Decrease of MMP-9 Activity Improves Soleus Muscle Regeneration

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The regeneration of skeletal muscles relies on the function of satellite cells that are quiescent myogenic precursors associated with adult skeletal muscle fibers. Upon injury, the satellite cells are activated, divide extensively, and differentiate into new myofibers. These events are accompanied by the remodeling of the surrounding extracellular matrix, which is mediated by variety of factors, including matrix metalloproteinases (MMPs). Regeneration of certain type of muscles, such as Soleus slow twitch muscle, is often inefficient and hindered by the development of fibrosis. Here, we studied the effect of inhibition of MMP-9 and MMP-2 activity on the Soleus muscle regeneration *in vivo* and on the *in vitro* differentiation of myoblasts derived from this muscle. Using *in situ* and in-gel zymography, we tested the activity of these two MMPs *in vivo*, during regeneration of the muscle, and *in vitro*, during differentiation of the myoblasts. We also analyzed the histology of regenerating muscles and morphology of differentiating myoblasts. All these analyses showed that treatment with doxycycline and anti-MMP-9, but not MMP-2 antibody, significantly improved Soleus muscle regeneration and ameliorated development of excessive fibrosis, as well as delayed myoblast proliferation and differentiation *in vitro*.

Introduction

SKELETAL MUSCLES have a high ability to undergo regeneration in response to mechanical damage or disease. The muscle regeneration involves two overlapping stages: myolysis and reconstruction. The myolysis encompasses active muscle degeneration, the extracellular matrix (ECM) remodeling, and the inflammation processes. The reconstruction includes activation and differentiation of the satellite cells; the synthesis of new ECM components, such as laminins, fibronectin, and collagen;^{1,2} and results in the formation of the fully functional skeletal muscle with normal histology. However, the repair process is often inefficient and hindered by the development of fibrosis. It has been shown that the efficiency of crush-induced repair and the extensity of fibrosis are related to the muscle type, and to the proportion of fast and slow fibers within the muscle. Analyses of the regeneration of fast and slow twitch muscles—induced by microlesion,³ anesthetic injection,⁴ or crush⁵, revealed differences in the progression and final outcome of the muscle repair. For example, the myolysis of crushed and denervated slow twitch Soleus muscle, in contrast to the fast twitch extensor digitorum longus (EDL), is rapid, extensive and accompanied by massive inflammation, and development of fibrosis.⁵

A proper outcome of the repair of the muscle and the development or lack of fibrosis are finely orchestrated processes involving the satellite cell differentiation into myoblasts and myotubes, as well as modification in the synthesis and degradation of various ECM components. Among the factors involved in remodeling and degradation of ECM are zinc-dependent matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9 (gelatinase A and B, respectively). Moreover, the MMP-9 and MMP-2 are involved in the migration of satellite cells and myoblasts, as well as in the myotube formation.^{6–8} Several studies showed that the activity of MMP-9 and MMP-2 is low in the intact muscles and increases under pathological conditions.^{9–14} We showed previously that during the crush-induced regeneration of Soleus muscle, the MMP-9 activity increases during the myolysis and reconstruction phases while the MMP-2 activity increases only at the reconstruction phase.⁸ In contrast, during the regeneration of EDL muscle, the MMP-9 activity decreases during myolysis while MMP-2 activity increases at the reconstruction phase.⁸ Thus, it is possible that the changes in the MMP-9 and/or MMP-2 expression and activity are responsible for the excessive development of fibrosis in such poorly regenerating muscles as the Soleus muscle. This, in turn, suggests that the intervention leading to the reduction of MMP-9 and/or MMP-2 activity may improve the repair

