicate statistical significance (Sigma-Aldrich).
Results

Impaired chemotaxis of uPA/−/− macrophages toward WT muscle extract

Macrophage accumulation is nearly absent following muscle injury in uPA/−/− mice (19); however, the reason for this impairment remains unclear. Previous studies have suggested an impaired ability of uPA/−/− cells to migrate through extracellular matrix (20). A modified Boyden chemotaxis chamber was used to test whether uPA is required for macrophage chemotaxis, even when invasion through matrix is not required.

We first compared spontaneous cell migration (cell movement from upper wells toward medium alone in lower wells) and found no significant difference between WT (10.2 ± 1.1 cells/field) and uPA/−/− (10.6 ± 1.2 cells/field) macrophages for this control condition. We next used muscle extract as a chemotactic agent to determine whether uPA/−/− macrophages exhibited impaired chemotaxis. When WT muscle extract was placed in the lower wells of the chemotaxis chamber, the number of migrated cells was increased ∼3-fold for WT macrophages compared with control wells, indicating that WT muscle extract possessed chemotactic activity (Fig. 1A). Compared with WT macrophages, uPA/−/− macrophages showed significantly impaired chemotaxis toward WT muscle extract. Exogenous uPA added to macrophage suspensions increased chemotaxis of both WT and uPA/−/− macrophages and eliminated the impairment observed in uPA/−/− compared with WT macrophage chemotaxis. These data indicate that macrophage uPA is required for macrophage chemotaxis, even when matrix invasion is not required.

FIGURE 1. Muscle extract-induced chemotaxis is impaired in uPA/−/− macrophages. A, WT muscle extract (WT-mx) induced chemotaxis of WT macrophages, but chemotaxis of uPA/−/− macrophages was impaired. Exogenous uPA (100 ng/ml) added to cell suspensions (upper wells) eliminated impairment in uPA/−/− macrophage chemotaxis. B, uPA/−/− muscle extract (uPA/−/−-mx) induced chemotaxis of WT macrophages but not uPA/−/− macrophages. uPA/−/−-mx was less effective than WT muscle extract in stimulating chemotaxis. Exogenous uPA (100 ng/ml) added to uPA/−/−-mx (lower wells) rescued chemotactic activity. C, uPA alone (100 ng/ml) induced chemotaxis of WT macrophages, but chemotaxis was significantly reduced in uPA/−/− and uPAR−/− macrophages compared with WT macrophages. Data are means ± SE (n = 12–64, number of wells). One-way ANOVA showed a significant difference in mean values among treatment groups (p < 0.05). *, Mean value significantly greater than medium only controls (within strain); †, mean value significantly greater than stimulus by WT muscle extract (A) or uPA/−/−-mx (B); ‡, mean value significantly different from WT for same experimental condition.

FIGURE 2. MCP-1-induced chemotaxis is impaired in uPA/−/− macrophages. A, MCP-1 (10 ng/ml) induced chemotaxis in WT and uPAR−/− macrophages but not uPA/−/− macrophages. B, WT and uPA/−/− macrophages were incubated with FITC-MCP-1 (10 μg/ml) and analyzed by flow cytometry to evaluate binding capacity. Both WT and uPA/−/− macrophages showed increased fluorescence, indicating that both bound labeled MCP-1. Data are means ± SE (n = 24–48, number of wells). One-way ANOVA showed a significant difference in mean values among treatment groups (p < 0.05). *, Mean value significantly greater than medium only controls (within strain); ‡, mean value significantly different from WT for same experimental condition.
Impaired chemotaxis of macrophages toward uPA−/− muscle extract

Muscle extract from uPA−/− mice was used next to determine whether uPA produced in injured muscle contributes to the chemotactic activity of muscle extract (Fig. 1B). When compared with WT muscle extract, uPA−/− muscle extract elicited a significantly reduced chemotactic response in WT macrophages. In addition, uPA−/− muscle extract-induced chemotaxis was nearly absent in uPA−/− macrophages. When exogenous uPA was added to uPA−/− muscle extract, chemotaxis of both WT and uPA−/− macrophages was enhanced. These data indicate that uPA contributes to the chemotactic activity of muscle extract.

