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THE PLASMINOGEN ACTIVATION SYSTEM IN SKELETAL MUSCLE REGENERATION: ANTAGONISTIC ROLES OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR (UPA) AND ITS INHIBITOR (PAI-1)

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1. ABSTRACT

The plasminogen activation (PA) system is an extensively used mechanism for the generation of proteolytic activity in the extracellular matrix, where it contributes to tissue remodeling in a wide range of physiopathological processes. Despite the limited information available at present on plasminogen activators, their inhibitors and cognate receptors in skeletal muscle, increasing evidence is accumulating on their important roles in the homeostasis of muscle fibers and their surrounding extracellular matrix. The development of mice deficient for the individual components of the PA system has provided an incisive approach to test the proposed muscle functions *in vivo*. Skeletal muscle regeneration induced by injury has been analyzed in urokinase-type plasminogen activator (uPA)-, tissue-type plasminogen activator (tPA)-, plasminogen (Plg)- and plasminogen activator inhibitor-1 (PAI-1)-deficient mice and has demonstrated profound effects of these molecules on the fibrotic state and the inflammatory response, which contribute to muscle repair. In particular, the opposite roles of uPA and its inhibitor PAI-1 in this process are highlighted. Delineating the mechanisms by which the different plasminogen activation system components regulate tissue repair will be of potential therapeutic value for severe muscle disorders.

2. INTRODUCTION

Mammalian adult skeletal muscle constitutes half of the body mass and is a stable tissue with very little turnover under normal circumstances. Muscle plasticity occurs in a number of physiopathological processes including embryonic myogenesis, adult muscle aging, inflammatory myopathies, muscular dystrophies and muscle hypertrophy (1). There is ample evidence that the extracellular matrix (ECM) sourrounding the skeletal muscle tissue plays an important role in maintaining the structure of the muscle and also in providing an environment in which the contractile muscle fibers can function. The plasminogen activation system is one of the main suppliers of extracellular proteolytic activity, thus contributing to ECM degradation and tissue remodeling, which occur in processes such as neurite outgrowth, wound healing, inflammation, angiogenesis and tumor cell invasion (2-4). This brief article aims to revise the contemporary knowledge on the role of the plasminogen activation system in skeletal myogenesis, with special emphasis on skeletal muscle regeneration, where extensive tissue remodeling occurs.

3. THE PLASMINOGEN ACTIVATION SYSTEM

Activation of the zymogen plasminogen into the active serine proteinase, plasmin, is a highly regulated and

widely employed mechanism for the generation of extracellular proteolytic activity. Activation of plasminogen is exerted by two distinct plasminogen activators (PAs), tPA (tissue-type plasminogen activator) and uPA (urokinase-type plasminogen activator) (5,6) (figure 1). uPA binds to cells through the well-characterized uPA receptor (uPAR, CD8), while alpha-enolase and annexin-II have been identified as cellular receptors for plasminogen and tPA, respectively (4,7-9), serving to localize PA and plasmin activities pericellularly. Plasmin is the major enzyme responsible for the dissolution of fibrin at both intravascular and extravascular sites. uPA and plasmin are additionally implicated in numerous non-fibrinolytic processes leading to extracellular matrix (ECM) degradation, either directly by proteolytic cleavage of ECM components such as fibronectin or laminin, or indirectly through the activation of latent matrix metalloproteinases (MMPs) (3,10). Furthermore, uPA and plasmin, as well as some MMPs, have been shown to activate several latent growth factors *in vitro*, including transforming growth factor beta (TGF-beta), hepatocyte growth factor/scatter factor (HGF/SF) and basic fibroblast growth factor (bFGF), whose activities are crucial for cell migration and tissue remodeling *in vivo* (11-14). Thus, several mechanisms account for the important implication of the plasminogen activation/plasmin system in different physiopathological processes involving extracellular ECM degradation, tissue remodeling and cell migration, including mammalian ovulation, trophoblast invasion, post-lactational mammary involution, neurite outgrowth, excitotoxic-induced neuronal death, nerve regeneration, skin wound healing, inflammation, glomerulonephritis, angiogenesis and tumor cell invasion (2-4,15). Because unrestrained generation of proteolytic activity may be hazardous to the cells, plasmin activity is tightly controlled at the level of PAs by plasminogen activator inhibitors (PAI-1 and PAI-2), and at the level of plasmin by □lpha2-antiplasmin (5,6). PAI-1 is the primary physiological inhibitor of uPA. It regulates the proteolytic activity of uPA directly via its serine proteinase activity, and indirectly by regulating the levels of uPA-uPAR complex through promotion of its endocytosis (16-18). uPAR and PAI-1 have also been implicated in non-proteolytic cellular processes. uPAR can directly promote integrin-mediated cell adhesion on a vitronectin (VN) substrate in the presence of uPA (19,20); through its avid interaction with VN, PAI-1 may inhibit VN-mediated cell adhesion and migration (21,22). These interactions also induce cell signaling (8,23,24). Thus, the plasminogen activation system exerts its biological functions not only through the catalytic activity of its components (leading to fibrinolysis and fibrin-independent proteolysis) but also through modulating the interaction between ECM-integrins, which does not necessarily involve catalytic activity of PAs.