process of such skeletal muscles. However, the impact of such directed inhibition of each of these enzymes at the Soleus muscle regeneration was not tested, so far. Doxycycline is one of the tetracycline antibiotics, which are potent inhibitors of MMPs. They have been shown to reduce tissue degradation in such pathological conditions as periodontal disease or arthritis,¹⁵ and to increase smooth muscle cell adhesion, growth, as well as migration.^{16,17} Doxycycline impacts at the remodeling of ECM during arterial injury,¹⁷ and reduces collagen IV proteolysis during skeletal muscle injury.¹⁸ Impact of doxycycline at the Soleus muscle regeneration was not tested. Due to its role in the MMP inhibition and the ECM remodeling, the doxycycline might be a promising treatment for the elimination of excessive fibrosis, and the improvement of the muscle repair. Thus, the aim of this study was to analyze the impact of the inhibition of MMPs, using specific anti-MMP-9 or anti-MMP-2 antibody as well as doxycycline, on the regeneration of the Soleus muscle and the differentiation of skeletal myoblasts.

Materials and Methods

Ethical issues

All procedures involving animals were approved by Local Ethics Committee no. 1 (Warsaw, Poland).

Muscle injury and anti-MMP-2/MMP-9 antibody or doxycycline treatment

The regeneration of Soleus muscles was induced in 3-month-old male Wistar rats as previously described.⁵ Briefly, the animals were anesthetized with pentobarbital, Soleus muscle was exposed, denervated by cutting the motor nerve of the muscle, and then the area between tendons was crushed with forceps. After the crush, muscles were injected with either 100 μ L of anti-MMP-2, or anti-MMP-9 antibody (10 mg/mL in phosphate-buffered saline [PBS]), or 50 μ L of doxycycline (300 μ M in PBS) (Millipore, Chemicon, Billerica, MA). Control muscles were injected with either 100 or 50 μ L of PBS, respectively. At day 1, 3, 7, or 14 of regeneration the animals were euthanatized with CO₂ and the regenerating muscles were removed. Muscles were then either directly used for the biochemical analyses or frozen in isopentane (precooled in liquid nitrogen), and then stored at -80°C pending histological studies. Each experimental group consisted of three rats and each experiment was repeated five times.

Histological analysis

The transversal, 10- μ m-thick, cross-sections of control and experimental Soleus muscles were obtained using cryostat (Microm HM 500N; Thermo Fisher Scientific—Microm International GmbH, Langensfeld, Germany), and stained with the Gomori staining (Merck KGaA, Darmstadt, Germany).

Isolation and culture of myoblasts

Satellite cells were dissociated with pronase from Soleus muscles of 3-month-old Wistar male rats, as previously described.¹⁹ Cells were seeded (2000 cells/cm²) on 35-mm $\varnothing$ dishes (Becton, BD Bio Sciences, Durham, NC) coated with

1% gelatin (Sigma, Schnellendorf, Germany), and cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 10% horse serum (Gibco, Invitrogen Ltd., Paisley, United Kingdom), in 5% CO₂ at 37°C. At day 4, 6, 8, 10, 12, or 14 of *in vitro* culture, cells were counted and the morphology of the cells was assessed using Nikon Eclipse TE200 microscope equipped with Hoffman contrast, and the culture medium was collected and saved for gelatin zymography.

Cell treatments

At days 4 (proliferation phase) and 8 (the beginning of differentiation) of *in vitro* culture, the myoblasts were treated with anti-MMP-2 antibody, anti-MMP-9 antibody (1 g/mL; Chemicon), or doxycycline (60 μ M), all dissolved in culture medium. Control myoblasts were cultured under the standard conditions as described previously. Each experiment was repeated five times.

Index of fusion

At day 4, 6, 8, 10, 12, or 14 of culture, the control and experimental myoblasts were stained with Giemsa-May-Grünwald (Merck KGaA) for myotube classification and determination of fusion index.¹⁹ Fusion index represented the percentage of nuclei found in the myotubes divided by the total number of nuclei visible in the field of view. Ten representative microscopic fields for each culture were analyzed. Each experiment was repeated five times. The myotubes were classified on the basis of the number of nuclei present within each myotube. We analyzed 40 fields of view for each culture from three independent experiments.

