Enhanced Arteriogenesis and Wound Repair in Dystrophin-Deficient mdx Mice

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Background—The absence of functional dystrophin in Duchenne muscular dystrophy (DMD) patients and in mdx mice results in progressive muscle degeneration associated with necrosis, fibrosis, and inflammation. Because vascular supply plays a key role in tissue repair, we examined whether new blood vessel development was altered in mdx mice.

Methods and Results—In a model of hindlimb ischemia on femoral artery dissection, hindlimb perfusion, measured by laser Doppler imaging, was higher in mdx mice (0.67±0.26) than in wild-type (WT) mice (0.33±0.18, P<0.03). In keeping with these data, a significant increase in arteriole length density was found in mdx mice (13.6±8.4 mm/mm²) compared with WT mice (7.8±4.6 mm/mm², P<0.03). Conversely, no difference was observed in capillary density between mice of the 2 genotypes. The enhanced regenerative response was not limited to ischemic skeletal muscle, because in a wound-healing assay, mdx mice showed an accelerated wound closure rate compared with WT mice. Moreover, a vascularization assay in Matrigel plugs containing basic fibroblast growth factor injected subcutaneously revealed an increased length density of arterioles in mdx (46.9±14.7 mm/mm³) versus WT mice (19.5±5.8 mm/mm³, P<0.001). Finally, serum derived from mdx mice sustained formation of endothelium-derived tubular structures in vitro more efficiently than WT serum.

Conclusions—These results demonstrate that arteriogenesis is enhanced in mdx mice both after ischemia and skin wounding and in response to growth factors. (Circulation. 2004;110:3341-3348.)

Key Words: muscles • genetics • vessels

Muscular dystrophies are a group of genetic diseases characterized by progressive degenerative changes in skeletal and cardiac muscle.1 The most common muscular dystrophies involve mutations of the dystrophin-glycoprotein (DGC) complex, which is constituted of the intracellular proteins dystrophin and syntrophin and the sarcolemmal proteins dystroglycan (α and β subunits) and sarcoglycans (α, β, γ, and δ subunits).2 The oligomeric complex spans the sarcolemma and links intracellular actin cytoskeleton to the extracellular matrix. DGC complexes are also present in a variety of nonmuscle cells.3 Dystrophin mutations are the most common cause of muscular dystrophy, accounting for both the Duchenne (DMD) and Becker phenotypes.4 The mdx mouse is a murine model of muscular dystrophy in which a non-sense mutation in the dystrophin gene abrogates dystrophin expression.5 Although the primary defect in dystrophic muscle is very well characterized, the relationship between the absence of the protein dystrophin and the pathogenic mechanisms of DMD is still unclear.

The lack of functional dystrophin protein destabilizes the DGC complex and alters signal transduction pathways, thereby rendering muscle fibers susceptible to damage.1 Recent evidence suggests that numerous factors could be involved in the pathogenesis of muscular dystrophies.

Growth factors, cytokines, and chemokines represent essential elements in the modulation of muscle cell regeneration and differentiation. Interestingly, many growth factors and chemokines, such as basic fibroblast growth factor (bFGF),6 monocyte chemoattractant protein-1 (MCP-1),7,8 and nerve growth factor,9 are upregulated in mdx mice. However, their role in DMD pathogenesis is still unclear. Moreover, disruption of muscle membrane repair machinery results in progressive muscular dystrophy in the presence of a functional DGC complex.10

Dystrophin protein is expressed not only in skeletal muscle cells but also in vascular smooth muscle and endothelial...
cells. In vascular smooth muscle cells, DGC disruption perturbs vascular functions, leading to ischemic lesions and exacerbating muscle damage. The molecular mechanism implicating dystrophin in vascular dysfunction may involve skeletal muscle–derived nitric oxide (neuronal nitric oxide synthase, nNOS), the enzyme that produces the freely diffusible signaling molecule nitric oxide (NO). In skeletal muscle, NO acts as an antiinflammatory molecule, preventing muscle damage. Moreover, NO regulates blood flow in exercising skeletal muscle by antagonizing α-adrenergic vasconstriction. It is conceivable that NO produced by nNOS in skeletal muscle fibers could diffuse to nearby arterioles, resulting in vasodilation and increasing blood flow. The dystrophin complex interacts with nNOS, anchoring this protein in the cytoplasm, and its expression is reduced. Clinical assessment of vascular control in DMD patients and experimental studies in dystrophin-deficient mice revealed an impairment of skeletal muscle contraction to attenuate α-adrenergic vasconstriction. More recently, it has been demonstrated that flow (shear stress)–mediated NO-dependent artery dilation is impaired in mdx dystrophin-deficient mice. Therefore, it was suggested that dystrophin deficiency, through a sustained vascular constriction and inadequate blood flow supply, causes chronic ischemia and contributes to muscle damage.