We next tested whether uPA alone possesses chemotactic activity as has been reported previously (30). uPA alone induced chemotaxis of WT macrophages (Fig. 1C), but chemotaxis of uPA−/− and uPAR−/− macrophages toward uPA was impaired. These data indicate that uPA produced in injured muscle may itself promote chemotaxis, and this is dependent on uPAR expressed on macrophages. Since we found previously that uPAR is not required for macrophage accumulation in damaged muscle in vivo (21), any uPAR-dependent mechanism identified for chemotaxis in vitro may not contribute following muscle injury in vivo.

In total, these results indicate that uPA may play dual roles in macrophage chemotaxis; first, macrophage-derived uPA is required for chemotaxis, even when invasion through matrix was not necessary, and second, uPA generated in injured muscle promotes macrophage chemotaxis to the site of injury, as a chemotactic agent itself and possibly through uPA-mediated proteolytic generation of chemotactic factors within the injured muscle.

Impaired chemotaxis of uPA−/− macrophages toward MCP-1

We further examined the role of macrophage uPA in chemotaxis using MCP-1 as a chemotactic agent (Fig. 2A). MCP-1 is a chemokine produced in damaged muscle and is thought to play an important role in attracting macrophages to the site of damage (33). As expected, MCP-1 (10 ng/ml) induced chemotaxis of WT macrophages. Compared with WT macrophages, uPA−/− macrophages showed significantly impaired chemotaxis toward MCP-1 and no difference compared with control wells. In contrast, MCP-1 induced chemotaxis of uPAR−/− macrophages at a level that was not different from WT macrophages. These results indicate that uPA is required for MCP-1-induced chemotaxis through an uPAR-independent mechanism.

To determine whether the impaired chemotaxis of uPA−/− macrophages toward MCP-1 was due to a reduced ability to bind MCP-1, fluorescently labeled MCP-1 was incubated with macrophages from WT and uPA−/− mice (Fig. 2B). Mean fluorescence of WT and uPA−/− macrophages incubated with labeled MCP-1 increased by a similar amount compared with unlabeled control cells, indicating no difference in the ability of WT and uPA−/− macrophages to bind MCP-1.
macrophages to bind MCP-1. These results suggest that impaired chemotaxis of uPA−/− macrophages toward MCP-1 is not a result of impaired binding of MCP-1.

Impaired chemotaxis of uPA−/− macrophages toward fMLP

To test whether the impaired chemotaxis of uPA−/− macrophages was a general phenomenon, we assessed fMLP-induced chemotaxis (34). As expected, fMLP-induced chemotaxis of WT macrophages (Fig. 3A). In contrast, fMLP did not increase migration of uPA−/− macrophages, similar to the results with muscle extract and MCP-1. Exogenous uPA increased chemotaxis of both WT and uPA−/− macrophages and eliminated the impairment observed in uPA−/− compared with WT macrophage chemotaxis. We next examined whether uPAR was required for uPA stimulation of fMLP-induced chemotaxis. fMLP-induced chemotaxis of uPAR−/− macrophages was similar to that of WT macrophages and was similarly enhanced following the addition of exogenous uPA to cell suspensions in upper wells (Fig. 3A). These results indicate that uPA promotes fMLP-induced chemotaxis through an uPAR-independent mechanism.

We also assessed whether alternative receptors for uPA were available on uPAR−/− macrophages that could induce signaling pathways for cell migration (27, 35, 36). Fluorescently labeled uPA was incubated with macrophages from WT and uPAR−/− mice (Fig. 3B). Mean fluorescence of WT macrophages incubated with labeled uPA increased significantly compared with unlabeled control cells, indicating that WT macrophages were able to bind uPA as expected. Mean fluorescence of uPAR−/− macrophages was not increased following incubation with labeled uPA, suggesting that alternative binding sites are not available on these cells.

To provide an indication of whether uPA regulates chemotaxis through proteolytic activity, we next examined chemotaxis in WT and uPAR−/− macrophages treated with aprotinin, a serine protease inhibitor (Fig. 3C). fMLP-induced chemotaxis of both WT and uPAR−/− macrophages demonstrated dose-dependent impairments with increasing concentrations of aprotinin (0.1–100 IU/ml), indicating that proteolytic activity is required for chemotaxis. These concentrations of aprotinin resulted in spontaneous cell migration that was 98% of that of cells exposed to normal medium alone, providing an indication that cell viability was not affected by aprotinin. In sum, the results indicate that uPA proteolytic activity promotes chemotaxis independent of uPAR binding and matrix invasion.