4. THE PLASMINOGEN ACTIVATION SYSTEM IN MYOGENESIS

Myogenesis refers to the formation of skeletal muscle either in embryonic development or in pathologies. A key feature of muscle regeneration is the proliferation of muscle precursor cells and their fusion into myotubes or with the ends of the damaged muscle fibers in a manner analogous to developmental myogenesis (25). Despite its complexity, myogenesis has been effectively recapitulated in cell culture *in vitro*; satellite cells (muscle stem cells) can be isolated from skeletal muscle and grown under permissive culture conditions to differentiate, fuse and form myotubes. Cells derived from chicken, mouse, rat and human muscle express most of the proteins of the plasminogen activation system, including uPA, uPAR and PAI-1 (26-31); alpha-enolase plasminogen receptor has also been reported in the C2C12 murine myoblast cell line (32). uPA is able to stimulate proliferation, migration and fusion of satellite cells derived from mice and humans (29,30,33). Conversely, specific inhibition of uPA and plasmin proteolytic activities abrogated migration, fusion and differentiation of murine myoblasts *in vitro* (29,31). Quax *et al*. (1992) demonstrated that the amino-terminal fragment of uPA, which retains its ability to bind to uPAR but has no proteolytic activity, inhibited human myogenesis *in vitro*, suggesting than uPA binding to its receptor is necessary for muscle differentiation (27). Likewise, antibodies against PAI-1, which block uPA-PAI-1 interaction, were able to inhibit human satellite cell migration and fusion (30,34). An antibody against alpha-enolase Plg receptor, which blocks cell surface-associated Plg activation on myoblasts, abrogated murine myoblast fusion and differentiation *in vitro* (32), indicating that uPAR and alpha-enolase may serve to concentrate and enhance uPA and plasmin activities, respectively, on the cell surface of migratory myoblasts, contributing to efficient myogenesis. Evidence of a direct role for uPAR/uPA/PAI-1 tripartite complex during human myogenic migration and cell fusion was also provided by several studies (30,34), suggesting an integrated function of the different components of the PA system in myogenesis, rather than individual requirements. Further studies have shown that growth factor (bFGF, TGF-beta and HGF/SC)-dependent proliferation and migration of satellite cells require the cell-associated plasminogen activation system (35).

5. DIFFERENTIAL ROLES OF THE PLASMINOGEN ACTIVATION SYSTEM COMPONENTS IN MUSCLE REGENERATION

Extracellular proteolysis takes place during skeletal muscle formation and muscle regeneration, in which muscle precursor satellite cells play a major role. In the absence of stress, skeletal muscle is a stable tissue with little turnover; however, after an injury, skeletal muscle has the remarkable ability to initiate a rapid and extensive repair process preventing the loss of the muscle mass (25). Skeletal muscle repair is a highly synchronized process involving the activation of various cellular responses. The initial phase of muscle repair is characterized by necrosis of the damaged tissue and activation of an inflammatory response. The infiltrating inflammatory cells will remove the necrotic tissue and promote revascularization (36). This phase is rapidly followed by activation of muscle satellite cells to proliferate as myoblasts, differentiate and fuse leading to new myofiber formation and reconstitution of a functional contractile apparatus. Activation of adult muscle satellite cells is therefore a key element in this process.