Gelatin zymography

Detection of enzymatic activity of MMP-2 and MMP-9 was performed for the regenerating muscles and *in vitro*-cultured cells. In the latter case, the cell-conditioned media were analyzed. Protein extracts of control and treated muscle were prepared as previously described.²⁰ Intact and regenerating muscles were homogenized in the buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 0.05% Brij 35, and 1% Triton X-100 (pH 7.6). Zymography was performed as previously described.²¹ In brief, homogenized muscles or cell-conditioned media were mixed with non-reducing sample buffer containing 62.5 mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 0.05% bromophenol blue (Sigma), and loaded onto 7.5% SDS-polyacrylamide gels containing 0.1% gelatin (Sigma). After electrophoresis, SDS was removed from the gels by washing in 2.5% Triton X-100 (2 $\times$ 20 min). Next, the gels were incubated for 48 h in the buffer containing 50 mM Tris-HCl, 5 mM CaCl₂, and 200 mM NaCl (pH 7.5), and stained with Coomassie blue (Bio-Rad, Hercules, CA). Resulting zymographs were scanned and analyzed using the GelDoc Quantity One software (Bio-Rad). The intensity of selected bands was expressed as the optical density and normalized using the value of control, nontreated cells or muscle as a 100%. Each experiment was performed five times. The MMP-9 proenzyme was detected as a 98-kDa band, and the activated form of MMP-9 was visible as the 82-kDa band. The MMP-2 proenzyme and the activated form were detected as the 68- and 62-kDa band, respectively.

In situ zymography

Localization of active forms of MMP-2 and MMP-9 was performed for the regenerating muscles (days 1, 7, and 14) and *in vitro*-cultured differentiating myoblasts (days 6, 8, and 10). *In vitro*-cultured myoblasts or muscle sections were permeabilized with 0.05% Tween20 in PBS. Detection of enzymatic activity in cells or muscle cross-sections was made according to the manufacturer's instructions (DQ™ gelatin from pig skin, fluorescein conjugate [Molecular Probes, Eugene, OR]). Actin filaments were visualized with rhodamine-phalloidine and nuclei with Draq-5 (BioStatus, Leicestershire, United Kingdom). Images of cells or muscle sections were captured using LSM 510 confocal system (Carl Zeiss, Inc., Jena, Germany) equipped with LSM 5 Rel. 3.2 software.

Results

Inhibition of MMP-9 improves muscle regeneration

Regeneration of the Soleus skeletal muscle (Fig. 1a) can be induced by crushing.^{22–24} Histological analysis of such muscle proves that the myolysis phase is completed by day 3 after the injury (data not shown) and, by day 7, the reconstructed muscle contains the newly formed, heterogeneous in size, fibers (Fig. 1b). At day 14 of the regeneration, the Soleus muscle undergoes an extensive fibrosis (Fig. 1c). To validate the hypothesis that MMPs play an important role in the regulation of fibrotic fibers, we decided to inhibit the activity of these enzymes. We injected the injured Soleus muscle with either nonspecific MMP inhibitor, that is, doxycycline, or anti-MMP-9 or anti-MMP-2 antibody. Doxycycline administered just after the crush improved the progress of regeneration and resulted in the reconstruction of muscle fibers that were larger and more homogenous in size than those observed in untreated regenerating muscles. Moreover, such treatment reduced development of fibrosis (Fig. 1h, i). Anti-MMP-9 antibody introduced to injured Soleus also significantly impacted at the regeneration process. At day 7 after the injury, the improvement in the regeneration was manifested by the presence of more myotubes containing centrally positioned nuclei, as compared with untreated muscles (Fig. 1d). At day 14, the majority of myotubes contained peripherally located nuclei. In contrast, in the untreated muscle, the majority of the myotubes contained centrally positioned nuclei (Fig. 1e). The injection of anti-MMP-2 antibody did not result in any visible improvement of muscle repair at day 7 after injury (Fig. 1f). At day 14 the restoration of muscular architecture of anti-MMP-2 antibody-treated muscles was similar to that of untreated muscle (Fig. 1g). Thus, the process of regeneration was improved in muscles treated with either doxycycline or anti-MMP-9 antibody but not anti-MMP-2 antibody. This indicates that the decrease of MMP-9 activity is crucial for the properly occurring repair process.