Phagocytosis and gene expression not impaired in uPA−/− macrophages

Macrophage phagocytosis and gene expression were compared between WT and uPA−/− macrophages to determine whether the defect in chemotaxis in uPA−/− macrophages was indicative of general
functional impairment. Macrophages from WT and uPA−/− mice were compared for their ability to ingest latex beads, a standard in vitro method to measure phagocytosis (Fig. 4A). The percentage of cells to ingest one or more beads was not different between WT and uPA−/− macrophages with or without LPS stimulation.

Expression of selected growth factors and cytokines by macrophages from WT and uPA−/− mice were examined next, either with or without LPS stimulation (Fig. 4B). LPS enhanced expression of the inflammatory cytokines inducible NO synthase (iNOS), IL-6, and IL-12 to a similar extent in WT and uPA−/− macrophages. uPA was expressed following LPS stimulation in WT macrophages, and, as expected, was not expressed in macrophages from uPA−/− mice. In summary, these data demonstrate that impaired chemotaxis in uPA−/− macrophages was a specific deficit and was not due to a general functional impairment.

Macrophage depletion delayed muscle regeneration

Macrophages were depleted from WT mice using clodronate liposomes to mimic the impaired accumulation of macrophages observed in uPA−/− mice following muscle injury (19, 20) and to determine whether macrophage depletion itself results in impaired muscle healing. To ensure that clodronate liposomes did not influence muscle cells, C2C12 myoblasts were cultured with or without clodronate liposomes (0–600 μM) for 24 h and counted to determine cell viability. In other experiments, C2C12 cells were allowed to expand until ~90% confluent, at which time they were stimulated to differentiate and clodronate liposomes were added. Neither cell viability nor the capacity to form multinucleated myotubes was impaired when comparing clodronate liposome-treated cells to untreated cells. In contrast, treating macrophages with clodronate liposomes resulted in the death of all cells within 24 h.

Administration of clodronate liposomes following cardiotoxin-induced muscle injury (Fig. 5A) effectively reduced macrophage accumulation in injured muscle from WT mice through 5 days post-injury compared with untreated controls (Fig. 5B). Some macrophage accumulation was observed at 5 days post-injury in mice treated with clodronate liposomes and was further increased at 10 and 12 days post-injury, suggesting that clodronate liposomes were exhausted following their final injection at 5 days post-injury and allowed macrophages to accumulate. Neutrophil accumulation was also quantified and was not significantly different between untreated and clodronate liposome-treated mice (not shown), supporting the specificity of clodronate liposomes on macrophages.

Cryosections stained with H&E demonstrated that the injury protocol elicited damage to greater than 95% of EDL muscle cross-sections from both untreated and macrophage-depleted mice. At 5 days post-injury, muscle from untreated mice showed a reduction in damaged area associated with formation of central-nucleated fibers (Fig. 5D). In contrast, muscles from macrophage-depleted mice were characterized by a lack of recovery of muscle morphology, the persistence of swollen and necrotic fibers, and the absence of central nucleated fibers. Muscles from untreated mice collected at later time points post-injury displayed progressive recovery of normal morphology, whereas muscles from clodronate liposome-treated mice showed delayed recovery. This delayed muscle regeneration was associated with exhaustion of clodronate liposomes and accumulation of macrophages.

To complement morphological assessment of muscle regeneration, cell proliferation and MyoD expression were assessed in muscle cryosections (Fig. 5E). The incorporation of BrdU into proliferating cell nuclei was significantly reduced at 3 and 5 days post-injury in muscle from macrophage-depleted mice compared with untreated mice. In addition, the number of cells that stained positively for the myogenic regulatory factor, MyoD, was also significantly reduced in sections of muscle from macrophage-depleted mice compared with untreated mice. Furthermore, RT-PCR analysis indicated that expression of IL-12, a proinflammatory cytokine expressed by macrophages, was impaired in macrophage-depleted compared with control mice, and expression of IGF-1 was delayed (Fig. 5F), suggesting that macrophages contribute to IGF-1 expression during muscle regeneration.