After ischemia, many factors regulate the consequent neoangiogenic response. In DMD, cytokines and growth factors produced by both resident and inflammatory cells recruited at sites of muscle degeneration may stimulate angiogenesis; conversely, a lack of NO because of nNOS deficiency may hamper this process. The present study assessed whether blood vessel development in response to an acute ischemic event was affected in dystrophin-deficient mice. To this aim, vascularization was examined in a model of hind limb ischemia in mdx mice. It was found that in mdx mice, arteriogenesis in response to ischemia was enhanced. Furthermore, bFGF-stimulated vessel formation in subcutaneously injected Matrigel was increased as well in mdx mice, and serum derived from mdx mice sustained in vitro endothelial cell–derived tubular structure formation more efficiently than the wild-type (WT) counterpart. Finally, faster skin wound healing was observed in mdx mice.

Methods

Animal Model of Hindlimb Ischemia

Male C57BL/10SnJ (WT) and mdx mice (The Jackson Laboratory, Bar Harbor, Me), 2 months of age and weighing 23 to 25 g, were used for all experiments. Animals were housed at constant room temperature (24±1°C) and humidity (60±3%). Hindlimb ischemia was induced by femoral artery dissection as described previously. Laser Doppler perfusion imaging (LDPI; Lisca) was used to record serial blood flow measurements for 14 days after surgery as described previously. All experimental procedures complied with the Guidelines of the Italian National Institutes of Health and with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md) and were approved by the Institutional Animal Care and Use Committee.

Histology, Immunohistochemistry, and Morphometric Analysis

Histology, immunohistochemistry, and morphometric analysis were performed as described in the online-only Data Supplement.

Angiogenesis Assays

In vivo and in vitro angiogenic assays are described in the Data Supplement.

Animal Wound Model

Wound healing was performed as described in the Data Supplement.

Hematopoietic Colony Assay

Peripheral blood cells were isolated by Ficoll-Histopaque (Sigma) gradient separation and then cultured in Methocult M3534 (Stem Cells Technologies). Briefly, 10⁵ cells were plated in triplicate in methylcellulose medium containing interleukin-6, interleukin-3, and stem cell factor. After 14 days, colonies containing granulocytes/macrophages (CFU-GM), granulocytes (CFU-G), macrophages (CFU-M), and endothelial cells (CFU-EC) were counted. To evaluate the expansion of stem cell–derived hematopoietic colonies, we grouped together CFU-GM, CFU-G, and CFU-M colonies as representative of colony-forming activity by clonogenic cells.

Statistical Analysis

All data are expressed as mean±SEM. A Student 2-tailed t test was performed, and a probability value of P≤0.05 was considered statistically significant.

Results

Angiogenic Response to Hindlimb Ischemia

Ischemia was induced in WT and mdx mice. LDPI was used to document changes in hindlimb blood flow at the indicated time points after the induction of ischemia. In both strains, blood flow was drastically reduced immediately after femoral artery dissection, and progressive recovery was detected between day 3 and day 14 (Figure 1). At day 7 after surgery, blood flow in the ischemic limb of mdx mice was markedly higher than in WT mice (0.67±0.26 versus 0.33±0.18; P<0.03). A significant difference was still evident at day 14 after surgery (0.82±0.2 versus 0.56±0.17; P<0.03). At day 21, blood flow was similar between WT and mdx mice (data not shown). Morphometric analysis was performed on adductor muscle sections from WT and mdx mice at day 7 after femoral artery dissection, when blood flow by LDPI exhibited the most significant difference between the 2 groups. Although capillary density was similar in both strains at the indicated time point (Figure 1B), the length density of arterioles 4 to 11 μm in diameter was significantly increased in mdx compared with WT mice (13.6±8.43 versus 7.8±4.6 mm/mm³; P<0.03) (Figure 1C). In nonischemic hindlimbs, capillary and arteriolar density was similar in both WT and mdx mice (Figure 1, B and C).

