Angiogenic Impairment of the Vascular Endothelium
A Novel Mechanism and Potential Therapeutic Target in Muscular Dystrophy


Objective—Dystrophin, the missing or defective protein in Duchenne muscular dystrophy, is expressed not only in muscle cells but also in vascular endothelial cells (ECs). In this study, we assessed the effects of dystrophin deficiency on the angiogenic capacities of ECs.

Approach and Results—We isolated vascular ECs from mdx mice, the murine equivalent of Duchenne muscular dystrophy in humans, and wild-type controls, and we found that mdx-derived ECs have impaired angiogenic properties, in terms of migration, proliferation, and tube formation. They also undergo increased apoptosis in vitro compared with wild-type cells and have increased senescence-associated β-galactosidase activity. Mdx-derived ECs also display reduced ability to support myoblast proliferation when cocultured with satellite cell–derived primary myoblasts. These endothelial defects are mirrored by systemic impairment of angiogenesis in vivo, both on induction of ischemia, stimulation with growth factors in the corneal model and matrigel plug assays, and tumor growth. We also found that dystrophin forms a complex with endothelial NO synthase and caveolin-1 in ECs, and that NO production and cGMP formation are compromised in ECs isolated from mdx mice. Interestingly, treatment with aspirin enhances production of both cGMP and NO in dystrophic ECs, whereas low-dose aspirin improves the dystrophic phenotype of mdx mice in vivo, in terms of resistance to physical exercise, muscle fiber permeability, and capillary density.

Conclusions—These findings demonstrate that impaired angiogenesis is a novel player and potential therapeutic target in Duchenne muscular dystrophy. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: angiogenesis inhibitors • endothelium, vascular • muscle diseases, inflammatory • nitric oxide synthase type III

In Duchenne muscular dystrophy (DMD), the protein dystrophin, which serves as a link between cytoskeletal actin and the extracellular matrix, is missing or defective.2,3 Dystrophin expression is not limited to muscle tissues but is present in many other cell types, including endothelial cells (ECs).3 The physiological importance of dystrophin in the vascular endothelium is demonstrated by the fact that mdx mice, the murine equivalent of DMD in humans, have vascular abnormalities that impair blood flow. This occurs through lower NO-dependent flow (shear stress)–induced endothelium-dependent dilation, reduced endothelial NO synthase (eNOS) and neuronal NOS expression, and decreased vascular density.3,4 Based on this evidence, it has been proposed that the dystrophic skeletal muscle works in a condition of functional ischemia, determined by a mismatch between metabolic demand and blood flow supply, and that such condition is required for, and an essential cause of, muscle damage in mdx mice.5 The functional importance of deficient vascularization in the setting of muscular dystrophy has been confirmed by the demonstration that Flt-1 haploinsufficiency ameliorates the dystrophic phenotype of mdx mice.6 Flt-1 is a decoy receptor for vascular endothelial growth factor (VEGF), a prototypical angiogenic agent, and Flt-1 heterozygous knockout (Flt-1(+/−)) mice have increased EC proliferation and vascular density.10 Interestingly, mdx:Flt-1(+/−) adult mice not only have increased muscle blood flow and capillary density compared with mdx mice but also display decreased fibrosis, calcification, and membrane permeability and increased...
force production. Additional evidence of an interdependent relationship between angiogenesis and muscular dystrophy is provided by the recent finding that, in mdx mice, reducing angiogenesis by ablating the MMP-2 gene results in impaired growth of regenerating muscle fibers. These data suggest that increasing the vasculature in DMD may ameliorate the histological and functional phenotypes associated with this disease and that, for an effective therapy of DMD, both the muscle and the vasculature should be addressed.

In this study, we used 4 distinct experimental models to demonstrate that angiogenesis is systemically impaired in vivo in mdx mice. Such impairment depends on the reduced angiogenic properties of the EC compartment, as indicated by the fact that, on stimulation with growth factors, mdx-derived ECs have reduced ability to migrate, proliferate, and form tubular-like structures in vitro. They also have reduced ability to stimulate the proliferation of myogenic precursor cells, when cocultured with satellite cell-derived primary myoblasts. We also demonstrate that dystrophic ECs have impaired ability to produce NO and form cGMP, compared with wild-type ECs, and that such impairment may be rescued by treatment with low-dose aspirin. Low-dose aspirin also ameliorates the phenotype of mdx mice in vivo, by increasing the vascular density of the dystrophic skeletal muscle, reducing muscle fiber permeability, and improving resistance to physical exercise.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Systemic Impairment of In Vivo Angiogenesis in mdx Mice
To fully assess the angiogenic properties of mdx mice, we used 4 distinct experimental models, which encompass multiple aspects of in vivo angiogenesis in various organs and tissues.