Inhibition of MMPs activity reduces gelatinolytic activity in regenerating Soleus muscle

Next, we analyzed the localization of active MMP-9 and MMP-2 within the regenerating Soleus muscle. Localization of gelatinolytic activity, that is, the activity reflecting the presence of active MMP forms, was assessed by *in situ* zymography (Fig. 2). Analysis of intact, that is, uninjured,

FIG. 1. Histological analysis of transversal sections of regenerating Soleus muscles at day 7 (**b, d, f, h**) and day 14 after crush (**c, e, g, i**). Gomori-stained intact muscle (**a**), regenerating control muscle (**b, c**), and regenerating muscle treated with anti-matrix metalloproteinase-9 (MMP-9) antibody (**d, e**), anti-MMP-2 antibody (**f, g**), or doxycycline (**h, i**), analyzed at the indicated days after the injury. Scale bar = 100 μ m.

muscle detected low gelatinolytic activity around the muscle fibers (Fig. 2a). Muscle damage resulted in the sustained increase of this activity between day 1 and day 14 after the injury (Fig. 2b–d). The gelatinolytic activity was detected at the myolysis (Fig. 2c) and reconstruction phases (Fig. 2c) within the endomysium containing infiltrating inflammatory cells (Fig. 2b). Injection of anti-MMP-9 (Fig. 2e–g) or anti-MMP-2 antibody (Fig. 2h–j) did not result in any significant changes. However, the doxycycline treatment significantly decreased the gelatinolytic activity of these two enzymes

FIG. 2. *In situ* zymography of transversal sections of regenerating Soleus muscles. Gelatinolytic activity was detected at day 1 (**b, e, h, k**), day 7 (**c, f, i, l**), and day 14 after the crush (**d, g, j, m**). Intact muscle (**a**), regenerating control muscle (**b–d**), and regenerating muscle treated with anti-MMP-9 antibody (**e–g**), anti-MMP-2 antibody (**h–j**), or doxycycline (**k–m**) were incubated with fluorescein-conjugated gelatin as described in the Materials and Methods section. Gelatinolytic activity detected in transversal muscle sections is shown in green, chromatin is shown in red. Scale bar = 50 μ m.

starting from day 1 of regeneration (Fig. 2k–m). Since, using *in situ* zymography we were not able to distinguish between MMP-9 and MMP-2 activities, we performed in-gel zymography.

The method of in-gel gelatin zymography provides reliable identification of gelatinases based on the molecular mass of their inactive and active forms. In-gel zymography allowed us to analyze MMP-2 and MMP-9 activation in intact and injured muscles, at day 3 and day 7 of regeneration. In control muscles we observed the elevation of MMP-9 activity at days 3 and 7 (Fig. 3). In contrast, the MMP-2 activity increased only at the reconstruction phase, that is, at day 7, which was in agreement with our previously published

data.⁸ At day 3 after the injury, the treatment with anti-MMP-9 antibody reduced the MMP-9 activity to 65%, and at day 7 to 80% of that in untreated muscle. Simultaneously, at day 3, the level of MMP-2 activity was not significantly affected, and later during regeneration, that is, at day 7, it was reduced to ~90%. Injection of anti-MMP-2 antibody resulted in 40% decrease in the MMP-2 activity at day 3 after injury (Fig. 3). However, at day 7, activity of this enzyme was not significantly different than in the untreated control. The activity of MMP-9 in the muscle treated with anti-MMP-2 antibody did not change significantly. Since, the injection of anti-MMP-9 antibody did not significantly influence the activity of MMP-2, and anti-MMP-2 antibody did not impact at MMP-9, we concluded that their action was highly specific. Analysis of MMP-2 and MMP-9 in the muscles injected with doxycycline showed that such treatment reduced exclusively the activity of MMP-9. At day 3 after the injury it decreased by ~50% in the muscle injected with doxycycline, in comparison to the noninjected control (shown as 100%). Further, at day 7, the activity of MMP-9 was still slightly reduced, to about 90% of activity detected in untreated muscle. All these data indicate that the decrease in the MMP-9 but not MMP-2 activity is responsible for the reduction in the development of fibrosis in Soleus muscle.

