Multifunctional roles of MT1-MMP in myofiber formation and morphostatic maintenance of skeletal muscle

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Summary
Sequential activation of muscle-specific transcription factors is the critical basis for myogenic differentiation. However, the complexity of this process does not exclude the possibility that other molecules and systems are regulatory as well. We observed that myogenic differentiation proceeded through three distinct stages of proliferation, elongation and fusion, which are distinguishable by their cellular morphologies and gene expression patterns of proliferation- and differentiation-specific markers. Treatment of the differentiating myoblasts with inhibitors of matrix metalloproteinases (MMPs) revealed that MMP activity at the elongation stage is a critical prerequisite to complete the successive myoblast cell fusion. The MMP regulated the myogenic differentiation independently from the genetic program that governs expression of the myogenic genes. Membrane-type 1 matrix metalloproteinase (MT1-MMP) was identified as a major contributor to this checkpoint for morphological differentiation and degraded fibronectin, a possible inhibitory factor for myogenic cell fusion. A MT1-MMP deficiency caused similar myogenic impediments forming smaller myofibers in situ. Additionally, the mutant mice demonstrated some central nucleation of the myofibers typically found in muscular dystrophy and MT1-MMP was found to cleave laminin-2/4 in the basement membrane. Thus, MT1-MMP is a new multilateral regulator for muscle differentiation and maintenance through processing of stage-specific distinct ECM substrates.

Key words: MT1-MMP, ECM remodeling, Myogenesis, Myopathy

Introduction
Mammalian skeletal musculature is essential for body movement, breathing and postural behavior. Although the tissue is stable under normal conditions, skeletal muscle can regenerate when a loss of muscle mass occurs after injury. Muscle satellite cells that reside in the skeletal musculature act as myoblast precursors and regenerate myofibers through a process similar to embryonic myogenesis (Schultz et al., 1985). Although muscle regeneration requires multiple aspects of tissue organization, only the genetic program that governs myoblast differentiation has been studied extensively. Muscle injury generates signals that activate quiescent satellite cells, leading them to proliferating myoblasts that are characterized by an up-regulation of MyoD, a basic helix-loop-helix transcription factor (Bischoff, 1986; Cooper et al., 1999). Upon differentiation, the myoblasts withdraw from the cell cycle and enter terminal differentiation, during which additional transcription factors, such as myogenin and myogenic regulatory factor 4 (MRF4), that regulate late phase myogenesis, are up-regulated (Nabeshima et al., 1993; Zhu and Miller, 1997). These MRFs also direct the expression of muscle-specific genes, such as myosin heavy chain (MHC) and muscle creatine kinase (MCK) by binding to their promoter sequences directly (Chakraborty et al., 1991; Wheeler et al., 1999).

Concomitant with the cellular genetic program are dramatic changes in cell morphology. Under in vitro culture conditions, the proliferating myoblast morphology resembles that of fibroblasts. Once shifted to a differentiation condition, the myoblasts cease proliferation and begin elongating and becoming spindle shaped with bipolar edges. Finally, the elongated myoblasts fuse to form large multinucleated myotubes that correspond to the myofibers found in situ in skeletal muscle tissues. The differentiation proceeds in the tissue environment surrounded by extracellular matrix (ECM) and the differentiated myofibers must be integrated into the skeletal muscle framework to form a functional contractile unit. Thus, it is not surprising that the ECM molecules also influence the differentiation process. For example, excess exogenous fibronectin, a glycoprotein that binds to ECM components including collagen, fibrin and heparin, inhibits myotube formation (Podleski et al., 1979). By contrast, myotube formation is enhanced by laminin, a component of the basement membrane (Foster et al., 1987). Thus, it appears that some ECM components play different roles during myocyte differentiation, and thus must be substituted with appropriate molecules, depending on the differentiation stages. Indeed, the fibronectin concentration detected on myoblast surfaces is reported to decrease during myoblast differentiation (Chen, 1977).
Substitution of the ECM components requires inhibiting their gene expression, and a concerted action of proteinases, such as the matrix metalloproteinases (MMPs) (Mott and Werb, 2004), to clear the previous ECM deposit in the outside matrix. However, information regarding MMPs during myogenesis and regeneration is quite limited. Previous studies indicate that satellite cells and myoblasts produce MMP-2, MMP-9, and MT1-MMP (Lluri and Jaworski, 2005). Expression of MMP-2 was also detected in the regenerating skeletal muscle after experimental injury was induced by tissue-damaging agents (Kherif et al., 1999). However, roles of these MMPs on skeletal muscle differentiation have not been characterized clearly.