First, we used the hindlimb ischemic model and found significant impairment of ischemia-induced angiogenesis in mdx mice compared with controls. In particular, Laser Doppler perfusion imaging demonstrated a progressive increase in the blood flow of ischemic hindlimbs in wild-type mice, with significant differences seen at day 28 compared with days 7 and 14 after surgery ($P<0.01$; Figure 1A). In contrast, there was no significant increase in the hindlimb blood perfusion of mdx mice during the 28 days of follow-up after ischemia. As a result, at day 28 after surgery, the Doppler flow ratio was significantly reduced in mdx mice in comparison with the wild-type group (0.37±0.12 versus 0.97±0.10; $P<0.01$; Figure 1A). Likewise, capillary density at day 28 after surgery was significantly higher in the adductor muscle of wild-type mice versus mdx mice (555.7±51.2 versus 332.4±72.1 capillaries/mm$^2$; $P<0.001$; Figure 1B and 1C).

Next, we used the corneal model of angiogenesis. The cornea is a nonvascularized tissue, thus it is commonly used to study the ability of a given molecule to induce neoangiogenesis. We implanted pellets containing VEGF, a prototypical angiogenic factor, in the corneas of mdx mice and wild-type animals. As expected and consistent with previous findings in the literature, pellets containing VEGF induced a robust angiogenic response in the cornea of wild-type animals, as detected by BSI lectin fluorescent staining 6 days after pellet implantation (Figure 1D). In contrast, VEGF-induced corneal neovascularization was strikingly reduced in mdx mice (Figure 1D). We performed a comprehensive quantification of prototypical parameters of corneal neovascularization and found that the number of vessels per cross-section (62.2±18.3 versus 130.2±25.4; $P<0.001$; Figure 1E), the average length of the neovessels (0.69±0.11 versus 0.23±0.05 mm; $P<0.001$; Figure 1F), and the circumferential extent of the neovascularization (52.5 versus 112.9±8.5 degrees; $P<0.001$; Figure 1G) were significantly reduced in mdx mice compared with wild-type controls.

We also used the in vivo matrigel subcutaneous angiogenic assay. Matrigel-containing VEGF was injected into the subcutaneous space along the dorsal midline of mdx mice and wild-type controls. Matrigel plugs were dissected and analyzed 14 days after implantation. As expected, the matrigel plugs containing VEGF that were implanted in wild-type mice exhibited a high number of vessels/field (78.2±14.3). In contrast, the number of neovessels/field was significantly lower in the matrigel plugs that were implanted in mdx mice (32.2±8.5; $P<0.01$; Figure 1H).

Finally, we used a model of tumor growth. Lewis lung carcinoma cells were injected subcutaneously in the proximal dorsal midline of mdx mice and wild-type controls. Tumor growth was measured every other day during a period of 20 days on tumor implantation. At the end of the follow-up period, tumor volume was significantly lower in mdx mice compared with controls (7015.3±1421.2 versus 11 052.8±1856.2 mm$^3$; $P<0.01$; Figure 1I). Also, the tumors that had grown in mdx mice had a significantly lower number of vessels/cross-section than those that have grown in control animals (28.3±2.8 versus 55.2±5.8 vessels/cross-section; $P<0.001$; Figure 1J).

To assess the proliferative index of the endothelium of mdx mice in vivo, we looked at the expression of the integrin αVβ3 in the mouse model of hindlimb ischemia. Among several integrins, αVβ3 is unique because this molecule is expressed exclusively by the angiogenic endothelium, whereas resting ECs do not display this integrin. Indeed, αVβ3 expression is indispensable for proliferation during angiogenesis. Ten days after induction of ischemia, abundant αVβ3-positive ECs were discernible in the vasculature of regenerating muscles of wild-type mice, indicating an active angiogenic response of the wild-type endothelium to ischemia. Compared with these mice, the vasculature of ischemic muscles of mdx animals was instead substantially poorer in terms of αVβ3-positive cells (Figure 1A in the online-only Data Supplement). We also performed a semiquantitative analysis of αVβ3 expression, which confirmed a significant decrease of the expression.

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of this integrin in the vessels of mdx mice (Figure 1b in the online-only Data Supplement).

Taken together, these data provide strong evidence that angiogenesis is impaired in mdx mice, and that such impairment is not limited to the skeletal muscle, but instead is a systemic phenomenon.

**EC Defects in mdx Mice**

The corneal model of angiogenesis, the in vivo matrigel angiogenic assay, and the tumor model of angiogenesis demonstrate that, in mdx mice, angiogenesis is impaired also in the presence of VEGF stimulation and tumor growth. These findings suggest that deficient angiogenesis in mdx mice does not depend on the lack of appropriate angiogenic stimuli. To further explore this issue, we measured local levels of VEGF in the skeletal muscle of mdx and wild-type mice after hindlimb ischemia, based on the notion that VEGF is physiologically upregulated in skeletal muscle on induction of ischemia. We found that in mdx mice, ischemia-induced VEGF upregulation occurs normally and does not differ from that observed in wild-type controls (Figure II in the online-only Data Supplement).