Modification of MMP-9 activity delays myoblast differentiation in vitro

As we described previously, the inhibition of MMP activity impacted Soleus muscle regeneration, and reduced fibrosis. To check the influence of these enzymes on the satellite-cell-derived myoblasts, we decided to take advantage of *in vitro* culture system. Isolation of the satellite cells from skeletal muscles causes their activation, which is followed by the phase of intensive proliferation of the resulting myoblasts, their fusion and the formation of multinucleate myotubes. Therefore, *in vitro* culture can be used to study the processes occurring in *in vivo*—regenerating muscles. During *in vitro* culture of activated rat myoblasts, the first significant increase in their number was observed at day 6 after plating (Figs. 4a and 5a). The myoblast number increased continuously until day 10 when it reached plateau (Figs. 4b and 5a). Differentiation, leading to the formation of myotubes, did not begin prior to day 6 of culture (Fig. 4c). The intensive myoblast fusion was observed at day 8, and finally, at day 14, the majority of myotubes contained more than 10 nuclei (Fig. 5b). We treated the myoblasts with either doxycycline, or anti-MMP-2 or anti-MMP-9 antibody twice, at days 4 and 8 of *in vitro* culture, that is, at the proliferation and fusion phases, respectively. Doxycycline treatment resulted in the decrease of myoblast proliferation and also prevented the formation of myotubes (Fig. 4j–l). The number of cells found at day 14 of culture was ~60% lower than in untreated control. Differentiation of experimental myoblasts, that is, formation of myotubes, was not observed at day 6 after plating, that is, at the time when control myoblasts already started fusion process (Fig. 5b, c). In doxycycline-treated cultures the smallest myotubes, that is, containing 2–4 nuclei, were observed at day 10. At the same time, in the control cultures, the myotubes contained about 2 to more than 15 nuclei (Fig. 5c). Even at day 14 the myotubes formed in doxycycline-treated culture were smaller and contained less

FIG. 3. MMP-9 and MMP-2 activity in regenerating Soleus muscles. Gelatin zymography of control regenerating muscles (C), and regenerating muscles treated with doxycycline (D), anti-MMP-9 (Ab9), or anti-MMP-2 antibody (Ab2). The intensity of selected bands was presented as an optical density. The data resulting from Coomassie-blue-stained zymograms were normalized. The intensity of the bands is presented as a percentage of control, untreated muscle (100%). Values are mean $\pm$ SE of five independent experiments.

FIG. 4. Morphology of *in vitro*-cultured control, doxycycline, anti-MMP-9, or anti-MMP-2 antibody treated Soleus muscle myoblasts. Myoblasts were analyzed at day 6 (a, d, g, j), day 10 (b, e, h, k), and day 14 (c, f, i, l) of *in vitro* culture. Morphology of control (a–c) and anti-MMP-9 antibody (d–f) or anti-MMP-2 antibody (g–i) or doxycycline (j–l) treated myoblasts was assessed using transmitted light microscopy. Scale bar = 100 μ m.