In this study, we firstly evaluate the role of MMPs in muscle differentiation using the C2C12 myoblast cell line. Differentiation of the C2C12 cells proceeded through three distinct stages of proliferation, elongation and fusion, as characterized by their morphologies and gene expression patterns. Pulse-treatment of the cells with the broad-spectrum MMP inhibitor batimastat (BB94) revealed that impaired MMP activity at the elongation stage was sufficient to induce significant reduction of myotube formation at fusion stage. Since BB94 did not affect the expression of the myogenic transcription factors or their target genes, ECM remodeling by MMPs at this particular stage regulates the morphological differentiation independently from the molecular differentiation event. We then focused on the function of MT1-MMP, a potent modulator of cellular functions though pericellular proteolysis and a specific activator of proMMP-2 (Seiki, 2003; Itoh and Seiki, 2006), since both MMP-2 and MT1-MMP were expressed in the elongation stage accompanying transient decrease of TIMP-2 expression. A specific role for MT1-MMP in the differentiation was evaluated using short hairpin RNA (shRNA) and it eventuated less myotube formation with an impaired fusion step. Inhibition of MT1-MMP expression or treatment of the cells with BB94 caused accumulation of fibronectin bound to the cells. Finally, we confirmed the role of MT1-MMP in myogenesis in vivo, using MT1-MMP-deficient mice. The deficient mice had smaller and heterogeneous myofibers compared with those in the wild-type mice, supporting our observation in vitro that myofiber formation is severely affected by the absence of MT1-MMP. In addition, we noticed that some centrally nucleated myofibers exist in the mutant mice. These fibers represent those that occur during muscle regeneration following tissue damage, and are typical in patients with muscular dystrophy. This observation suggests that, apart from its role in myogenesis, MT1-MMP also maintains myofiber integrity. Muscular dystrophy is caused by defects in the laminin-based cell adhesion system (Wewer and Engvall, 1996; Hayashi et al., 1998; Cohn and Campbell, 2000). Indeed, laminin-2/4 was directly processed by MT1-MMP and we found abnormal processing of laminin-2/4 in the mutant mice. Thus, this study illuminates new roles for MT1-MMP in muscle tissue development and its maintenance.

Results
Myogenesis in vitro proceeds through three distinct stages
We employed a well-characterized in vitro myogenic differentiation model by using the C2C12 mouse myoblast cell line. We added insulin-like growth factor 1 (IGF-1) into the serum-free culture medium of the C2C12 cells to cease proliferation and induce differentiation. Under our culture conditions, the proliferating cells resembled fibroblasts (Fig. 1A, 0 hour) initially, and started to elongate into bipolar structures characteristic of the differentiation process (Fig. 1A, 48 hours). Then they fused together, forming multinucleated myotubes (Fig. 1A, 96 and 144 hours). In addition to tracking these morphological changes, we monitored with immunostaining the expression levels of the proliferating cell nuclear antigen (PCNA) and the myosin heavy chain (MHC), which are proliferation and differentiation protein markers, respectively. Before administering IGF-1 (0 hours), the myoblasts expressed PCNA (Fig. 1A); PCNA expression decreased sharply afterward, and was not detectable after 48 hours. Conversely, the expression level of MHC was not detectable during this first period (0 and 48 hours), when the cells typically proliferate and elongate. The MHC staining was detectable at 96 hours, when the elongated cells began fusing, and was measurable thereafter (144 hours). These results define three distinct morphological and molecular stages of proliferation, elongation and fusion. We measured cellular proliferation by counting the nuclei, and evaluated the cell fusion ratio with the number of nuclei in multinucleated myotubes. The cellular proliferation activity ceased after 24 hours, which coincided with the observed decrease in PCNA expression (Fig. 1B). A few multinucleated myotubes appeared at 72 hours, and gradually increased thereafter. The number of fused cells reached 40-50% at 144 hours. Thus, the time windows of the three stages roughly correspond to 0-24 hours, 24-72 hours, 72-144 hours, for the proliferation, elongation, and fusion stages, respectively.

MMP-dependent checkpoint in the elongation stage regulates successive cell fusion
To evaluate the role of MMPs during myogenic differentiation, we added the synthetic broad-spectrum metalloproteinase inhibitor BB94, and monitored cell fusion by counting the numbers of nuclei in the multinucleated cells at 144 hours. The commitment of the cells to terminal differentiation was confirmed by immunological detection of myogenin, a transcription factor acting as a differentiation switch. Initially, we administered BB94 throughout the differentiation process, including the elongation and fusion stages (Fig. 2A, +/+). BB94 inhibited the cell fusion events significantly, even though the cells were committed to terminal differentiation. The cell viability was not affected by BB94, as determined by the nuclear count at 144 hours. To explore whether the MMPs are required during a defined time period, we pulse-treated the differentiating cells with BB94, during the elongation (+/-) and fusion (-/+) stages. As demonstrated in Fig. 2B, treating the cells during elongation was sufficient to abrogate the fusion step, while cell fusion itself was not affected by BB94. Thus, we demonstrated that the elongation stage, characterized by cell morphology and marker gene expression, is a time during which MMP activities are critically required to prepare the extracellular environment for cell fusion.

