Matrix metalloproteinase-2 ablation in dystrophin-deficient mdx muscles reduces angiogenesis resulting in impaired growth of regenerated muscle fibers

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common severe X-linked muscular disorder, characterized by progressive muscle wasting and weakness (1). It is caused by a mutation in the DMD gene encoding the large cytoskeletal protein dystrophin (2). Dystrophin localizes to the sarcolemma of muscle fibers and forms a dystrophin–glycoprotein complex (DGC) with dystroglycans (DGs), sarcoglycans and syntrophin–dystrobrevin complexes, and DGC links the cytoskeletal protein actin to the basal lamina of muscle fibers (3). DGC may play a role in membrane stabilization during muscle contraction or act as a transducer of signals from the extracellular matrix (ECM) to the muscle cytoplasm via its interactions with intracellular signaling molecules (4). The loss of dystrophin leads to a condition in which the membrane is leaky under mechanical stress, and the subsequent increase in Ca2+ permeability results in activation of various proteases and alteration of the expression or function of dystrophin-associated plasma membrane proteins, such as neuronal nitric oxide synthase (nNOS), aquaporin-4 and ion channels (5). The pathology of the dystrophic muscle includes

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degeneration, necrosis with inflammatory cell invasion, regeneration and fibrous and fatty changes. However, the underlying mechanisms of ECM degradation, inflammation and fibrosis remain poorly understood.

ECM components have important roles in homeostasis and maintenance of muscle fiber functional integrity. Matrix metalloproteases (MMPs), a family of zinc-dependent endopeptidases, are key regulatory molecules in the formation, remodeling and degradation of ECM components in both physiological and pathological processes (6,7), such as tumor progression and metastasis, cerebrovascular and cardiovascular diseases and rheumatoid arthritis (8). Among the MMPs, MMP-2 (also called gelatinase A) and MMP-9 (also called gelatinase B) are involved in ECM remodeling of the skeletal muscle and in the dystrophic pathology of mdx, a mouse model for DMD (9). MMP-9 is produced by neutrophils and macrophages and is able to process β-DG, resulting in disruption of the link between the ECM and cell membrane in the dystrophin-deficient skeletal muscle from DMD patients (10–12). We previously reported that MMP-9 overexpresses in the mdx skeletal muscle after physical exercise (13) and may primarily be involved in the inflammatory process during muscle degeneration (14). Recently, it was reported that deletion of the MMP9 gene in mdx mice reduces muscle inflammation and fibronecrosis, resulting in an improvement in muscle function (15). Based on these reports, MMP-9 may play an important role in ECM degradation in the dystrophin-deficient muscle.

MMP-2 is derived from vascular endothelial cells as well as smooth muscle cells and has a role in angiogenesis, based on observation of impaired angiogenesis in an ischemic model in MMP-2 knockout mice (16). We and other researchers have also demonstrated that MMP-2 may be associated with ECM remodeling during muscle regeneration and fiber growth (9,14). Moreover, MMP-2 may be involved in myogenesis (17) and muscle regeneration (18). However, the precise role of MMP-2 has not been fully elucidated in the dystrophic muscle. To better understand the role of MMP-2 in dystrophic pathology, we generated mdx/MMP-2−/− mice by crossing mdx with MMP-2 knockout mice (MMP-2−/−) and examined the muscle pathology. Here, we demonstrate that MMP-2 ablation in mdx mice results in impairment of muscle fiber growth related to down-regulation of vascular endothelial growth factor-A (VEGF-A) when compared with mdx/MMP-2+/+ mice. Moreover, expression of nNOS in 3-month-old mdx/MMP-2−/− mice was significantly lower than that in mdx mice. These results imply that MMP-2 may be required for growth of regenerated muscle fibers through angiogenesis by VEGF-A (hereafter, VEGF) in the dystrophin-deficient muscle.