Exogenous uPA restored macrophage accumulation and muscle regeneration in uPA−/− mice

Because exogenous uPA restored chemotaxis of uPA−/− macrophages in vitro (Figs. 1 and 3), exogenous uPA was administered to injured uPA−/− muscle in an effort to restore macrophage chemotaxis in vivo. Immunohistochemical analysis revealed that uPA administration restored macrophage accumulation in uPA−/− muscle to a level comparable with macrophage accumulation in WT muscle at 5 days post-injury (Fig. 6). Associated with the restored macrophage accumulation, muscle regeneration was also rescued by uPA administration as assessed using H&E stained muscle sections. Muscles from untreated uPA−/− mice were characterized by a lack of recovery of muscle morphology, the persistence of swollen and necrotic fibers, and the absence of central nucleated fibers. Exogenous uPA restored the formation of regenerating fibers and the recovery of muscle morphology to WT levels.

WT BM transfer to uPA−/− mice rescued macrophage accumulation and muscle regeneration

The transfer of WT BM to uPA−/− recipient mice following lethal irradiation (W-U mice) was used to further test the hypothesis that macrophage-derived uPA regulates macrophage chemotaxis into injured muscle and efficient muscle regeneration. WT mice reconstituted with WT BM (W-W) and uPA−/− mice with uPA−/− BM...
(U-U) served as controls. The BM transfer protocol resulted in over 85% engraftment of donor BM as determined by flow cytometry (Fig. 7A). Uninjured muscles from mice that received lethal irradiation and BM transfer demonstrated no obvious differences in morphology 30 days post-irradiation compared with nonirradiated muscles as visualized with H&E staining (not shown).

As expected, at 5 days post-injury, macrophages were prevalent in injured muscles of W-W mice, and macrophage accumulation was nearly absent in injured muscles of U-U mice. However, transfer of WT BM to uPA−/− mice restored macrophage accumulation in injured muscle of these U-U mice to levels observed in W-W mice (Fig. 7B). By 10 days post-injury, macrophage accumulation had declined in muscles of W-W and W-U mice and remained depressed in U-U muscles.

At 5 days post-injury, the number of regenerating fibers in muscle of W-W mice was less than that observed in nonirradiated WT muscle, whereas at 10 days, the number of regenerating fibers in W-W mice equaled or exceeded that found in nonirradiated WT muscle (Fig. 7C and D). The delayed formation of central nucleated fibers in irradiated compared with nonirradiated muscle is likely due to the well-known deleterious effects of radiation on satellite cells (37). Similar to nonirradiated uPA−/− muscle, formation of regenerating fibers was absent in U-U mice at both 5 and 10 days post-injury. In contrast, transfer of WT BM to uPA−/− mice resulted in formation of regenerating fibers in these W-U mice equal to that observed in W-W mice, indicating that WT BM transfer was sufficient to restore muscle regeneration in uPA−/− mice. Damaged area continued to decline in muscle from both W-W and W-U mice with central nucleated fibers occupying greater than 85% of the area of each muscle section by 20 days post-injury. In contrast, muscles from U-U mice at 20 days post-injury were characterized by the absence of central nucleated fibers.
Donor cells contributed to muscle regeneration to levels similar to WT controls. Injured muscle, expression of repair-promoting factors, and subsequent contribution of BM-derived cells to regenerating fibers. Muscle fibers were positive for GFP (none shown), indicating the rare contribution of BM-derived cells to regenerating fibers.

Donor cells did not contribute to muscle fiber formation

Recent publications have suggested that BM-derived stem cells can contribute directly to the formation of muscle fibers during regeneration (38). Muscle fibers at 20 days post-injury from lethally irradiated recipient mice reconstituted with GFP BM cells were also analyzed to determine the contribution of BM-derived stem cells to the formation of regenerating fibers. Only two regenerating fibers in >3000 muscle fibers analyzed were GFP-positive (<0.1%). Therefore, BM cells appear to contribute very rarely to the formation of regenerating fibers observed in W-U mice, and thus, the restored macrophage accumulation in these mice appears sufficient to rescue regeneration.