In spite of its ability to inhibit morphological differentiation, BB94 did not significantly impact the number of myogenin-positive cells (Fig. 2B). Examination of differentiation gene marker expression levels revealed that the MCK and
embryonic myosin heavy chain (eMyHC) levels were not altered (Fig. 2C). Immunostaining of the cells with anti-MHC antibody (Fig. 2D) also confirmed that inhibiting cell fusion does not impact the expression of MHC. Since myogenic differentiation proceeds by successive activation of multiple transcriptional factors, including myogenic regulatory factors (MRFs), myocyte enhancer factor 2 (MEF2) and serum response factor (SRF) (Duprey and Lesens, 1994; Rudnicki and Jaenisch, 1995; Naya and Olson, 1999), we further tested effect of BB94 on the activity of these endogenous transcription factors using the reporter luciferase gene with their binding sites cloned into the Luc promoter region. These results yielded no significant effect as demonstrated in Fig. 2E. All of these results suggest that BB94-inhibited morphological differentiation does not require differentiation-related gene expression modulation. Thus, the BB94-sensitive step in the elongation stage appears to regulate the morphological and genetic differentiation processes independently.

Expression of MMPs in the differentiating myoblasts

MMP-2 is reportedly expressed in C2C12 cells (Kherif et al., 1999; Lluri and Jaworski, 2005). We monitored expression of MMP-2 mRNA in the cells during differentiation with semi-quantitative RT-PCR. The MMP-2 mRNA levels were low in the myoblasts, and increased upon induction of differentiation, particularly during elongation (Fig. 3A). The expression level peaked at the end of elongation (72 hours), and then declined to basal levels. By contrast, the TIMP-2 mRNA levels rapidly decreased straddling the transition from proliferation to elongation, gradually recovered reaching to the former state only at the end of elongation, and then tended to decrease again during fusion.

The activity of MMP-2 was also monitored by gelatin zymography (Fig. 3B, upper panel), and the expression of MT1-MMP, a specific activator of proMMP-2, was assessed by western blotting (middle panel). According to the induction of MMP-2 mRNA, the gelatinolytic activities corresponded to the latent precursor (pro), intermediate (int), and activated MMP-2 (act), which increased during elongation. Consistent with MMP-2 activation was the detection of cellular MT1-MMP. The active form of MT1-MMP (60 kDa, closed arrow head) was detected throughout the differentiation process, it increased during the elongation (48 and 72 hours) and gradually declined to basal level during fusion stage (72–144 hours). An auto-degradation product (43 kDa indicated by the open arrowhead) was only faintly observed throughout the differentiation process. Thus, the BB94-sensitive elongation stage is the period during which MMP-2 and MT1-MMP expression levels increase, accompanying a transient drop in TIMP-2 levels. The balance between TIMP-2 and the MMPs in elongation stage appears to be preferable for MMP-dependent proteolysis.

MT1-MMP contributes to the MMP-dependent checkpoint

Since BB94 can inhibit metalloproteinase family members other than the MMPs, we used two natural MMP-specific inhibitors, TIMP-1 and TIMP-2 (Baker et al., 2002) to confirm the involvement of MMPs in the elongation stage. TIMP-1 inhibits all soluble MMPs and some ADAM (a disintegrin and metalloproteinase) family members, though it does not inhibit MT-MMPs. TIMP-2...
inhibits all MMPs including MT-MMPs but not ADAMs. For these experiments, we pulse-treated differentiating C2C12 cells with the TIMP inhibitors during elongation, and we monitored the resulting cell fusion events (Fig. 4A). BB94 inhibited the fusion ratio by about 60%, compared with the non-treated control (NT) and solvent alone (DMSO). TIMP-1 and TIMP-2 inhibited myoblast cell fusion significantly, and the combination of TIMPs 1 and 2 inhibited cell fusion as effectively as BB94 (Fig. 4A). Thus, the target proteinases that were inhibited by BB94 are most likely MMPs.

After determining that MMP-2 and MT1-MMP are expressed in the elongation stage, we tried to evaluate whether these MMPs are the enzymes preparing the ECM environment for the successive fusion stage. The membrane-bound MT1-MMP appears particularly important for cell elongation because it is a potent pericellular modulator, and is the upstream activator of MMP-2. Thus, we focused on determining the function of MT1-MMP. Constitutive expression of the anti-MT1-MMP shRNA (shMT1-MMP), under the control of the human U6 promoter in C2C12 cells, reduced MT1-MMP protein levels throughout the differentiation process. The knockdown efficiency of MT1-MMP was over 90% at 0 hour (Fig. 4B), and was comparable even at 144 hours (unpublished observation). A reduction in MT1-MMP expression rendered the formation of fewer myotubes, compared with the control shRNA against lacZ (Fig. 4B). Thus, MT1-MMP is likely one of the major MMP-dependent checkpoint regulators for myotube formation.

Fibronectin has been implicated as a negative regulator of myogenic differentiation (Podleski et al., 1979), and a major ECM component of proliferating myoblasts. Fibronectin levels are reduced significantly as myogenic differentiation proceeds at the transcription and translation levels. Fibronectin is also a substrate of MT1-MMP, as demonstrated in previous biochemical studies (Ohuchi et al., 1997). These findings prompted us to measure the relative amounts of fibronectin in...
differentiating C2C12 cells, which we accomplished by western blotting (Fig. 4C). The fibronectin levels decreased markedly to roughly 10% by the end of elongation (Fig. 4C, 72 hours; NT, DMSO, and shLacZ). By contrast, treatment of the cells with MMP inhibitors or shRNA to down-regulate MT1-MMP, prevented fibronectin level reductions significantly (72 hours; BB94, TIMP-1, TIMP-2, and shMT1-MMP). It is interesting to note that the relative amount of the remaining fibronectin at 72 hours correlates inversely with the inhibition rate of cell fusion (Fig. 4A,B). Thus, fibronectin appears to represent the ECM environment that is modulated by MMPs during elongation.