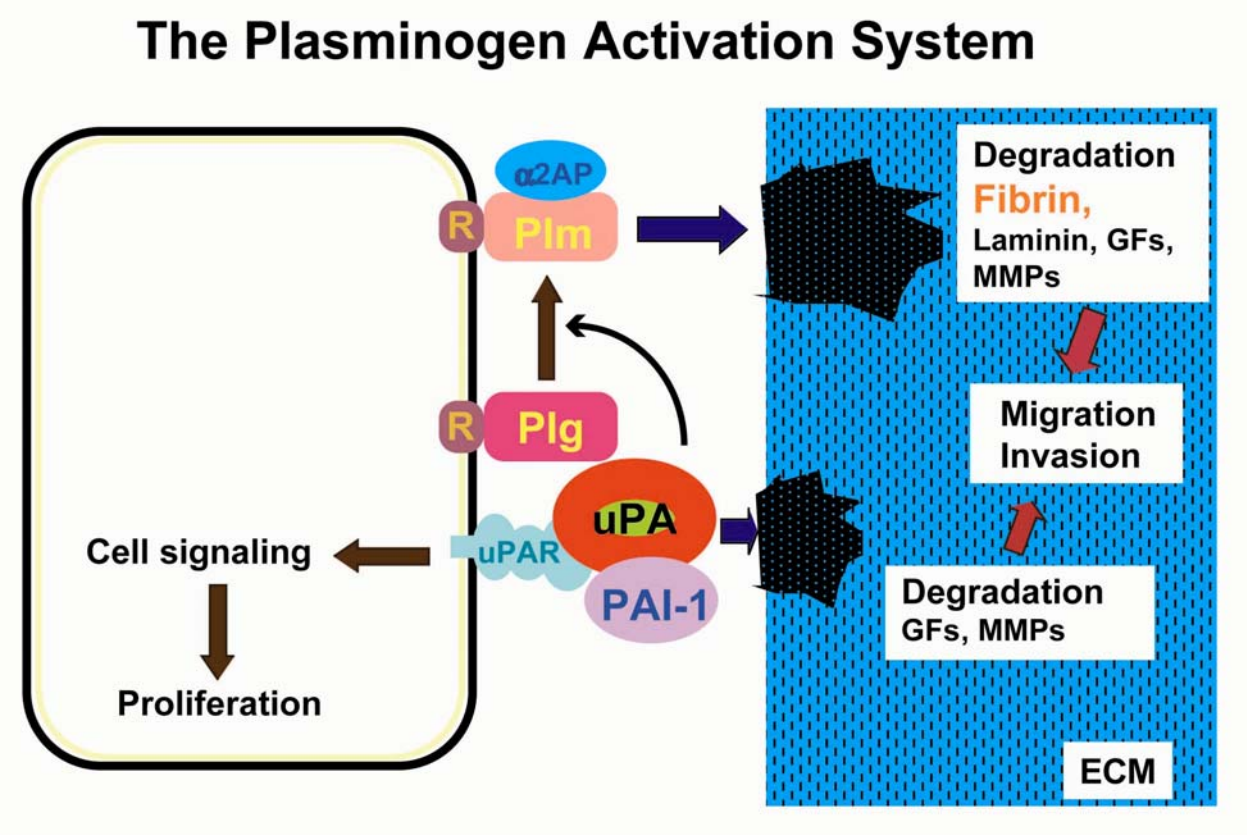

Figure 1. The plasminogen activation system. Active plasmin (Plm) is generated from its zymogen plasminogen (Plg) by urokinase-type plasminogen activator (uPA), which, in turn, can be inhibited by plasminogen activator inhibitor (PAI-1). uPA binds to its specific receptor uPAR while Plg can associate with alpha-enolase Plg receptor on the cell surface. Besides fibrinolysis, uPA and plasmin activities induce different cellular processes, including migration, proliferation and ECM degradation.