FIG. 5. Analysis of proliferation and fusion of *in vitro*-cultured control, doxycycline, anti-MMP-9, or anti-MMP-2 antibody treated Soleus muscle myoblasts. **(a)** Growth curves. Values are mean $\pm$ SE of five independent cultures. **(b)** Index of fusion. Analyses were performed at the indicated days of *in vitro* culture as described in the Materials and Methods section. Values are mean $\pm$ SE of five independent cultures. **(c)** Classes of myotubes: containing 2–4, 5–8, 9–14, and more than 15 nuclei. Analyses were performed at the indicated days of *in vitro* culture as described in the Materials and Methods section. One hundred percent indicates all myotubes analyzed.

nuclei, and the fusion index was five times lower than in nontreated controls (Fig. 5b). Anti-MMP-9 but not anti-MMP-2 antibody visibly affected myoblast proliferation and myotube formation (Figs. 4d–f and 5a, b). Interestingly, anti-MMP-2 antibody treatment resulted in the deformation of myotubes. Newly formed myotubes were irregular, waved, and twisted (Fig. 4h–i). At day 14 of culture, anti-MMP-9 antibody treatment reduced number of cells to $\sim$ 30% (Fig. 5a). Also the fusion index was $\sim$ 25% lower than in control (Fig. 5b). Anti-MMP-9 antibody treatment also impacted the proportion of the myotube classes. At day 10 of culture, the majority of myotubes in anti-MMP-9 antibody-treated cultures contained 2–4 nuclei, while in control cultures the

myotubes containing more than 15 nuclei were already present (Fig. 5c). However, these differences were ameliorated at day 14 of culture. The same changes in the fusion index were observed when myoblasts were treated with either doxycycline, or anti-MMP-9 or anti-MMP-2 antibody only once, at day 8 of culture, that is, only at the differentiation stage (data not shown). Thus, the modification of MMP-9 but not MMP-2 activity is crucial for the proliferation and fusion of the myoblasts.

Inhibition of MMP activities reduces gelatinolytic activity associated with in vitro-cultured myoblasts

To analyze the activity of MMPs studied in *in vitro*-cultured myoblasts, we performed *in situ* and in-gel zymography. In control cultures gelatinolytic activity was found in the ECM surrounding the mononucleated myoblasts and the forming myotubes (Fig. 6a–c). Doxycycline treatment administered twice, that is, at days 4 and 8 of culture, resulted in the reduction of MMP activity associated with the myoblasts, both at the proliferation (Fig. 6j–k) and at the differentiation phases (Fig. 6l). The treatment with anti-MMP-9 (Fig. 6d–f) or anti-MMP-2 antibody (Fig. 6g–i) did not affect gelatinolytic activities of MMPs when analyzed by *in situ*

FIG. 6. *In situ* zymography of *in vitro*-cultured control, doxycycline, anti-MMP-9, or anti-MMP-2 antibody treated Soleus muscle myoblasts. Myoblasts were analyzed at day 6 (**a**, **d**, **g**, **j**), day 8 (**b**, **e**, **h**, **k**), and day 10 of *in vitro* culture (**c**, **f**, **i**, **l**). Control myoblasts (**a**–**c**). Myoblasts treated with anti-MMP-9 antibody (**d**–**f**), anti-MMP-2 antibody (**g**–**i**), doxycycline (**j**–**l**). Gelatinolytic activity, green; actin filaments, red. Scale bar = 50 μ m.

FIG. 7. MMP-9 and MMP-2 activity in *in vitro*-cultured Soleus muscle myoblasts. Gelatin zymography of *in vitro*-cultured control (C), anti-MMP-2 antibody (Ab2), anti-MMP-9 (Ab9) antibody, or doxycycline (D) treated Soleus muscle myoblasts. The intensity of selected bands was expressed as optical density. Data from Coomassie-blue-stained zymograms were normalized to the control. Bands are presented as a percentage of control, nontreated muscle (100%). Values are mean $\pm$ SE of five independent experiments.