Expression of MT1-MMP and MMP-2 in regenerating skeletal muscle

To confirm the MMP expression patterns during myogenic differentiation in vivo, we injured the muscle tissues of wild-type mice by injecting cardiotoxin (CTX) at the tibialis anterior (TA) muscle, and examined any subsequent tissue regeneration (Fig. 5). Within 24 hours after tissue damage, we observed the infiltration of numerous cells in the interfiber spaces containing necrotic myofibers (Fig. 5A, day 1). The number of the infiltrating polymorphonuclear leukocytes (PMNs) increased until 3 days post-injury, and decreased thereafter. Following their disappearance, newly formed myofibers appeared at day 4 (arrow head), and were fully apparent at 5 days post-injury. Subsequently, the small myofibers increased in diameter gradually, and formed morphologically normal myotubes at 14 days, although their nuclei remained localized at the center of the cytoplasm.

The MMP-2 activity in the regenerating tissue was monitored by gelatin zymography (Fig. 5B). No apparent gelatinolytic activity was observed in the untreated control tissue (0 day). Most of the extensive gelatinolytic activities observed at day 1 appear to represent undefined proteases released from the necrotic cells. With this assay, the gelatinolytic activity of the latent and active forms of MMP-2 was detected at 60 kDa (closed arrowhead) and 55 kDa (open arrowhead), respectively. Both forms of MMP-2 activities were induced in the regenerating muscle, and reached their maximal activity levels with frequent activation 5 and 6 days later, after which time they decreased slightly. This observation is consistent with that reported previously by Kherif et al. (Kherif et al., 1999) and further confirmed by their loss in the MMP-2 null mice used for the same experiment (unpublished observation). Concomitant with the MMP-2 activation was the active-form of MT1-MMP (60 kDa) that we detected by immunobloting during days 4-10 (Fig. 5B, closed arrowhead). An auto-degraded form (43 kDa), which represents the active state of MT1-MMP (Osenkowski et al., 2004), was also detected at day 4 (Fig. 5B, open arrowhead). Thus, MMP-2 and MT1-MMP are expressed in the C2C12 cells and in the mouse tissue, with similar time courses during myogenic differentiation. However, we do not exclude the possibility that MT1-MMP expressed by some other types of cells such as inflammatory macrophages have some contribution to the result.

Morphologically altered myofibers in MT1-MMP-deficient mice

Our cell culture assays indicated that MT1-MMP contributes, at least partially, to the MMP-dependent myogenesis checkpoint. To evaluate this effect in vivo, we analyzed the skeletal muscle of MT1-MMP-deficient mice. These mutant mice are viable for only several weeks post-gestation, presenting with severe bone deformation and joint fibrosis defects. The mutant tissues appeared normal, with no adverse histology at 1 week post-gestation (unpublished observation). However, we observed tissue abnormalities 4 weeks after...
The wild-type mice possessed morphologically homogeneous myofibers (Fig. 6a) attached together via the surrounding ECM through cell adhesion molecules, such as those visualized with the anti-β-dystroglycan antibody assay (Fig. 6c; anti-β-DG). By contrast, the myofiber sizes in the mutant mice were heterogeneous, including smaller less well-developed, sometimes angulated, and faintly hypertrophied muscle fibers (Fig. 6b,d). In summary, we found that all tissues at 1 week post-gestation were indistinguishable among the genotypes. Difference in myofiber diameter among the genotypes gradually became evident at 2-3 weeks later (unpublished observation), and therefore, myofiber heterogeneity observed in the mutant mice at 4 weeks post-gestation appears to represent the impaired postnatal myofiber development accumulated this period. Thus, we think the phenotype of the mutant mice reflects the defect of myotube formation caused by knockdown of MT1-MMP in C2C12 cells. These deficits were never observed among the MMP-2 deficient mice at the same age (unpublished observation) suggesting that MMP-2 is dispensable for the myotube formation.

To examine the accumulated ECM components deposited among the myofibers in situ, we compared the tissue sections using elastica van gieson (EVG) staining of the interstitial matrix, and reticulin silver impregnation (silver staining) of the basement membrane. The EVG-staining revealed apparent peripheral fibrosis in the mutant mice, and a slightly accumulated deposition of the interstitial matrix between the myofibers (Fig. 6f), compared with the wild-type mice (Fig. 6e). A slightly thickened basement membrane was also observed with the silver staining (Fig. 6g,h).