RESULTS

MMP-2 ablation in mdx mice impairs growth of regenerated muscle fibers

To elucidate the role of MMP-2 in the dystrophin-deficient muscle, we crossed MMP-2 knockout (MMP-2−/−) mice (19) with mdx mice with the genetic background of C57 BL/6J to generate mdx/MMP-2−/− mice. MMP-2 mRNA and its protein activity were absent in the skeletal muscle of both MMP-2−/− and mdx/MMP-2−/− mice, but not in wild-type (WT) and mdx mice (Supplementary Material, Fig. S1A and C). There was no statistically significant difference in the MMP-9 mRNA level in the skeletal muscle between the groups at 1 and 3 months of age (Supplementary Material, Fig. S1B). Although we could not detected MMP-9 protein activity on gelatin zymography in mice aged 3 months (Supplementary Material, Fig. S1C), the levels of both the pro- and active forms of MMP-9 protein levels were found to be significantly increased in mdx and mdx/MMP-2−/− mice when compared with the WT and MMP-2−/− mice at both 1 and 3 months of age (Supplementary Material, Fig. S1D–F). The discrepancy between the mRNA and protein levels of MMP-9 in the dystrophic skeletal muscle is speculated to be due to post-transcriptional regulation (20). MMP-3 mRNA levels were significantly elevated in the skeletal muscle of mdx and mdx/MMP-2−/− mice at 1 and 3 months of age when compared with that of the WT mice; the level was greatly increased in mdx/MMP-2−/− mice at 3 months of age (Supplementary Material, Fig. S1G). The mRNA levels of tissue inhibitor of metalloproteinase (TIMP)-1, an intrinsic inhibitor of MMP-9 and -3, were significantly up-regulated in the skeletal muscle of mdx and mdx/MMP-2−/− mice when compared with that of the WT mice at 1 and 3 months of age (Supplementary Material, Fig. S1H). There was no difference in the mRNA levels of TIMP-2, an intrinsic inhibitor of MMP-2, among the mouse groups at 1 or 3 months of age (Supplementary Material, Fig. S1I). In both MMP-2−/− mice (19) and mdx/MMP-2−/− mice, fertility and development were grossly normal. There were no differences in body weight at 1 or 3 months of age between mdx and mdx/MMP-2−/− or between WT and MMP-2−/− mice (Supplementary Material, Fig. S2A). Muscle power, evaluated using the hanging wire test and serum creatine kinase (CPK) levels, in mdx/MMP-2−/− at 3 months of age was not statistically different from that of mdx at the same age (Supplementary Material, Fig. S2B and C).

We then examined the histopathology of the skeletal muscle from mdx/MMP-2−/− mice. A previous study of mdx mice reported muscle necrosis with infiltration of neutrophils or macrophages at around 2 weeks of age, massive muscle degeneration/necrosis at around 1 month of age and completion of muscle necrosis with substitution of many regenerated fibers at 3 months of age (21). Histopathological findings for the tibialis anterior (TA) muscle from mdx/MMP-2−/− mice at 1, 2 and 3 months of age were roughly comparable to those of mdx mice at each age (Fig. 1A). However, we found that the muscle fibers, especially the centronuclear regenerated fibers, in mdx/MMP-2−/− mice at 3 months of age were significantly smaller than in mdx mice at the same age (Fig. 1B–E). Almost all of the small regenerated fibers in mdx/MMP-2−/− mice at 3 months of age were type II fibers, and a similar tendency was observed in mdx mice at the same age (Supplementary Material, Fig. S2D). We also analyzed diaphragm, quadriceps and gastrocnemius muscles in mdx and mdx/MMP-2−/− mice at 1 and 3 months of age. Like the TA muscle, these skeletal muscles in mdx/MMP-2−/− mice at 3 months of age exhibited impaired growth of regenerated fibers (Supplementary Material, Fig. S3). Furthermore, we monitored the muscle regeneration process as a result of cardiotoxin injury in the skeletal muscle of MMP-2−/− mice at 6 weeks of age. The result clearly
indicated that the centronuclear regenerating fibers 7 days after cardiotoxin injection in the skeletal muscle of MMP-2−/− mice were smaller than those of the WT (Supplementary Material, Fig. S4). These results suggest that ablation of MMP-2 may impair the growth of regenerated muscle fibers after the damage.

MMP-2 deficiency impairs angiogenesis in regenerated skeletal muscles of mdx

It has been reported that MMP-2 may be associated with angiogenesis, based on inhibition studies of MMP-2 (22,23). MMP-2 knockout mice have shown impairment of angiogenesis in an ischemia-induced model (16) and in a tumor model (24). To determine whether angiogenesis was impaired in the skeletal muscle of mdx/MMP-2−/− mice, we examined vessels in the TA muscle, through immunohistochemistry using an antibody to PECAM-1: an epithelial cell marker (Fig. 2A). We found that the mean size of vessels was significantly smaller in the skeletal muscle of mdx/MMP-2−/− mice at 3 months of age than that of age-matched mdx mice (Fig. 2B). Although the number of vessels per square millimeter was not different, the vessel counts per myofiber were significantly higher in mdx mice at 3 months of age, but decreased in age-matched mdx/MMP-2−/− mice (Fig. 2C and D). We also determined the endothelial area, which is
equivalent to vascular bed, by multiplying the size of vessels by their number in the TA muscle. The endothelial area in mdx/MMP-2−/− mice at 3 months of age was significantly lower than that of the mdx mice at the same age (Fig. 2E). These results clearly indicate that MMP-2 ablation impairs angiogenesis in the mdx skeletal muscle.
MMP-2 ablation in mdx mice down-regulates VEGF