Muscle satellite cell activation resembles embryonic myogenesis in several ways including *de novo* induction of myogenic regulatory factors, such as MyoD and myogenin, and embryonic forms of myosin heavy chain (25). A number of proteolytic enzymes have been proposed to play a role during muscle regeneration, either in the inflammatory response, in the activation of satellite cells and/or in the migration of myoblasts across the basal lamina and in their further fusion to form the terminal muscle fiber (37,38). Metalloproteinases such as MMP-2 and MMP-9, meltrin-alpha and cathepsin B seem to be required for myotube formation *in vitro* (39,40). Moreover, the expression of MMP-2 and MMP-9 has been reported in the degeneration-regeneration process of myofibers *in vivo* (40,41). The mechanism of MMP activation in most cell types involves a proteolytic activation cascade initiated by uPA/plasmin (10). Muscle injury induces the expression of uPA, Plg and PAI-1 during the initial regeneration phase (31,42-45). Alpha-enolase Plg receptor and uPAR expression are also upregulated in regenerating muscle (32,46) (Suelves and Munoz-Canoves, unpublished results). Using a genetic approach, our group recently demonstrated that uPA, but not tPA, activity is required for efficient skeletal muscle regeneration *in vivo* (43). Similarly to wild-type mice, tPA-deficient mice completely repaired experimentally-injured skeletal muscle, while uPA-deficient mice were unable to repair the damage (43) (figure 2). Moreover, the muscle regeneration capacity of Plg-deficient mice was severely impeded, indicating that uPA-dependent plasmin activity is necessary for skeletal muscle regeneration *in vivo* (31,47). Interestingly, muscle satellite cells derived from Duchenne Muscular Dystrophy (DMD) patients produce more uPAR and PAI-1 and less uPA than normal satellite cells, being its invasion capacity affected by the differential expression of these PA system components (33,35). Here, we provide evidence that the muscle regeneration process following cardiotoxin-induced injury is accelerated in PAI-1-deficient mice, as evidenced by the reduced extent of degeneration and increased percentage of centrally-nucleated fibers (CNF) -a marker of regeneration- at early times after injury with respect to wild-type mice (figure 2). On the basis of the distinct muscular alterations observed in the individual knock-out mice of the PA system after injury, we conclude that the PA system components play differential roles in muscle regeneration: uPA and plasmin activities are necessary for this process, whereas that of tPA is dispensable, indicating that no redundancy exists between both PAs in muscle; in contrast, PAI-1 deficiency accelerates the muscle regeneration process.