Angiogenic Response to bFGF in the Matrigel Assay

To explore the angiogenic response to bFGF, Matrigel supplemented with bFGF was injected subcutaneously into the mid-lower abdominal region of control and mdx mice. In this model, host endothelial cells and smooth muscle cells migrate and form a vascular network in the Matrigel implants. The number of blood vessels within the implants was first determined in histological sections by Masson’s trichrome staining 8 days after implantation (Figure 2A). Quantitative
analysis of the Matrigel plugs revealed that although the absence of bFGF in the Matrigel plugs failed to induce an angiogenic response in both WT and mdx mice, the total number of vessels increased in the presence of bFGF in a similar manner in both strains (Figure 2B). However, analysis of histological sections performed by staining vessels with α-smooth muscle actin antibody, which specifically identifies smooth muscle cells, revealed a significantly higher length density of arterioles (4 to 41 μm in diameter) in bFGF-supplemented Matrigel plugs excised from mdx mice compared with WT mice (46.9±14.7 versus 19.5±5.8 mm/mm³) (Figure 2, C and D).

Angiogenic Response in Wound Repair

Because neovascularization is a critical component in wound healing and regeneration, we examined whether skin wound closure was accelerated in mdx mice. Full-thickness skin wounds were surgically created on the middle back of WT and mdx mice. Analysis of wound diameter was performed through digital processing of pictures at the indicated time.
points (Figure 3A), and the rate of wound healing was expressed as a percentage of closure (Figure 3B). At day 3 after skin injury, areas were reduced by 34±15.9% in \( mdx \) and by 16.9±12.6% in WT mice \((P<0.005)\). Statistically significant differences in the percentages of wound closure between the 2 groups persisted at day 5 (56±14.9% versus 38±19.3%, \( P<0.005 \)) and day 7 (79.4±10.3% versus 73±10.8%, \( P<0.02 \)) (Figure 3B). Complete wound closure in both strains was evident by day 14 (Figure 3, A and B). To assess whether the difference in wound healing between WT and \( mdx \) mice was associated with enhanced skin neovascularization, morphometric analysis was performed on normal skin and on 5-day wounded skin in both WT and \( mdx \) mice. No difference in capillary density was found between strains before and after wounding (Figure 3C). In contrast, the length density of arterioles 4 to 41 \( \mu \)m in diameter was increased in \( mdx \) compared with WT mice (32.8±1 versus 21.5±3.4 mm/mm\(^3\), \( P<0.02 \)) (Figure 3D), and this difference persisted at day 5 after wounding (30.9±2.7 versus 19.6±2.4 mm/mm\(^3\), \( P<0.02 \)) (Figure 3D).

Figure 3. Angiogenic response to wound healing. Accelerated healing of full-thickness wounds in \( mdx \) mice compared with WT mice. A, Representative examples show wound healing in \( mdx \) and WT mice. Wounds were photographed at time indicated from day 0 to 10. Day 0 pictures were taken immediately after wounding. B, Average results showing that wound closure rate was different between WT (wt) and \( mdx \) mice. Results are presented as percentage of closed wound area vs total area at time 0 after wound. Each time point represents average result from 12 mice. *\( P<0.02 \). \( P<0.005 \) at day 3, \( mdx \) vs wild-type. Capillary (C) and arteriole length density (D) in skin of WT and \( mdx \) mice before (–) and at day 5 (+) of wound healing (WH). No difference was detected in capillary density in 2 animal groups. Statistically significant increase in arteriole length density per mm\(^3\) was observed in \( mdx \) vs WT mice, both in normal and in wounded skin (n=4 per group; *\( P<0.02 \) vs WT).