We found that ablation of MMP-2 caused growth impairment of regenerated fibers and of angiogenesis in the mdx skeletal muscle at 3 months of age. We hypothesized that the growth impairment might be associated with the abnormality in angiogenesis; therefore, we examined the mRNA levels of various angiogenesis-related factors such as angiopoietin-1 and -2, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β) and VEGF. No statistical differences in the levels of angiopoietin-1 or -2, PDGF or FGF were detected between mdx and mdx/MMP-2−/− mice at 3 months of age (Fig. 3A–C and E). TGF-β mRNA levels were increased in mdx and mdx/MMP-2−/− mice when compared with WT, but did not significantly change with or without MMP-2 (Fig. 3D). However, we found a significant decrease in the VEGF mRNA level in mdx/MMP-2−/− mice when compared with mdx mice at 3 months of age (Fig. 3F). We also found a significant decrease in the mRNA level of Flt-1 (VEGF receptor 1), but not Flk-1 (VEGF receptor 2), in mdx/MMP-2−/− mice at 3 months of age (Fig. 3G and H). The VEGF protein level was significantly lower in the skeletal muscle of mdx/MMP-2−/− mice when compared with mdx mice at 3 months of age (Fig. 3A and B). VEGF was localized at neural cell adhesion molecule (NCAM)-positive muscle satellite cells and at the sarcolemma in some muscle fibers (Fig. 4C), but not at Mac-3-positive macrophages (Supplementary Material, Fig. S5). Flt-1 was also localized at most of the NCAM-positive muscle satellite cells. Flk-1-positive cells were identified as vascular endothelial cells by immunoreactivity of PECAM-1 (Fig. 4C).

It has also been reported that MMP-2 is up-regulated in muscle regeneration in an experimentally injured model (9) and plays a role in myogenesis (17). To determine whether myogenic or growth factors are involved in pathogenesis in mdx/MMP-2−/− mice, we investigated the mRNA levels of the myogenic transcription factors Pax-3 and -7, MyoD, Myf5, myogenin and MEF2 in the skeletal muscle. However, there were no statistical differences in the levels of these factors between mdx and mdx/MMP-2−/− mice or between WT and MMP-2−/− mice at 1 or 3 months of age (Supplementary Material, Fig. S6A–F). There were also no differences in the mRNA levels of the growth factors such as insulin-like growth factor, myostatin or follistatin in the TA muscles between the two groups (Supplementary Material, Fig. S6G–I).

Overexpression of S100 proteins and cytokines in the skeletal muscle of mdx/MMP-2−/− mice at 3 months of age

As reported above, we examined the expression levels of various factors related to angiogenesis, myogenesis and growth in the skeletal muscle of mdx/MMP-2−/− mice. To determine whether other genes affect pathology in mdx/MMP-2−/− mice, we conducted microarrays to comprehensively identify differentially expressed genes in the skeletal muscle of mdx and mdx/MMP-2−/− mice at 1 and 3 months of age. Based on the profiles of all the genes, we found 113 genes that were differentially (>2- or <0.5-fold) affected by MMP-2 ablation (Supplementary Material, Table S1). Thirteen genes were up-regulated (Supplementary Material, Table S2) and 18 genes were down-regulated (Supplementary Material, Table S3) at 1 month of age, and 69 genes were up-regulated (Supplementary Material, Table S4) and 18 genes were down-regulated (Supplementary Material,
Table S5) at 3 months of age in mdx/MMP-2<sup>−/−</sup> mice when compared with mdx mice (Supplementary Material, Fig. S7A and B). The genes that were up-regulated at 3 months of age in mdx/MMP-2<sup>−/−</sup> mice were mainly involved with protein binding, cytokine and its receptor or cell growth. Among the genes up-regulated at 3 months of age, S100 calcium-binding protein A8 (S100A8) and A9 (S100A9), cytokines such as chemokine C-C motif ligand (CCL)-2 and-7, chemokine C-C motif receptor (CCR)-1 and -2, and chitinase 3-like 3 were highly up-regulated in mdx/MMP-2<sup>−/−</sup> mice (Supplementary Material, Table S4). We confirmed these results by using quantitative real-time polymerase chain reaction (RT-PCR; Supplementary Material, Fig. S7C–I).