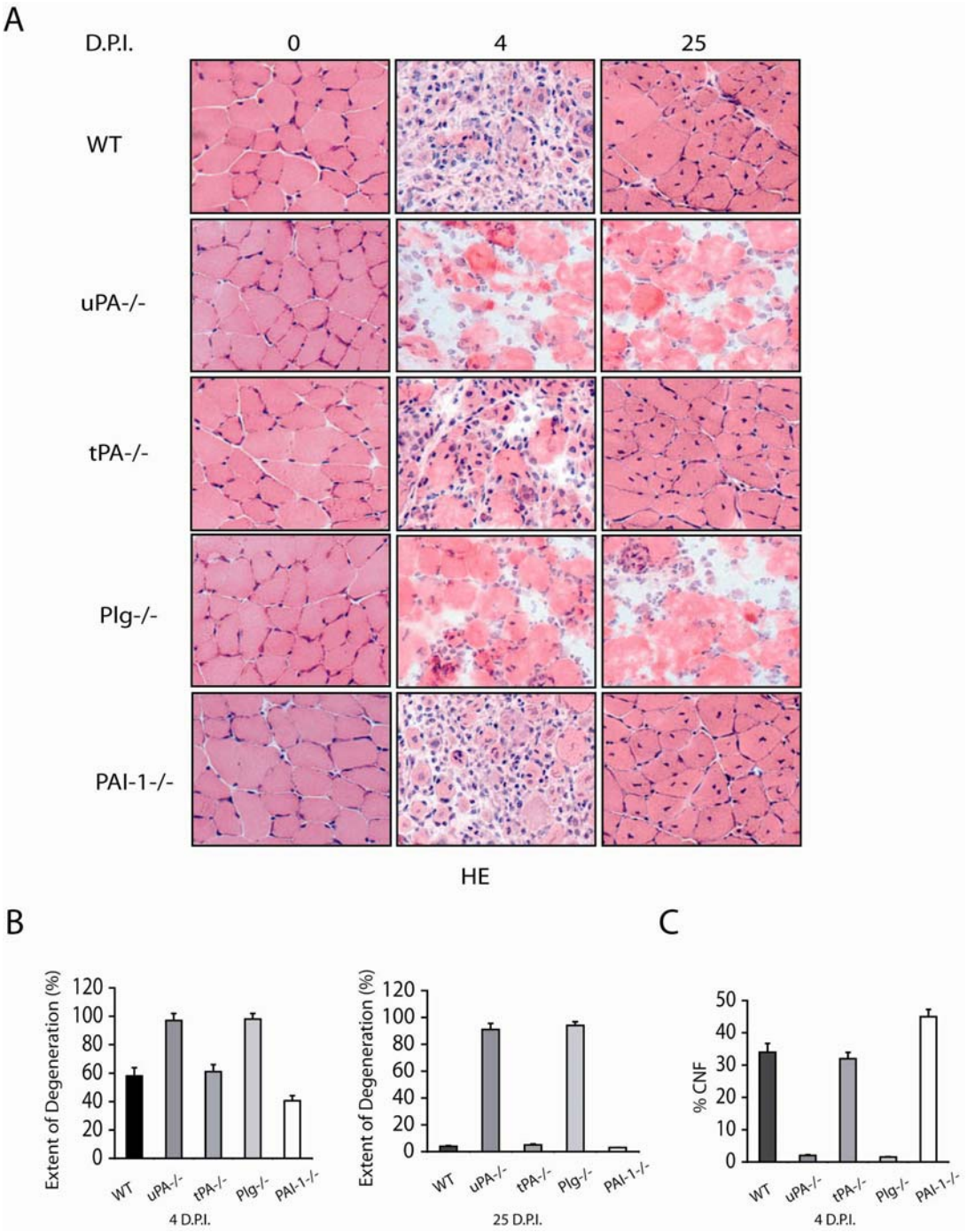

Figure 2. uPA- (but not tPA-) and Plg-deficient mice show a persistent regeneration defect following muscle injury while PAI-1-deficient mice exhibit an accelerated regeneration, with a reduced extent of myofiber degeneration and increased percentage of centrally-nucleated fibers (CNF). A. Frozen sections of injured muscles from wild-type (WT), uPA-, tPA-, Plg- and PAI-1-deficient mice, respectively, were stained with Hematoxilin/Eosin (HE) at 4 and 25 days post injury (P.I.). Contralateral control muscles were also stained with HE (0 days P.I.). In WT, tPA- and PAI-1-deficient mice, regeneration is complete after 25 days, as evidenced by the presence of centrally nucleated fibers (CNF). In uPA- and Plg-deficient mice, however, a regeneration defect is still visible 25 days post injury. B. Analysis of muscle fiber degeneration (%) was determined microscopically and expressed as a percentage of the total muscle area. C. Assessment of muscle fiber regeneration. Muscle fiber regeneration (%) was quantified on micrographs and expressed as the percentage of total muscle fibers containing central nuclei present in the entire cross-section of the muscle. PAI-1-deficient mice show a reduced extent of degeneration and increased regeneration with respect to WT mice 4 days post injury.

6. MECHANISMS IMPLICATED IN THE MYOGENIC FUNCTION OF THE PLASMINOGEN ACTIVATION SYSTEM

The fact that loss of uPA and plasmin activities impedes while PAI-1 accelerates muscle regeneration raises the question of how the plasminogen activation system is involved in tissue repair. Previous studies have shown an accumulation of extravascular fibrin in the regenerating muscle of uPA- and Plg-deficient mice (31,43), indicating the importance of uPA-mediated plasmin activity in muscle fibrin clearance. Extravascular fibrin deposition is a key feature in pathologies characterized by inflammation and tissue repair, including impaired skin wound healing, nerve remyelination and glomerulonephritis (48-51). Fibrin accumulation in the extracellular basal membrane may have deleterious effects, such as the impediment of normal nutrition to the muscle tissue. The hypothesis that increased fibrin levels may contribute to impaired muscular regeneration was further explored by systemic fibrinogen depletion of uPA- and plasminogen-deficient mice. Administration of the defibrinogenating agent ancrod reduced plasma fibrinogen levels in both knock-out mice, resulting in an almost complete restoration of the normal muscle regeneration process, demonstrating that fibrin accumulation has a pathogenic role in sustaining muscle degeneration (31,43).