Serum From \( mdx \) Mice Accelerates Vascular Structure Formation In Vitro

The following experiments were aimed at determining whether the improved neovascularization in \( mdx \) mice could be associated with enhanced release of angiogenic growth factors into the systemic circulation. Human umbilical vein ECs (HUVECs) were plated on Matrigel in the presence of increasing concentrations of serum obtained from either WT or \( mdx \) mice. In all experimental conditions, HUVECs formed tubular structures, and 90 minutes after plating, branching points were counted to compare the angiogenic potential of serum from WT and \( mdx \) mice. At the indicated time point, a significant increase in the number of branching points was detected when cells were cultured in the presence of serum obtained from \( mdx \) mice (Figure 4, A and B). This difference persisted when increasing serum concentrations were used (Figure 4B). To investigate whether the enhanced tubular structure formation was mediated by an increased accumulation of growth factors in serum from \( mdx \) mice, the concentrations of 4 well-known angiogenic factors, vascular endothelial growth factor, bFGF, stromal-derived factor-1 (SDF-1), and MCP-1, were evaluated by an ELISA method. Although levels of vascular endothelial growth factor, bFGF, and MCP-1 were similar in serum from both strains (data not shown), SDF-1 levels were significantly increased in serum from \( mdx \) mice (1214±181 versus 745±154 pg/mL in WT mice, \( P<0.03 \)) (Figure 4C). Blocking antibody to the SDF-1 receptor CXCR4 strongly inhibited tubular structure formation by HUVECs in the presence of 1% of serum from both
WT and mdx mice. Under these experimental conditions, mdx and WT serum induced the formation of a similar number of branching points (Figure 4D).

Involvement of Stem Cell Mobilization in Ischemia-Induced Angiogenesis

Recent experimental results showed that bone marrow–derived stem cells as well as circulating progenitor cells incorporate into foci of damaged tissue, augmenting both skeletal muscle regeneration and neovascularization.23,24

In light of these studies, we sought to investigate whether stem cells are involved in the enhanced neovascularization observed in mdx mice. To this end, we first analyzed the level of hematopoietic progenitor cells in the peripheral blood of mdx mice. By a colony-formation assay using methylcellulose stem cell medium, a 4-fold increase in the number of circulating hematopoietic progenitors capable of forming CFU colonies (CFU-GM, CFU-G, and CFU-M) was found in the peripheral blood obtained from mdx mice compared with WT mice (Figure 5A). Then, we tested the possibility that stem cells contributed to the increased vascularization observed in ischemic muscle of mdx mice. Despite the increased number of stem cells in the peripheral blood of mdx mice, immunohistological analyses performed to detect stem cells expressing c-kit antigen in hindlimb skeletal muscle tissue did not evidence any significant differences between the 2 strains (data not shown). However, 3 days after ischemia, skeletal muscle from mdx mice revealed a 10-fold increase of c-kit–positive (c-kit+) cells compared with those detected in WT mice (Figure 5B). These cells were located close to both the muscle fibers and the vessel walls. Interestingly, some c-kit+ cells associated with vessel walls expressed smooth
The present study identifies a link between dystrophin and smooth muscle cells. Muscle actin (Figure 5B), indicating their differentiation into smooth muscle cells.

**Discussion**

The present study identifies a link between dystrophin and neovascularization. Mdx mice, lacking functional dystrophin, showed enhanced vascularization of ischemic hindlimbs, accelerated wound healing, and improved vessel growth in bFGF-infiltrated Matrigel plugs.

In the absence of ischemia, there was no detectable difference in the vascularization of hindlimb muscle between control and mdx mice. However, after ischemia, evidence of enhanced perfusion was obtained from blood flow measurements, and at day 7 after surgery, blood flow was 50% higher in mdx limbs than in limbs from control mice. Consistent with this result, ischemic hindlimbs from mdx mice presented, at day 7 after surgery, a significant increase in small arteriolar length density, whereas no change in capillary density was detected. Vascularization plays a key role in tissue repair, and skeletal muscle tissues of mdx mice show degeneration and regeneration between 2 and 12 weeks of age. Increased vasculogenesis may represent an adaptive response of the organism to pathological levels of muscle regeneration.

The enhanced number of arterioles after ischemia and in the in vivo bFGF Matrigel plug assay indicates that mdx mouse neovascularization occurs through the activation of an arteriogenic response. In contrast, at least under the experimental conditions of the present study, capillary number was not affected.

Acceleration of the regenerative process is not restricted to skeletal muscle, because wound closure was also improved in mdx skin. Indeed, efficient wound repair is a coordinated process that requires not only an increase in the synthesis and deposition of collagen and migration of fibroblasts and keratinocytes into the wound area but also neovascularization. In contrast to skeletal muscle, a significant increase in arteriole length density was found in mdx mouse normal skin versus WT mouse skin, and this difference persisted at 5 days after wounding.