MMP-2 ablation further decreases nNOS expression in the skeletal muscle of mdx mice

Nitric oxide (NO) is a vasodilator produced by nitric oxide synthase (NOS), and inhibition of NOS activity abolishes capillary proliferation in electrically stimulated skeletal muscles (25). In electrically stimulated skeletal muscles, expression of endothelial NOS (eNOS) and nNOS increase in the early and late stages of angiogenesis, respectively (25–27). nNOS is linked to DGC, and dystrophin deficiency causes a reduction in nNOS expression at the sarcolemma, resulting in modification of dystrophic pathology (28,29). Thus, we examined the mRNA levels of nNOS, eNOS and inducible NOS (iNOS) produced by invaded inflammatory cells in the skeletal muscle of mdx/MMP-2<sup>−/−</sup> mice. mRNA expression of nNOS, but not of eNOS and iNOS, was significantly lower in mdx/MMP-2<sup>−/−</sup> than in mdx mice at 3 months of age (Fig. 5A–C). Western blotting also revealed that the nNOS protein level was significantly lower in mdx/MMP-2<sup>−/−</sup> than in mdx mice at 3 months of age (Fig. 5D and E), although there were no apparent differences in nNOS immunoreactivity between the mdx/MMP-2<sup>−/−</sup> and mdx mice (Fig. 5F). These results suggest that down-regulation of nNOS in the regenerated skeletal muscle of mdx/MMP-2<sup>−/−</sup> mice may influence impairment of angiogenesis.

MMP-2 does not affect the degradation of DGC components in the process of dystrophic muscle regeneration

DGs comprise two subunits, a highly glycosylated ECM protein, α-dystroglycan (α-DG), and a transmembrane
protein, β-dystroglycan (β-DG). DGs are membrane receptors involved with the complex of glycoproteins associated with dystrophin (30), and their interaction is crucial in maintaining the integrity of the plasma membrane. In the dystrophin-deficient muscle, the interaction between the two DG subunits may be disrupted by the proteolytic activity of MMPs (12). Previous research has examined the proteolytic activities of human MMP-9 and MMP-2 on the recombinant extracellular domain of β-DG and characterized a cleavage site by MMP-9 on β-DG (31). However, the molecular mechanism underlying the effect of MMP-2 is still unknown. We examined whether MMP-2 ablation in mdx mice affects processing of DGC in the skeletal muscle. In the skeletal muscle of both 

![Figure 5](https://academic.oup.com/hmg/article-abstract/20/9/1787/882577)

3 months of age (Fig. 6B and C). Similarly, expression of β-sarcoglycan did not change in the skeletal muscle of mdx/MMP-2/−/− mice at 1 or 3 months of age when compared with mdx of the same age (Supplementary Material, Fig. S8). In the process of regeneration of the dystrophic muscle, MMP-2 may not have a critical role in the degradation of DGC components.

**DISCUSSION**

In this study, we investigated the role of MMP-2 in the dystrophic skeletal muscle using mdx mice with MMP-2 ablation (mdx/MMP-2/−/−). The histopathology of the skeletal muscle in mdx/MMP-2/−/− mice at 3 months of age showed small regenerated muscle fibers and an impairment of angiogenesis when compared with mdx mice at the same age. The impaired growth of regenerating muscle fibers was also observed in the
cardiotoxin injury model of MMP-2<sup>−/−</sup> mice, suggesting that MMP-2 may play an important role in the muscle regeneration under certain disease conditions. MMP-2, a primary MMP derived from vascular endothelial cells and smooth muscle cells, degrades various ECM proteins (32,33) and is implicated as a key player in vascular development and angiogenesis (34). MMP-2 knockout mice have demonstrated a reduction in angiogenesis and corresponding tumor growth (24) and impairment of ischemia-induced neovascularization through a decreased number of endothelial cells and endothelial pro-genitor cells (16). Therefore, we hypothesized that the close relationship between impairment of regenerated myofiber growth and reduction in angiogenesis in the mdx/MMP-2<sup>−/−</sup> mice might allow us to identify factors related to angiogenesis or growth in these mice. We subsequently found a reduction of VEGF expression in the regenerated skeletal muscle of the mice at 3 months of age.