Discussion

Loss of uPA results in impaired inflammatory and healing responses in several different tissues, including lung (22, 39), liver (17, 18, 40), and skin (16). The attenuated inflammatory response has been suggested to be at least partly responsible for the impaired healing observed in uPA−/− mice. Specific to the present study, macrophage accumulation in injured skeletal muscle and subsequent muscle regeneration are severely impaired in uPA−/− mice (19, 20). These data provide the rationale for the current hypothesis that macrophage-derived uPA plays an essential role in macrophage chemotaxis into damaged muscle and is required for efficient muscle regeneration. Our data indicate that macrophage-derived uPA is required for chemotaxis, even when invasion through matrix is not necessary. The mechanism by which macrophage-derived uPA promotes chemotaxis appears to be independent of receptor binding but dependent on proteolytic activity. In addition, we demonstrate that macrophage depletion is sufficient to impair macrophage chemotaxis, confirming the importance of macrophages in muscle healing. Furthermore, transfer of WT BM cells to uPA−/− mice is sufficient to restore macrophage accumulation and muscle regeneration. In this rescue, transferred WT cells appeared to contribute to IGF-1 expression but did not fuse to regenerating fibers. These data indicate that WT leukocytes, including macrophages, that express uPA are sufficient to rescue impaired muscle regeneration in uPA−/− mice. In total, the results indicate that uPA plays fundamental roles in macrophage chemotaxis and that macrophage-derived uPA promotes efficient muscle regeneration.

uPA−/− macrophages demonstrated impaired chemotaxis toward injured muscle extract, MCP-1, and fMLP, indicating that macrophage-derived uPA is required for chemotaxis toward all of these chemotactic agents. In these experiments, chemotaxis was measured through an uncoated polycarbonate membrane, and thus, matrix invasion was not required. The impaired chemotaxis of uPA−/− macrophages did not appear to be due to a general functional impairment because phagocytosis of latex beads and expression of inflammatory cytokines was not impaired. The impaired chemotaxis of uPA−/− macrophages also did not appear to be due to reduced ability to bind chemotactic agents, as WT and uPA−/− macrophages bound similar amounts of MCP-1. The mechanism by which macrophage-derived uPA regulates chemotaxis appears to be independent of uPAR binding because chemotaxis toward MCP-1 and fMLP was not different between WT and uPAR−/− macrophages. uPA proteolytic activity may be required for chemotaxis because the serine protease inhibitor aprotinin significantly reduced chemotaxis of both WT and uPAR−/− macrophages. The target(s) of uPA-mediated proteolytic activity remain(s) to be elucidated. Hepatocyte growth factor (HGF), a well-known motogenic factor, is secreted as an inactive profactor, and uPA can proteolytically activate HGF (29, 41). HGF can be

and the persistence of necrotic tissue. Supporting these morphological data, BrdU incorporation and MyoD expression was nearly absent in muscle of U-U mice. In contrast, the restored muscle regeneration in W-U mice was associated with levels of BrdU incorporation and MyoD expression similar to those seen in W-W mice (Fig. 7E), indicating that WT BM transfer was sufficient to restore cell proliferation and satellite cell accumulation in muscle of uPA−/− mice.

Muscle from WT mice demonstrated expression of IL-12 and IGF-1 at 5 days post-injury (Fig. 7, F and G). In contrast, muscle from uPA−/− mice demonstrated significantly reduced expression of IL-12 and IGF-1, associated with the impaired accumulation of macrophages. When WT BM was transferred to uPA−/− mice, expression of IL-12 and IGF-1 was restored to near WT levels associated with restored macrophage accumulation and muscle regeneration. Taken together, the transfer of WT BM to lethally irradiated uPA−/− mice effectively restored macrophage chemotaxis into injured muscle, expression of repair-promoting factors, and subsequent muscle regeneration to levels similar to WT controls.

Donor cells contributed to the inflammatory response in recipient mice

BM from GFP-transgenic mice was transferred to lethally irradiated uPA−/− mice to assess the contribution of donor BM to muscle inflammation and muscle fiber formation. Serial muscle cross-sections were obtained from these mice at 5–20 days post-injury. Numerous small GFP-positive cells were observed in injured muscle at 5 days post-injury (Fig. 8). By 10 days post-injury, GFP-positive cells were reduced, and by 20 days they were nearly absent. Few Ly-6G-positive cells were evident in the muscle cross-sections at 5 days post-injury, and none were present at 10 days, indicating that the majority of the GFP-positive cells were not neutrophils. Cells staining positive for the F4/80 Ag showed similar localization as GFP staining and similar changes over time, indicating that the majority of donor BM-derived cells were likely macrophages.
produced by macrophages, but whether uPA is required for activation of macrophage-derived HGF and whether HGF is required for macrophage chemotaxis remains to be determined. Another possibility is that uPA is required for proteolytic processing of molecules that are required for cell attachment/detachment during chemotaxis. This possibility also remains to be tested. In total, the in vitro data indicate that uPA plays a fundamental role in macrophage chemotaxis, apart from matrix invasion.