Another prominent histological feature in the mutant mice was the centrally located nuclei in the myofibers (Fig. 6b). The nuclei of the normal mature myofibers tended to localize at the periphery (Fig. 6a), while those undergoing regeneration were more centrally localized, as illustrated in Fig. 5A. The pattern observed in the mutant mice also resembles that in muscular dystrophy mice, whereby continuous tissue destruction and regeneration occurs in skeletal muscle. Thus, the myofibers of the mutant mice appeared fragile, and some of the fibers appeared to be undergoing regeneration after postnatal injury. These centrally nucleated myofibers first appeared at 4 weeks after birth. Because of the different timing of appearance of the defects, underlying mechanisms that cause aberrant myotube formation and centrally nucleated myofibers seem to be different. Thus, MT1-MMP may also play a role in maintaining muscle tissue integrity.

**MT1-MMP-deficient mice accumulate abnormally processed laminin-2/4 in the skeletal muscle**

An analysis of the gene perturbations underlying hereditary human muscular dystrophy has revealed dysfunctions of the laminin-based cell adhesion molecule system. Since MT1-MMP reportedly cleaves laminin-1 and -5, the similar muscle-enriched laminin-2/4 complex in the basement membrane is also a possible target of this protease. Thus, we examined laminin-2/4 in the skeletal muscle of the mutant mice, focusing on the laminin α2 chain that directly mediates adhesion to laminin receptors on myotubes.

Skeletal muscle extracts from 4-week old wild type and mutant mice were analyzed by immunoblotting with the anti-300K antiserum raised against the LG2 domain of the laminin α2 subunit (Fig. 7A,B). Laminin α2 was detected as three molecular species in the control blots. The largest band (a) represents the well-characterized 300 kDa N-terminal fragment generated by a single cleavage within the LG3 domain.
(Fig. 7A) (Smirnov et al., 2002). Other 250 kDa (b) and 230 kDa (c) bands are thought to be N-terminally truncated products. By contrast, the MT1-MMP-deficient mice contained only the 300 kDa and 230 kDa bands, and accumulated the larger one to a slightly higher degree. Thus, generation of the 250 kDa fragment appears to depend on MT1-MMP function.

To confirm this hypothesis in vitro, we used purified human laminin-2/4, which shares a high sequence identity with the mouse molecule (97% amino acid identity). The purified human laminin contained 300 kDa and 230 kDa bands, though the intensity of the 250 kDa band was barely visible (Fig. 7C, anti-300K). Two additional fragments of 170 and 140 kDa were also detected. Incubating this laminin preparation with the catalytic fragment of the recombinant MT1-MMP increased the amount of the 250 kDa band significantly, and produced a new 40 kDa band. Production of these bands by MT1-MMP was completely inhibited by BB94.

We used another antiserum raised against the IVa domain (Fig. 7A), located in the N-terminal portion, to provide a more extensive characterization of laminin processing by MT1-MMP (Fig. 7C, anti-IVa). We detected two 300 kDa and 230 kDa bands in the original preparation, and 250 kDa and 280 kDa bands upon incubation with MT1-MMP. Since the 250 kDa fragment reacts to both the anti-300K and -IVa, antibodies, it is probable that this processing occurs at the N-terminal portion (Fig. 7A, open arrow). These results also suggest that additional MT1-MMP cleavage sites exist. The 40 kDa band was detected with the anti-300K antibody, but not with anti-IVa. Conversely, the 280 kDa band was detected with anti-IVa, but not with anti-300K. Thus, we predict that the additional processing site is located in close upstream proximity to the anti-300K recognition site (Fig. 7A, closed arrow). In conclusion, the 250 kDa fragment observed in the mouse skeletal muscle appears to be dependent on the direct proteolytic activity of MT1-MMP.
myogenesis, as demonstrated by conditional environment is important for ensuring successful intrinsic genetic program regulated by MRFs, the ECM fused myoblasts (Nabeshima et al., 1993). In addition to the differentiated myofibers in their limbs, and accumulate non-differentiation. Myogenin mutant mice do not develop well-transcription factors, such as myogenin, to complete terminal a myogenic linage, and require additional myogenic MyoD- and Myf5-expressing myoblasts are committed to Myotube formation is controlled by two checkpoints, Discussion Myotube formation is controlled by two checkpoints, myogenin expression and ECM switching MyoD- and Myf5-expressing myoblasts are committed to a myogenic linage, and require additional myogenic transcription factors, such as myogenin, to complete terminal differentiation. Myogenin mutant mice do not develop well-differentiated myofibers in their limbs, and accumulate non-fused myoblasts (Nabeshima et al., 1993). In addition to the intrinsic genetic program regulated by MRFs, the ECM environment is important for ensuring successful myogenesis, as demonstrated by conditional β1 integrin knockout studies, or by competition of ECM binding with RGD peptides (Osses and Brandan, 2002; Schwander et al., 2003). In addition, the ECM composition must be changed during this process. For example, the initial interstitial ECM environment required by myoblasts must be cleared, and ultimately supplemented with components of the basement membrane that surround the myofibers (Rao et al., 1985). Thus, the ECM has to be remodeled in a dynamic manner, according to myogenic differentiation procedures. In this study, we demonstrated that muscle cells require a critical MMP-dependent step in order to complete myotube formation, and the checkpoint exists during the elongation stage, where the expression levels of MMP-2 and MT1-MMP increase with a concomitant down-regulation of TIMP-2. Pulse treatment of the C2C12 cells with an MMP inhibitor, BB94, at this stage was sufficient to disturb morphological differentiation, as evaluated by our cell fusion analysis. However, this was not the result of down-regulating the late phase MRFs, such as myogenin, because MMP inhibition did not alter the expression level and transcriptional activity of the MRFs. Thus, the cells committed to terminal differentiation appear to be regulated by two independent mechanisms, the late phase MRF-dependent and the MMP-dependent checkpoints. We identified MT1-MMP as a major MMP checkpoint regulator by inhibiting its expression with shRNA. However, it is likely that other MMPs also contribute to this checkpoint, since shMT1-MMP inhibited muscle cell fusion only in part compared with BB94 treatment, and TIMP-1, which does not inhibit MT1-MMP, was also an effective inhibitor for the cell fusion.