VEGF plays an important role in mediating both physiological and pathological angiogenesis via inducing vasodilation or vascular permeability, and by stimulating the proliferation, migration and survival of endothelial cells (35). In a previous study, both cultured satellite cells and myoblasts expressed VEGF and VEGFR-1 and -2; furthermore, administration in vitro stimulates myoblast migration and survival, protects myogenic cells from apoptosis and promotes myogenic cell growth (36). In normal muscles, VEGF and its receptors are expressed in vascular structures and not in muscle fibers; however, they are expressed in satellite cells and regenerating muscle fibers after experimental muscle damage, suggesting the operation of an autocrine pathway that may promote the survival and regeneration of myocytes (37). This study also demonstrates that the introduction of VEGF by using a virus vector promotes regeneration via angiogenesis, resulting in the decrease in muscle damage as well as the promotion of muscle regeneration and function in mdx mice (37). Meanwhile, another study shows that VEGF administration by using viral vectors injected in the normal mouse skeletal muscle results in the appearance of a notable subset of muscle fibers exhibiting muscle regeneration. Moreover, the delivery of VEGF markedly promotes muscle fiber regeneration with a dose-dependent effect after experimental muscle damage with ischemia, glycerol or cardiotoxin (38). Furthermore, the increased density of satellite cells has been observed adjacent to capillaries, suggesting a possible role of VEGF in homing circulating progenitor germ cells to specific muscle location and/or in regulating the satellite cells pool (39). Taken together, VEGF might function during regeneration not only through neovascularization, but also by directly acting on muscle cells and on the recruitment of progenitor cells from bone marrow during dystrophic pathology. VEGF was down-regulated in an ischemic-induced model using MMP-2 knockout mice due to a reduction in the number of invasive macrophages producing VEGF (16). However, our data indicate that VEGF is localized in NCAM-positive satellite cells and the sarcolemma in certain muscle fibers (Fig. 4C), but not in Mac-3-positive macrophages (Supplementary Material, Fig. S5). In a recent report, MMP-2 transcriptional inactivation by using an siRNA-based approach both in in vitro and in vivo significantly reduced integrin-αVβ3-mediated phosphoinositide 3-kinase/AKT-induced VEGF expression, which ultimately decreased tumor cell-induced angiogenesis (40). Similar mechanism might also underlie the relationship between MMP-2 and VEGF in the

Figure 6. β-DG degradation in the mdx/MMP-2<sup>−/−</sup> mice skeletal muscle. (A) Western blot analyses of β-DG in the skeletal muscle of WT, MMP-2<sup>−/−</sup>, mdx and mdx/MMP-2<sup>−/−</sup> mice. Full-length β-DG (βDG<sub>43</sub>, upper bands) and degraded 30-kDa proteins (βDG<sub>30</sub>, lower bands) were observed in both mdx or mdx/MMP-2<sup>−/−</sup> mice at 1 and 3 months of age. Relative (to GAPDH) levels of βDG<sub>43</sub> (B) and βDG<sub>30</sub> (C) revealed an increase in βDG<sub>43</sub> and a decrease in βDG<sub>30</sub> in mdx/MMP-2<sup>−/−</sup> when compared with mdx at 1 month of age. Bar: mean ± S.E.M.; *P < 0.05, **P < 0.01, significantly different from WT; #P < 0.05, ##P < 0.01, significantly different from MMP-2 ablation alone.
dystrophic muscle. Although VEGF is known to be located upstream of MMP-2, we suggest that MMP-2 and VEGF may regulate each other in the skeletal muscle.

We conducted microarrays to comprehensively identify differentially expressed genes in mdx/MMP-2<sup>-/-</sup> mice, because other genes could affect the phenotype. Among the genes up-regulated at 3 months of age in mdx/MMP-2<sup>-/-</sup> mice, S100A8 and A9 mRNA levels were significantly increased. S100 proteins are involved in the pathogenesis of cellular stress condition such as wound healing or inflammatory disorders (41). In particular, S100A8 and A9 are required for transcriptional activation of the MMP-2 gene (42). We suggest, therefore, that overexpression of S100A8 and S100A8/A9 heterodimer (43). The up-regulation increased in macrophages stimulated by recombinant S100A8 in knee joints of normal mice and was increased in macrophages stimulated by recombinant S100A8 or S100A8/A9 heterodimer (43). The up-regulation of MMP-3 in the skeletal muscle of mdx/MMP-2<sup>-/-</sup> mice at 3 months of age may be caused by overexpression of S100A8 and A9.

We also found that expression of nNOS in the mdx/MMP-2<sup>-/-</sup> mice was significantly lower than in mdx mice at 3 months of age. This further reduction in nNOS may have been caused by MMP-2 ablation in the mdx mice, or impairment of angiogenesis may secondarily induce a further decrease in nNOS. NO is increased in the ischemic hindlimb and eliminating NO impairs the revascularization process (44). It has been reported that an nNOS transgene in mdx mice ameliorated muscular dystrophy (45) and that mdx mice expressed dystrophin only in smooth muscle cells, restoring vascular nNOS expression and NO-dependent vasoregulation and resulting in improvement in dystrophic pathology (46). Taken together, these studies show that MMP-2 ablation in mdx mice may result in further reduction of nNOS in the dystrophic muscle, with a detrimental effect on the function and regeneration of the dystrophic muscle. It is reported that nNOS levels are reduced in inflammatory conditions (47). Actually, our data showed that the expression of some cytokines (e.g. CCL-2) was significantly increased in the skeletal muscle of mdx/MMP-2<sup>-/-</sup> at 3 months of age. Taken a report that S100A8/A9 enhances the gene expression of pro-inflammatory proteins such as CCL-2 (48), S100A8/A9 up-regulation may enhance pro-inflammatory genes, resulting in the down-regulation of nNOS expression in mdx/MMP-2<sup>-/-</sup> mice at 3 months of age. Interestingly, nNOS<sup>-/-</sup> mice in an acute lung injury model show reduced expression of VEGF protein (49). The decrease in nNOS levels via increased cytokines may also reduce VEGF expression in the skeletal muscle of mdx/MMP-2<sup>-/-</sup> mice at 3 months of age.