Our in vitro and in vivo data indicate that macrophage-derived uPA, but not uPAR, is required for chemotaxis of mouse macrophages toward injured muscle (Figs. 1–3) (21). Other studies have produced data that contrast with our data in some respects. In the original publication characterizing the uPA−/− mice, peritoneal macrophage numbers were not different between WT and uPA−/− mice, whether untreated or treated with thioglycollate (42). Thus, the impaired macrophage accumulation in injured muscle of uPA−/− mice likely depends on the specific local environment in the injured muscle. In another study, chemotaxis of human monocytes toward fMLP was impaired following treatment with anti-uPAR Abs whereas anti-uPA Abs had no effect (43). Human satellite cells, in contrast, showed impaired chemotaxis toward uPA and FGF after treatment with either anti-uPA or anti-uPAR Abs (30).

The current study is unique in that we used macrophages derived from transgenic mice deficient in uPA or uPAR. Reasons underlying the differences between the current study and previous studies that used Abs to block uPAR remain unclear but may be due to unintended effects of blocking Abs, adaptive mechanisms in knockout cells, and/or species-related differences.

Macrophages appear to be critical for healing of various tissues. Macrophage depletion using clodronate liposomes resulted in increased ischemia-reperfusion injury of the lung (1), reduced vascular repair after mechanical arterial injury (44), and delayed liver regeneration after partial hepatectomy (4). In the present study, clodronate liposomes were used to deplete macrophages and prevent their accumulation in skeletal muscle following injury; this resulted in impaired muscle regeneration similar to that observed in uPA−/− mice. These results indicate that macrophages are required for efficient muscle regeneration following injury and are consistent with another recent study that reported impaired muscle healing with clodronate liposome treatment following freeze injury (8). In addition, depletion of macrophages in transgenic mice that allow specific, conditional ablation of macrophages resulted in impaired healing following notexin injection (10). Our data are consistent with these findings and further indicate that macrophage-derived uPA plays fundamental roles in macrophage accumulation following muscle injury and subsequent regeneration.

Macrophages that accumulate following muscle injury are thought to be derived predominantly from the circulation and not from resident cells in injured muscle or neighboring tissue (5, 10). We therefore introduced WT macrophages into uPA−/− mice by transplanting BM from WT donor mice to uPA−/− recipient mice. This procedure resulted in the restoration of macrophage accumulation in injured muscle of uPA−/− mice and efficient muscle regeneration. This rescue was associated with the restored expression of IGF-1 in uPA−/− mice, indicating that macrophages contribute to IGF-1 expression, among other factors, during muscle regeneration. IGF-1 has been reported to stimulate satellite cell proliferation and differentiation, as well as promote growth of mature muscle fibers (45–47). In addition to IGF-1, macrophages can produce other growth factors, cytokines, and proteinases that have been implicated to help regulate the sequence of events for muscle regeneration (13, 14). The contribution of these factors from macrophages in vivo remains largely unknown.

BM cells can contribute to the formation of regenerating fibers by direct fusion, and this has been reported to occur with frequencies from 0.005 to 5.0% following tissue injury (38, 48). In our experiments, only two GFP-positive fibers were observed after examining >3000 fibers from 10 muscles in recipient mice that received GFP-positive BM. Thus, the transferred BM cells did not directly contribute to formation of regenerating fibers by fusing with these fibers. Instead, uPA expressing macrophages appear to be sufficient to restore activity of resident satellite cells in muscle of uPA−/− mice and rescue muscle regeneration.

Taken together these results indicate macrophage-derived uPA plays a fundamental role in macrophage chemotaxis into injured muscle, in addition to its role in matrix invasion, and is required for efficient muscle regeneration. These data indicate that enhancing macrophage activity or uPA activity in damaged muscle may provide a novel therapeutic approach for improving muscle regeneration.

**Note Added in Proof.** During the review and publishing of this manuscript, another group published findings (49) that are consistent with some of those presented here.

**Disclosures**

The authors have no financial conflict of interest.

**References**