Effect of metalloproteinase inhibitors on myotube formation The effects of metalloproteinase inhibitors on differentiating myoblasts have been studied previously, with some confounding results probably coming from different inhibitor spectrums (Couch and Strittmatter, 1983; Huet et al., 2001). The inhibitor that we used herein, particularly TIMP-2, is MMP-specific, though TIMP-1 inhibits some ADAMs, and these inhibited myotube formation efficiently. By contrast, Huet et al. (Huet et al., 2001) found that some hydroxamate-based inhibitors promote myotube formation of C2C12 cells without blocking cell fusion, and they discussed that these inhibitors used might recruit ADAMs rather than MMPs as inhibitor targets. Consistent with this consideration, we also experienced that transient expression of TIMP-3, which inhibits many ADAMs compared with TIMP-1 and TIMP-2, in our C2C12 cells promoted myotube formation (unpublished data). Thus, there is a possibility that ADAMs and MMPs regulate myoblast differentiation at different steps, though additional studies are required to support this hypothesis.

Another point is whether myoblast cell fusion into myotube is just delayed or irreversibly inhibited by inhibition of MMPs. For the C2C12 culture model, we could not check the later time point after 144 hours because the myotubes do not retain their physiological morphology beyond this time point. However, we still think there is a possibility that inhibition of MMPs causes delayed myotube formation, particularly when the inhibition is incomplete and ECM turnover proceeds slowly by the residual proteinases. The phenotype observed with MT1-MMP-deficient might be explained by the delayed myotube formation with accumulating ECM. Even these discussions were taken into account, it seems clear that the protease-dependent ECM switching is a critical part of the morphological differentiation and MT1-MMP is surely playing a role in this process.
Role of fibronectin in myotube formation

During myoblast differentiation, the ECM surrounding the cell must be remodeled from an interstitial to a basement membrane type of matrix, accompanied by a switching of the corresponding integrin. The cell surface fibronectin that is abundant in proliferating myoblasts gradually decreases, as differentiation proceeds and reaches the basal levels during the final stages of myotube formation (Chen, 1977). Such a decrease is also observed for its receptor, α5β1 integrin (Gullberg et al., 1995). Fibronectin appears to play important regulatory roles in myogenic differentiation. Addition of exogenous fibronectin to rat myoblasts in culture blocks cell fusion (Podleski et al., 1979), and genetically modified myoblasts that constitutively express α5β1 integrin cannot form myotubes (Sastry et al., 1999). Thus, proper switching of the cell-ECM interaction is of crucial importance to cellular differentiation. When fibronectin is utilized as a cell-scaffolding agent, it is assembled into fibril structures via binding to the cell surface receptors, where it is cross-linked by tissue transglutaminases (Akimov and Belkin, 2001). MMP-mediated proteolysis is vital for clearing the assembled matrix, even after its expression declines. Our MMP inhibitor studies showed a good correlation between cell fusion suppression and lingering fibronectin concentrations. Fibronectin is a substrate utilized by both MT1-MMP and MMP-2 (Okada et al., 1990; Ohuchi et al., 1997). Tissue transglutaminase is also inactivated through MT1-MMP-mediated cleavage (Belkin et al., 2001). Indeed, down-regulation of MT1-MMP increased the amount of residual fibronectin and inhibited the cell fusion capacity in the present study. Although the results are not sufficient to conclude the causal relationship, fibronectin appears to typify the type of ECM molecules that have to be replaced before initiation of the fusion step.