We assessed differences in the degradation patterns of DGC in the skeletal muscles between mdx and mdx/MMP-2<sup>-/-</sup> mice. The β-DG degradation was reduced by MMP-2 ablation in mdx mice at 1 month of age. Nevertheless, at 3 months of age, β-DG degradation was unchanged in the presence of MMP-2. Previous reports documented that MMP-2 as well as MMP-9 were able to degrade β-DG (50) and that macrophage-derived MMP-2 in a mouse model of experimental autoimmune encephalomyelitis participated in tissue injury via β-DG degradation through proteolytic activity (10). However, our data indicated that β-DG degradation by MMP-2 was not apparent in the process of muscle regeneration. The expression of β-sarcoglycan remained unchanged in the skeletal muscle of mdx/MMP-2<sup>-/-</sup> mice at 1 and 3 months of age when compared with mdx of the same age. These results suggest that the ablation of MMP-2 does not significantly influence the degradation of β-DG and β-sarcoglycan in the regeneration process of the dystrophic muscle.

In this study, we found that reduction in angiogenesis via decreased VEGF and nNOS expression may impair regeneration in the skeletal muscle of mdx mice with MMP-2 ablation (Fig. 7). Corticosteroids are promising agents for the prevention of progression in various diseases including DMD; however, they inhibit VEGF and MMP-2, resulting in a reduction in tissue angiogenesis (51,52). Normal rats treated with corticosteroids exhibit muscle atrophy and weakness with a concomitant reduction in VEGF expression (53,54); therefore, the decrease in MMP-2 and VEGF by corticosteroids might be associated with the pathogenesis in steroid myopathy.

**MATERIALS AND METHODS**

**Mice**

Control (strain: C57 BL/6J) and MMP-2 knockout (strain: C57BL/6J-Mmp2tm) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Dystrophin-deficient (mdx) mice (strain: C57BL/6J-DMDmdx) were a gift from the Institute of Neuroscience, National Research Center of Neurology and Psychiatry (Tokyo, Japan). MMP-2 knockout (MMP-2<sup>-/-</sup>) mice were crossed with mdx mice to generate littermate WT, MMP-2<sup>-/-</sup>, mdx/MMP-2<sup>+/-</sup> and mdx/MMP-2<sup>-/-</sup> mice. All genotypes were determined using PCR.
analysis of mice tail DNA. An amplification-resistant mutation system assay was used to identify control and mdx mice (55). MMP-2 knockout and control mice were identified using the primer sets suggested by the Jackson Laboratory. Mice were housed in a plastic cage in a temperature-controlled environment with a 12-h light/dark cycle and free access to food and water. All experiments with animals were carried out in accordance with the institutional guidelines and approved by the Institutional Review Board of Shinshu University, Japan.

Muscle tissue extraction and preparation

TA muscles were carefully dissected and frozen in isopentane cooled by liquid nitrogen for histological and immunohistochemical analyses and protein and RNA isolation, and were stored at −80°C. Ten-micrometer transverse cryostat sections were cut in the center of the TA muscle belly to obtain the largest cross-sectional area (CSA), placed on slides, air-dried and stained with hematoxylin and eosin (H&E). Serial sections were stained to demonstrate myofibrillar ATPase activity. Diaphragm, quadriceps and gastrocnemius muscles were also dissected and frozen, and were stained with H&E as noted above. The sections were viewed and photographed using a digital camera system (Leica Microsystems, Wetzlar, Germany).