Inflammation is a process frequently associated to tissue repair, since degenerating tissues are invaded by inflammatory cells. Accordingly, in response to muscle injury neutrophils, macrophages and T lymphocytes accumulate near the injury site in mice and rats during the inflammatory response (25,36,52). Mice with a specific deficit in uPA and Plg show a reduced staining for Mac-1- and T11-positive cells two days after injury, indicating that the number of macrophages and T lymphocytes reaching the injury site is reduced in the absence of uPA and Plg (31,43), suggesting that uPA/plasmin activity may have a profound effect on inflammation and inflammation-related muscular disease. Previous studies with uPA-deficient mice demonstrated that uPA is required for the pulmonary inflammatory response to *Cryptococcus neoformans*, since a lack of uPA resulted in inadequate macrophage recruitment, uncontrolled infection and death (53). Similarly, monocyte and lymphocyte recruitment was significantly diminished in Plg-deficient mice after thioglycollate-induced acute peritoneal inflammation (54). Additionally, components of the PA system may regulate the expression or/and activity of cytokines involved in inflammatory processes. Plasmin has been shown to release macrophage derived interleukin-1 and to activate TGF-beta (55,56). Thus, the reduced presence of macrophages and T lymphocytes in the injured muscles of uPA- and Plg-deficient mice might be due either to a reduction in the migration capacity of inflammatory cells devoid of plasmin activity, or to a reduced potential of these cells to traverse fibrin-rich matrices. Furthermore, it has been shown that activated macrophages (equivalent to those which accumulate at the site of muscle damage) produce soluble factors (FGF and PDGF) which are highly chemoattractant and also mitogenic for muscle precursor cells (57). Thus, the activated macrophages which accumulate in response to muscle damage will not only phagocytose necrotic tissue but also facilitate the repair of damaged myofibers. In this model, one major matrix component within damaged areas that may represent a particular impediment to inflammatory cell migration in the absence of Plg is fibrin. However, based on the existing data, we cannot exclude that plasmin deficiency may impede cell migration because of the lost contribution of plasmin to degrade other matrix components or the lack of other key matrix proteinases or of growth factors. Since most MMPs then can be directly activated by cleavage to a lower molecular weight protein by plasmin (10), it is temping to speculate that activation of MMP-2 and MMP-9 in regenerating skeletal muscle after cardiotoxin injury might also be mediated by plasmin proteolysis. Furthermore, uPA/plasmin can cleave and activate latent forms of growth/angiogenic factors such as bFGF, TGF-beta and HGF/SF (14,25,58), which are expressed within injured muscle and are believed to promote the activation of quiescent satellite cells after injury *in vivo* (59-61). Therefore, modulation of plasminogen activation activity may indirectly influence satellite cell recruitment and the growth and differentiation of cellular constituents in regenerating muscle. Based on this, future experiments in the field will surely be directed toward further definition of the benefit of defibrinogenating, anticoagulant, or fibrinolytic agents in muscle pathologies.

7. PERSPECTIVE

The results discussed in this article support a role for uPA-mediated plasmin generation in myogenesis *in vitro* and in regeneration of skeletal muscle *in vivo*. Moreover, evidence is here provided that muscle degeneration is reduced and regeneration accelerated in PAI-1-deficient mice after injury. Thus, a potential collaboration among the different components of the plasminogen activation system in this complex biological process can be envisioned. Cellular penetration of fibrin-containing matrices during skeletal muscle regeneration may be facilitated by the local conversion of plasminogen to plasmin and subsequent fibrin degradation. Independent studies have reported that the uPA/uPAR/PAI-1 tripartite complex can alter cell migration by modulating the interaction of the integrin $alpha_v beta_3$ with its ligand vitronectin. Thus, protease-dependent and –independent activities may account for the role of the plasminogen activation system components in muscle function. Therefore, delineating the mechanisms by which the plasminogen activation system regulates myogenesis and muscle tissue repair will be the aim of future studies.

8. ACKNOWLEDGEMENTS

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