zymography. Similar results were observed when myoblasts were treated only once, that is, at day 8 of culture (differentiation phase) and then analyzed at day 10 of culture (data not shown). To analyze the activity of MMPs we used the ingel zymography. Again doxycycline was administered at days 4 and 8 after plating and culture medium was collected at day 6 (proliferation phase) and at day 10 (differentiation phase). The level of MMP-9 or MMP-2 activity observed in the control cultures at days 6 and 10 was taken as 100% (Fig. 7). Doxycycline treatment led to the decrease of MMP-9 activity at both day 6 and day 10 of culture, by $\sim 60\%$ and 80% , as compared with control, respectively. MMP-2 activity was reduced by $\sim 75\%$ at day 6 (proliferation phase), but was comparable to control at day 10 (differentiation phase) (Fig. 7). Anti-MMP-9 antibody treatment resulted in the decrease in MMP-9 activity to $\sim 75\%$ at day 6, and to $\sim 40\%$ at day 10 of culture, as compared with control. MMP-2 activity was comparable to control (Fig. 7). The anti-MMP-2 antibody resulted in the reduction of the MMP-2 activity to $\sim 70\%$ at day 6, and to $\sim 60\%$ at day 10 of culture, as compared with control. Activity of the MMP-9 either did not change (day 6) or was reduced to 80% (day 10), as compared with control (Fig. 7). Thus, during *in vitro* culture, doxycycline blocked both MMPs analyzed. Also specific antibodies decreased the activity of their targets. Doxycycline dramatically impacted at both proliferation and differentiation of myoblasts. Drop in MMP-9 activity resulted in the significant decrease in proliferation and also affected myoblast differentiation, while reduction in MMP-2 activity mildly affected myoblast differentiation.

Discussion

Several lines of evidence document that during skeletal muscle regeneration increased expression of MMPs may re-

sult in the degradation of the ECM components leading to sarcolemmal damage and muscle fiber necrosis.^{8,25} It may also lead to the development of fibrous scar tissue perturbing myoblast migration and fusion. Such scar may also limit vascularization of the damage site restricting proper tissue repair.²⁵ Thus, the regeneration of crush-injured rat slow twitch Soleus muscle leads to the formation of a dysfunctional fibrotic muscle, characterized by the accumulation of ECM components, such as fibronectin.²⁴ Our previous study showed that MMP-9 and MMP-2 are significantly upregulated during crush-induced Soleus muscle regeneration.⁸ Rise in MMP-9 activity was associated with both the myolysis and the reconstruction phases, whereas the activity of MMP-2 increased at the reconstruction phase.⁸ The increased MMP-9 activity was also shown to be involved in inflammation and in the necrosis of muscles in DMD patients and in *mdx* mice (which are the animal models of this disease).^{9,12,26} Also cardiotoxin injury of tibialis anterior muscle leads to the immediate upregulation of MMP-9 expression.¹⁴ Our current study confirms the crucial role of MMP-9 in the development of fibrosis in poorly regenerating Soleus muscle. This finding is consistent with the data showing that the inhibition of MMP-9 activity significantly improves the structure and contractile functions of the dystrophic muscles in the Duchenne's muscular dystrophy patients as well as in the *mdx* mice,^{12,14} and delays progressive muscle weakness in the mouse model of oculopharyngeal muscular dystrophy.²⁷ Similarly, the doxycycline or minocycline improves postnatal growth, delays the onset of hind-limb paralysis, and increases the life span of mouse suffering from congenital muscular dystrophy.²⁸

The MMP-9 activity was previously shown to rise during the myoblast proliferation, whereas the MMP-2 activity reached its highest level during myoblast fusion.^{8,29,30} Our current study confirmed the different involvement of MMP-9