Cardiotoxin muscle injury and histochemistry

We injected 100 μl of cardiotoxin (10 mM in 0.9% NaCl) (Sigma, St Louis, MO, USA) into the TA muscle of WT and MMP-2−/− mice at 6 weeks of age using a 27-gage needle and a 1-ml syringe. The needle was inserted deep into the TA muscle longitudinally toward the knee from the ankle. The needle was held in place for a few seconds and then slowly withdrawn along the long axis of the anterior tibial muscle with a little pressure to allow the cardiotoxin to permeate throughout the muscle. The TA muscles were isolated before the injection, and 3 and 7 days after; the muscles were cut in the center of the TA muscle belly to obtain the largest cross-sectional area (CSA), placed on slides, air-dried and stained with hematoxylin and eosin (H&E). Serial sections stained with anti-PECAM-1 antibody. Sections were viewed and photographed using a fluorescent microscope (Olympus, Tokyo, Japan), and images were captured using a VB-7010 camera (Keyence, Osaka, Japan). The primary antibody dilutions and sources were as follows: rat monoclonal anti-PECAM-1 (1:50; BD Transduction Laboratories, San Jose, CA, USA), rabbit polyclonal anti-VEGF-A (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-Flt-1 (1:100; Santa Cruz Biotechnology), rabbit monoclonal anti-Flk-1 (1:100; Cell Signaling Technology, Danvers, MA, USA), rat monoclonal anti-laminin α2-chain (1:100; Enzo Life Sciences, Plymouth Meeting, PA, USA), rabbit polyclonal anti-nNOS (1:200; Invitrogen, Carlsbad, CA, USA), rabbit polyclonal anti-NCAM (1:100; Millipore, Billerica, MA, USA), rabbit monoclonal anti-Mac3 (1:50 BD; Transduction Laboratories), mouse anti-β-sarcoglycan (1:200; Leica Microsystems). Alexa Fluor® 488 or Alexa Fluor® 568-conjugated secondary antibodies were obtained from Invitrogen and used at 1:500 dilution.

Morphometric analysis

Morphometric analysis was performed to determine the CSA of each muscle fiber by using the H&E-stained TA, diaphragm, quadriceps and gastrocnemius muscle sections, separately recording the CSAs for perinuclear fibers and centronuclear regenerated fibers. Necrotic fibers, when present, were discarded. The distribution of muscle fiber CSAs was examined using National Institutes of Health (Bethesda, MD, USA) images. At least 1000 fibers were analyzed for each muscle, and muscle fiber boundaries were determined to count the size and number of the fibers. All images were obtained under identical conditions and at the same magnification. For the CSA histogram, histological parameters were evaluated and treated as previously described (56). Variability in fiber size was determined by the mean ± S.E.M. values.

Study for vessels was performed on 6-μm-thick TA muscle sections stained with anti-PECAM-1 antibody. Serial H&E-stained sections were used to count the number of muscle fibers. The number and size of each vessel and number of muscle fibers were counted under identical conditions and at same magnification. We determined the number of vessels per myofiber and endothelial area of PECAM-1-positive vessels in total area.

Immunohistochemical analysis

For immunofluorescent staining, serial cross-sections (6-μm thick) from frozen skeletal muscle tissues were mounted on glass slides. The sections were air-dried and blocked in 10% goat serum in phosphate-buffered saline (PBS) for 30 min and incubated with primary antibodies in blocking solution at 4°C overnight. The sections were washed briefly with 1 × PBS before incubation with secondary antibodies for 1 h at room temperature and then washed three times for 30 min with 1 × PBS. The slides were mounted using a fluorescence medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA), visualized using a fluorescent microscope (Olympus, Tokyo, Japan), and images were captured using a VS-2000Plus camera (Keyence, Osaka, Japan). The primary antibody dilutions and sources were as follows: rat monoclonal anti-PECAM-1 (1:50; BD Transduction Laboratories, San Jose, CA, USA), rabbit polyclonal anti-VEGF-A (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-Flt-1 (1:100; Santa Cruz Biotechnology), rabbit monoclonal anti-Flk-1 (1:100; Cell Signaling Technology, Danvers, MA, USA), rat monoclonal anti-laminin α2-chain (1:100; Enzo Life Sciences, Plymouth Meeting, PA, USA), rabbit polyclonal anti-nNOS (1:200; Invitrogen, Carlsbad, CA, USA), rabbit polyclonal anti-NCAM (1:100; Millipore, Billerica, MA, USA), rabbit monoclonal anti-Mac3 (1:50 BD; Transduction Laboratories), mouse anti-β-sarcoglycan (1:200; Leica Microsystems). Alexa Fluor® 488 or Alexa Fluor® 568-conjugated secondary antibodies were obtained from Invitrogen and used at 1:500 dilution.