To date, we do not know whether enhanced vasculogenesis is a direct consequence of the lack of functional dystrophin. The use of animal models of muscular dystrophy with a functional dystrophin and DGC complex, as well as dystrophin-overexpressing mdx mice, could help understand the role of dystrophin in neovascularization. Secondary mechanisms, such as the inflammatory response to muscle damage, could be responsible for increased arteriogenesis and regeneration in mdx mice. Conversely, a systemic imbalance of cytokines and growth factors may stimulate regenerative processes and inhibit others, eg, skeletal muscle fiber maturation. It is well accepted that the inflammatory response to myofiber damage is a compelling candidate mechanism for the exacerbation of DMD. Cytokines and chemokines released by DMD muscle may play an important role in recruiting leukocytes and macrophages, which in turn can act as a source of more cytokines and growth factors. Indeed, bFGF, MCP-1, nerve growth factor, and PDGF are upregulated in mdx mouse skeletal muscle, and some of these factors are also enhanced in the serum of DMD patients. These factors are all potent stimulators of arteriogenesis and can act as chemoattractants for satellite cells and stem cells. Consistent with these findings, we showed that serum obtained from mdx mice significantly enhanced HUVEC reorganization in tubular structures on Matrigel. Interestingly, we detected increased levels of the chemokine SDF-1 in mdx mouse serum. Blocking antibody to the SDF-1 receptor CXCR4 inhibited tubular structure formation on Matrigel assay, demonstrating that the increased levels of SDF-1 may be responsible for the enhanced vascularization in mdx mice. It is noteworthy that SDF-1 is essential for mobilization and recruitment of stem cells and smooth muscle progenitor cells into damaged tissues. This may explain why enhanced arteriogenesis was evidenced in mdx mice.
Indeed, we found an increased number of circulating bone marrow progenitor cells in serum from mdx mice. In the absence of ischemia, c-kit+ cells were rarely detected in skeletal muscle of both WT and mdx mice (data not shown). However, after ischemia, the number of stem cells expressing c-kit antigen increased in ischemic hindlimb of mdx mice. Interestingly, in mdx mice, stem cells were located close to muscle fibers and vessel walls. Thus, in mdx mice, stem cell mobilization and recruitment were exacerbated after ischemia, possibly contributing to increased vascularization.

The NOS pathway plays an important role in the regulation of muscle and endothelial cell proliferation, survival, and differentiation.33 Our results suggest that NO does not influence vascularization in the absence of functional dystrophin. In mdx mice, decreased levels of NO exacerbate inflammation and muscular injury. Normalization of NO production attenuated skeletal muscle damage and inflammation without affecting angiogenesis.34 Recently, Loufrani et al35 demonstrated that endothelial-dependent vascular smooth muscle relaxation was altered in mdx mice, resulting in attenuation of flow-induced vasodilation. Moreover, the same authors showed that vessel density was decreased in gracilis muscle of mdx mice. This result is apparently in contrast with our results showing the lack of differences in capillary and arteriolar length density between the adductor muscle from mdx and control mice. Indeed, different contributions of fast and slow fibers in skeletal muscle composition may explain this discrepancy. In rodents, nNOS is specifically expressed in fast- but not in slow-twitch muscle,36 and unlike the discrepancy. In rodents, nNOS is specifically expressed in fast- but not in slow-twitch muscle,36 and unlike the adductor muscle examined in our study, gracilis is a fast-twitch muscle. Thus, the diminished arteriolar density in gracilis muscle of mdx mice may represent the result of a local decrease of NO bioavailability that may not be present throughout the organism. Furthermore, in the present study, arteriolar and capillary densities were evaluated in 2-month-old animals, ie, when maximal skeletal muscle degeneration and regeneration are expected to occur.37 In contrast, Loufrani et al used 3.5-month-old mdx mice in which only small patches of necrosis and regeneration were seen. This suggests that the active inflammatory response in the acute phase of ongoing muscle degeneration may play a role in the enhanced arteriogenic response observed under the experimental conditions of our study. Thus, enhanced arteriogenesis may be attenuated at earlier or later periods when the pathological manifestations are less severe.

In conclusion, we propose that DMD is characterized by an imbalance of cytokine and growth factors; this imbalance may play an important role in DMD pathogenesis, disrupting normal neovascularization and enhancing recruitment of inflammatory cells.

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