Muscular dystrophy and MT1-MMP

Our study also revealed that MT1-MMP is required to maintain muscular integrity, and its deficiency causes morphological abnormalities that resemble muscular dystrophy. As evidenced by positional cloning of muscular dystrophy-causing genes in human patients, such as mutated genes encoding laminin α2, integrin α7, and dystrophin, the analysis of naturally occurring diseases and the targeted disruption of candidate murine genes, it is clear that muscular dystrophy arises from an impaired BM-cytoskeleton linkage (Wewer and Engvall, 1996; Hayashi et al., 1998; Cohn and Campbell, 2000). Thus, we posit that MT1-MMP is required to maintain this linkage appropriately by cleaving certain extracellular components. In previous studies, β-DG and laminin-2/4 are reportedly processed by proteinases, though the responsible enzymes have not been identified (Ehrig et al., 1990; Yamada et al., 2001). While β-DG was not affected by a MT1-MMP-deficiency (unpublished data), the processing of laminin-2/4 in muscle tissue was impacted in the mutant mice. The laminin α2 chain is the largest subunit of the heterotrimeric laminin-2/4 complex, and contains multiple functional domains. The globular domains at the C-terminus bind directly to the receptor molecules on the myofibers, while the N-terminal short arm interacts with components of the basement membrane. MT1-MMP was found to cleave the linker portion between the functional domains (Fig. 7A, open arrow), and this cleavage is reputedly important for maintaining the dynamic BM-cytoskeleton linkage and its defect that causes the muscle abnormalities.

Multifunctional involvement of MT1-MMP in muscle tissue development and maintenance

In this paper, we characterized a novel checkpoint in myogenic differentiation that regulates myotube formation during the elongation stage, as summarized in Fig. 8. This checkpoint requires MMP activity presumably to degrade the pre-existing interstitial components of the ECM that prevent cell fusion. MT1-MMP is one of the MMPs required for this checkpoint, as it can degrade the interstitial fibronectin molecules that have inhibitory effect for myotube formation. In mature muscle

Fig. 8. Requirement of MT1-MMP in multiple phases of skeletal muscle tissue development. Myoblast differentiation proceeds through three distinct stages. During elongation, the MMP-dependent ECM remodeling that is critical for the successive fusion step occurs. Time course of MMP-2, MT1-MMP and TIMP-2 expression supports the idea that MMPs are active in the elongation stage. Blocking of MMP activities by inhibitors or specific down-regulation of MT1-MMP by shRNA caused an accumulation of cell-associated fibronectin. Thus, fibronectin may represent ECM components having inhibitory effect for myoblast fusion and have to be cleared by MT1-MMP at this stage. A MT1-MMP deficiency also causes self-destruction of the muscle tissue, which resembles muscular dystrophy. MT1-MMP is responsible for laminin α2 processing in vivo and in vitro, and appears to regulate laminin-based cell adhesion.
tissue, we determined that MT1-MMP acts as a processing enzyme for the muscle-specific laminin-2/4 complex, and that this processing appears to be important for maintaining the integrity of laminin-based cell adhesion, thus preventing muscular dystrophy. Therefore, our study elucidated the MMP-dependent regulation of myogenic differentiation, and the previously unknown multifunctionality of MT1-MMP in muscle development and maintenance.

Materials and Methods

C2C12 cell culture and metalloprotease inhibitor treatment

Murine myoblasts of the C2C12 cell line were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics [growth medium (GM)]. For the differentiation experiments, 5 × 10⁵ cells were plated on type I-collagen-coated 35 mm Petri dishes, and cultured in GM for 24 hours. The culture medium was then substituted with differentiation medium [DM; DMEM with 10 ng/ml IGF-I, 1% bovine serum albumin (BSA), and antibiotics], which was changed every 24 hours thereafter.

A synthetic metalloprotease inhibitor BB94 was used at 10 μM. The vehicle control was 0.1% DMSO solution. Recombinant TIMP-1 and TIMP-2 were used at 1 μg/ml. Before DM replacement at 72 hours, the cells were washed three times with PBS to avoid inhibitor carry-over.

Indirect immunocytochemistry

The C2C12 cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS and then blocked with 3% BSA and 5% goat serum in PBS for 2 hours at room temperature. After incubation with an anti-PCNA antibody with blocking buffer (3% BSA, 5% goat serum in PBS), and then incubated with either fluorescein-conjugated antibody or an HRP-conjugated antibody in combination with diaminobenzidine (DAB) as the chromogen. Counterstaining was performed with hematoxilin or Hoechst dye.

Semi-quantitative RT-PCR

Total RNA was purified using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and quantified at OD260. Equal amounts of the RNA samples were reverse transcribed using random hexamer primers (Invitrogen) and Super Script II reverse transcriptase (Invitrogen). Semi-quantitative RT-PCR was performed, as described previously (Yamanouchi et al., 2000). The sequences of the intron-spanning primer pairs were as follows: MCK forward: 5’-AGC ACA GAC GAC ACA CTC T-3’, reverse: 5’-GGC TTG TAG TTC AGC TTG-3’. The RT-PCR amplicons were 2% agarose gel electrophoresed, quantified with NIH imaging software in the public domain (version 1.61, US National Institutes of Health) and normalized using glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as an internal control. The primer pairs for G3PDH were purchased from Clontech (Palo Alto, CA, USA).

Luciferase reporter assay for myogenic transcriptional factors

All luciferase (Luc) constructs were assembled on the pGL3 vector backbone, and translated the firefly luciferase gene. The 4RE-TK-Luc construct was kindly provided by Eric Olson (University of Texas Southwestern Medical Center). Luciferase reporter assay for myogenic transcriptional factors with PBS to avoid inhibitor carry-over.