Total protein extract and western blotting

Muscle tissues (20 mg) were homogenized in 150 μl of 5% sodium dodecyl sulfate (SDS) sample buffer (50 μl/l Tris–HCl, pH 8.0, 10 μmol/l ethylenediaminetetraacetic acid, 5% SDS and 5% β-mercaptoethanol). After centrifugation (10 min at 15 000g), the protein concentration was estimated in the supernatant using the BCA Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Protein homogenates recovered from the supernatant from each sample were denatured for 5 min at 95°C in reducing buffer (50 μl of SDS buffer containing 5% SDS, 0.01% bromophenol blue, 10% glycerol and 5% β-mercaptoethanol). Protein extracts (10 μg/lane) were submitted to SDS-polyacrylamide gel electrophoresis (7.5 or 12.5%) with pre-stained standard proteins (Bio-Rad) to achieve more accurate molecular weight determination. The resulting gel was transferred onto a 0.2-μm nitrocellulose membrane (Millipore) using a transfer buffer (25 mmol/l Tris–HCl, pH 8.3, 192 mmol/l glycine and 20% methanol). The membranes were blocked with Tris buffer, 0.1% Tween 20 (TBST) containing 5% milk (w/v) for 1 h at room
temperature. All membranes were incubated with primary antibodies at 4°C overnight followed by several washes with TBST. The membranes were incubated with peroxidase-conjugated secondary antibodies (Bio-Rad) for 1 h, washed several times with the washing buffer described above and visualized using an enhanced chemiluminescence system according to the manufacturer’s protocol (Amersham, Little Chalfont, UK). Protein signals were quantified by scanning densitometry using the program package of the National Institutes of Health. The results from each experimental group were expressed as integrated intensities relative to the control samples. Equal loading of proteins was assessed on stripped blots by immunodetection using the anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody. The primary antibody dilutions and sources were as follows: goat anti-VEGF-A (1:200; Santa Cruz Biotechnology), mouse anti-nNOS (1:1000; BD Transduction Laboratories), rabbit anti-MMP-9 (1:1000; Millipore), mouse anti-β-DG (1:800; Leica Microsystems), mouse anti-β-sarcoglycan (1:400; Leica Microsystems) and mouse anti-GAPDH (1:3000; Millipore).

**Gelatin zymography**

Frozen skeletal muscles were homogenized in an extraction buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS and 10% glycerol), and total protein content was assessed using a BCA Protein Assay Kit (Bio-Rad). Each extract (50 μg) was dissolved in a loading buffer provided by the manufacturer and subsequently electrophoresed through a gelatin-containing SDS-polyacrylamide gel provided as part of the Gelatin Zymography Kit (Invitrogen). The gel was washed with regenerating buffer and subsequently incubated for 24 h at 37°C in developing buffer that was also provided by the manufacturer. The gels were stained in Coomassie Brilliant Blue (CBB) and destained with a destaining solution (Bio-Rad). Gelatinolytic activity was identified as clear bands on a blue background. Gelatin zymography detects the activity of both pro- and active forms of gelatinolytic MMPs. This is because exposure to SDS during gel electrophoresis activates the pro-form active forms of gelatinolytic MMPs. This is because exposure to SDS during gel electrophoresis activates the pro-form MMPs without proteolytic cleavage of the prodomain. Equality of the protein concentration was confirmed by CBB staining. Myosin heavy chain was used as a loading control.

**RNA isolation and gene expression profiling**

Frozen tissues (20 mg) for each muscle were homogenized, and the total RNA was isolated using a RNeasy Fibrous Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA synthesis, biotin-labeled target synthesis, Mouse Genome 430 2.0 Array Gene Chip (Affymetrix, Santa Clara, CA, USA) array hybridization, staining and scanning were performed according to the standard protocols supplied by Affymetrix. The quality of the data was controlled using Microarray Suite MAS 5.0 (Affymetrix). The MAS-generated raw data were uploaded to GeneSpring GX software version 10 (Silicon Genetics, Redwood City, CA, USA). The software calculated signal intensities, and each signal was normalized to the median of its values in all samples or the 50th percentile of all signals for a specific hybridization experiment. Fold ratios were obtained by comparing normalized data for mdx and mdx/MMP-2−/− mice.

**Analyses by RT-PCR**

Single-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). The levels of mRNA and 18S rRNA were quantified using fluorescent dye SYBR-green detection (Roche Diagnostics, Basel, Switzerland) with 10 nM of each primer at a final volume of 10 μl, and the reactions were carried out in duplicate using the StepOnePlus RT-PCR system (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions for all primers were 10 min at 95°C, then 40 cycles each of 15 s at 94°C, 30 s at 48°C, 1 min at 72°C and a final extension of 10 min at 72°C. For each gene, all samples were amplified simultaneously. Each RNA quantity was normalized to its respective 18S rRNA mRNA quantity. Primer sequences for RT-PCR are shown in Supplementary Material, Table S1.

**Statistical analysis**

Results are expressed as mean ± S.E.M. Statistical analysis was performed using an unpaired t-test for two-group comparisons, and multiple comparisons were performed using a one-way ANOVA. Intergroup comparison was carried out using the Bonferroni correction. Statistical significance was set at P < 0.05. Statistical analyses were carried out using the software SigmaStat, version 2.0 (Aspire Software, Ashburn, VA, USA).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**Conflict of Interest statement.** None declared.

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