and MMP-2 in myoblast proliferation and differentiation *in vitro*. We showed that specific inhibition of MMP-9 decreased the number of myoblasts and the index of fusion. The MMP-2 inhibition did not influence the proliferation, but impacted the differentiation of myoblasts, and decreased the myoblast fusion. The doxycycline treatment led to the dramatic decrease in the cell number and index of fusion. Since the cells were subjected to the treatments twice, that is, at day 4 (proliferation phase) and day 8 (beginning of differentiation phase), we considered the possibility that the observed decrease in the index of fusion resulted from the lower number of myoblasts. We excluded this possibility by showing that fusion index of myoblasts subjected to a single (at day 8 of culture only, that is, at the differentiation phase) antibody or doxycycline treatment was the same as that characterized for cells treated twice. *In vitro* studies showed that the increase in MMP-2 activity resulted in the stimulation of mouse myoblast migration, whereas the overexpression of MMP-9 had no such effect.³¹ However, studies on human myoblasts showed that the treatment with anti-MMP-9 antibody resulted in the inhibition of myoblast migration.²⁹ Interestingly, results of our *in vivo* studies did document significant negative effect of MMP inhibition on myoblast proliferation and differentiation. Both the MMP-9 and doxycycline did not impair the formation of myofibers. Moreover, by reducing fibrosis, these factors had visibly beneficial effect on the muscle regeneration. Thus, the response to MMP inhibition observed *in vitro* may not reflect the behavior of satellite cells and myoblasts subjected to doxycycline or anti-MMP-9 antibody *in vivo*. Moreover, *in vivo* skeletal muscle regeneration can be modulated by fibroblasts, inflammatory cells, as well as other nonmuscle cells, which affect the behavior of satellite cells and myoblasts.^{32–35} Thus, such microenvironment may result in different response of these cells to the MMP inhibition than that observed during *in vitro* culture.

During Soleus muscle regeneration the MMP-9 activity is detectable for prolonged period of time, as compared with the properly regenerating EDL muscle.⁸ Inhibition of MMP-9 activity leading to the improvement of regeneration once more proves the negative effect of this enzyme on the repair process. The treatment of crushed muscle with anti-MMP-2 antibody did not significantly influence regeneration. This lack of impact suggests that MMP-2 does not play a crucial role in slow twitch muscles. Indeed, its expression was shown to increase in regenerating muscles in which fast twitch fibers dominate (e.g., gastrocnemius and quadriceps).³⁶ Therefore, it is possible that MMP-2 plays greater role in the regeneration of fast twitch than in slow twitch muscle.

The MMPs are also involved in plethora of other processes that are crucial for skeletal muscle regeneration, such as the synaptic remodeling, neuronal regeneration, and remyelination.³⁷ The differences in the reinnervation between fast and low twitch fibers were suggested by the results of the experiments showing that after peripheral nerve transection, the fast fibers, that is, the fibers characterized by decreased MMP-9 levels during myolysis phase, regenerate better.^{8,38} Moreover, the reinnervation of Soleus muscle resulted in the abnormal structure and function of the motor endplate.³⁹ Because the basement membrane components

play a critical role in the formation of the neuromuscular junctions, their premature degradation by MMP-9, that is, before they fulfill their role as the neuron-guiding scaffolds, might affect the muscle fiber reinnervation. Not surprisingly, the MMP-9 inhibition was shown to improve this process.⁴⁰ Thus, improvement of regeneration found in our study may also rely, at least partially, on the correction of the reinnervation process. Next, rise in MMP expression and activity is associated with inflammation, including chronic inflammation observed in skeletal muscles of mdx dystrophic mice.^{41–44} In this case regeneration of skeletal muscles affected by inflammatory process is disordered by the development of fibrosis.^{25,26,43} In our experimental model, Soleus myolysis is rapid, extensive, and accompanied by massive inflammation. Consequently, the inhibition of MMP-9 might result in the reduction of inflammation and the improvement of muscle repair.

In summary, our results show that despite the negative effect of doxycycline, or anti-MMP antibodies at *in vitro*—cultured myoblasts, these factors *in vivo* decrease development of fibrosis and improve Soleus muscle regeneration. This strongly suggested that MMP-9 plays a crucial role in the repair process. We believe that modulation of the microenvironment of injured slow twitch Soleus muscle by decreasing the MMP-9 activity enhances the efficiency of regeneration and also prevents the development of fibrosis. An appropriate combination of ECM component biosynthesis and degradation could improve survival, proliferation, and differentiation of myogenic cells.

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Disclosure Statement

No competing financial interests exist.

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