Western blotting and gelatin zymography

Western blotting and gelatin zymography were conducted as described previously (Uekita et al., 2001). The C2C12 cells were lysed directly in SDS sample buffer, and the tissue samples were extracted in SDS extraction buffer (0.2% SDS, 50 mM Tris-HCl, pH 7.6). The protein concentrations of the samples were determined with a kit provided from Pierce (Rockford, IL, USA), using mouse IgG as a standard. The following primary antibodies were used for to detect the proteins: anti-MT1-MMP (222-1D8, Daichi Fine Chemical, Toyama, Japan), anti-fibronectin (rabbit polyclonal, Sigma) and anti-tubulin (E7, DSHD at Iowa University). The reacted antibodies were detected with anti-mouse or rabbit HRP-conjugated secondary antibodies (Amersham, Tokyo, Japan), in combination with the ECL-Plus reagent (Amersham). For the gelatin zymography assay, the conditioned medium of the cells cultured in the DM was harvested at the culture period indicated in Fig. 3B, and the samples were prepared and analyzed from an equal number of cells. The samples were mixed with SDS sample buffer without a reducing agent, and were separated in 7.5% acrylamide gels containing 0.1% gelatin. The gels were incubated at 37°C after removing the residual SDS by washing the gel with 2.5% of a Triton X-100-containing buffer. The gels were stained with Coomassie Brilliant Blue R250 (Merck, Darmstadt, Germany), and the gelatinolytic activities were detected as clear bands against a blue background.

Establishment of MT1-MMP-knockdown C2C12 derivatives

Short-hairpin RNA (shRNA) expression vectors were constructed and packaged into the recombinant lentivirus using the BLOCK-it Lentiviral RNAi Expression System (Invitrogen), following the manufacturer’s instructions. The target sequence, 5’-GGA CTG AGA TCA AGG CTA ATG-3’, was selected with an on-line program provided by Invitrogen. The pLenti-U6-MiTR and packaging plasmid mixture were introduced into 293FT packaging cells provided within the kit. The recombinant lentiviruses in the conditioned medium were harvested, concentrated by ultracentrifugation, and titrated using the C2C12 cells. Transductions were performed at the MOI (multiplicity of infection) of 50, and the cells were propagated and maintained with 10 μg/ml blasticidin thereafter. The target gene knockdown was confirmed by western blotting.

Experimental skeletal muscle regeneration

Three-month-old C57BL/6J mice (CREA, Japan) were anesthetized by intraperitoneal injection of pentobarbital sodium solution (10 μg/g body weight, Dai-Nihon Seiyaku, Osaka, Japan), and the tibialis anterior (TA) muscles were directly injected with 3 μg of cardiotoxin (CTX) in 50 μl PBS. The CTX was released by pulling out the needle to be delivered along the muscle mass. To assess the tissue regeneration time course, the TA muscles dissected at 1, 3, 7, 10 and 14 days after injection, were used for histological analysis. All animal experiments were performed according to the Guide for Care and Use of Laboratory Animals, the University of Tokyo.

MT1-MMP knockout mice

The C2C12-MMP knockout mice were generated by homologous recombination in embryonic stem (ES) cells (Yana et al., manuscript in preparation). To obtain these MT1-MMP-deficient mice, ES cells were electroporated with the MT1-MMP-deficient allele, and the heterozygous mice were backcrossed to the C57BL/6J strain for at least eight generations. These heterozygous mice were then intercrossed to generate homozygous mice. The genotypes of all mice were confirmed by genomic PCR analysis.

Histology and immunohistochemistry

The dissected muscles were fixed in 10% neutral buffered formalin. The paraffin sections of 4 μm were dewaxed in xylene, rehydrated through an alcohol series, and then stained with hematoxylin and eosin, elastica-van gieson staining, and reticulin silver impregnation. The sections were also stained for β-Dystroglycan (β-DG), as follows: The rehydrated sections were antigen-retrieved with proteinase K, treated with blocking buffer (3% BSA, 5% goat serum in PBS), and then incubated with anti-βDG antibody (NCL-βDG, Novocatsra, Newcastle upon Tyne, UK). The staining signals were visualized with Cy3-conjugated secondary antibody with counterstaining with Hoechst dye.

Digestion of laminin-2/4 in vitro

A partially purified laminin-2/4 complex from human placenta was purchased from Chemicon (Temecula, CA, USA). The recombinant human MT1-MMP catalytic domain was described previously (Kajita et al., 2001). One μg of purified laminin was incubated with the recombinant enzyme (1.100 and 1.50 mg/ml in 100 μl of digestion buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl₂, 0.005% Brij-35, pH 7.5). After 18 hours of incubation at 37°C, the samples were mixed with SDS sample buffer containing 2-mercaptoethanol, and boiled. The cleaved pattern of the substrate was analyzed by western blotting using α2 subunit specific antisera. The anti-LG domain antibody designated as anti-309K (Ehrig et al., 1990) was a kind gift from Engvall (The Burnham Institute for Medical Science, CA), and the antiIVA domain antibody was raised against the recombinant protein expressed in E. coli.

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