Although impairment of other angiogenic molecules necessary for the assembly of vascular unit cannot be excluded, the demonstration that VEGF production in response to ischemia is normal in mdx mice indicates that most likely the impairment of angiogenesis in these animals does not depend on insufficient production of angiogenic factor(s), suggesting instead that the problem may lie within the EC compartment. To test this hypothesis, we isolated ECs from the inguinal adipose tissues of mdx mice and wild-type controls. After isolation, ECs were cultured under rigorously standardized conditions and used for the following set of in vitro assays. First, we performed a migration monolayer denudation assay and found that the distance migrated, relative to untreated cells, was significantly reduced in ECs derived from mdx mice compared with those derived from wild-type controls (1.51±0.6 versus 2.85±0.5; P<0.01; Figure 2A). Next, we performed a cell proliferation MTT assay and found that ECs derived from mdx mice had significant impairment of proliferation compared with nondystrophic ECs (0.48±0.12 versus 0.88±0.06 OD; P<0.01; Figure 2B). Then, we determined that ECs derived from mdx mice had significantly increased apoptosis compared with those derived from wild-type controls (P<0.05; Figure 2C). Senescence-associated β-galactosidase activity is determined by the overexpression and accumulation of endogenous lysosomal β-galactosidase specifically in senescent cells and is a widely used biomarker for senescent and aging cells. We found significantly increased senescence-associated β-galactosidase activity in ECs derived from mdx mice compared with those derived from wild-type controls.
activity in ECs isolated from *mdx* mice compared with wild-type animals (Figure 2D). Finally, we used a tube formation assay and found that the number of capillary-like structures was significantly lower in the assays containing *mdx*-derived ECs compared with those containing ECs isolated from wild-type mice (7.3±1.5 versus 10.6±1.2; *P* <0.05; Figure 2E).

It is known that ECs interact, both directly and indirectly, with myoblasts and promote proliferation of myogenic precursor cells. Because this phenomenon may be important in the setting of muscular dystrophy, we examined whether ECs isolated from *mdx* mice have impaired ability to support myoblast proliferation compared with nondystrophic ECs. Accordingly, ECs isolated from *mdx* mice or control mice were cocultured with satellite cell–derived primary myoblasts, as previously described. In all cases, satellite cell–derived primary myoblasts were from wild-type mice. After 3 days of culture in myoblast growth media, we quantified the number of desmin-positive cells, which is representative of myoblast proliferation and survival. The number of desmin-positive cells detected in the coculture assays was compared with that observed in cultures containing myoblasts alone. This analysis demonstrated that the relative number of desmin-positive cells was significantly lower when satellite cell–derived primary myoblasts were cocultured with ECs isolated from *mdx* mice (no. of cell lines=5) compared with ECs isolated from wild-type mice (no. of cell lines=5).

**Figure 2.** Angiogenic (and myogenic) defects in the endothelial cells (ECs) of *mdx* mice. ECs were isolated from the adipose tissue of *mdx* mice and wild-type controls. **A**, Migration was significantly reduced in ECs derived from *mdx* mice (n of cell lines=5) compared with controls (n of cell lines=5). **B**, A cell proliferation assay shows that ECs derived from *mdx* mice (n of cell lines=5) have impaired proliferation compared with ECs derived from wild-type animals (n of cell lines=5). **C**, ECs derived from *mdx* mice had significantly increased apoptosis compared with nondystrophic ECs. **D**, Senescence-associated β-galactosidase (SA-βgal) activity was significantly increased in ECs isolated from *mdx* mice compared with wild-type animals. **E**, Tube formation assay demonstrating significantly lower number of capillary-like structures in the assays containing *mdx*-derived ECs (no. of cell lines=5) compared with those containing ECs isolated from wild-type mice (no. of cell lines=5). **F**, The number of desmin-positive cells was significantly lower when satellite cell–derived primary myoblasts were cocultured with ECs isolated from *mdx* mice (no. of cell lines=5) compared with ECs isolated from wild-type mice (no. of cell lines=5).
controls and that this results in impaired NO-dependent arterial dilation. Here, we tested the hypothesis that ECs isolated from mdx mice have reduced ability to produce NO. To test this hypothesis, we assessed citrulline formation, which is representative of NOS activity, in ECs and found significant lower levels of L-[3H]-citrulline in the supernatant of culture dishes containing dystrophic ECs than in those containing wild-type ECs (7.2±2.1 versus 14.5±1.2 fmol/well; P<0.01; Figure 3A). We also assessed the accumulation of cGMP in ECs because cGMP is both the second messenger of NO in many biological systems and a sensitive marker of intracellular NO formation.20 We found significantly lower intracellular levels of cGMP in dystrophic ECs compared with control cells (2.85±0.5 versus 4.9±0.2 pmol/well; P<0.01; Figure 3B).

Association of Dystrophin With eNOS and Cav-1 in ECs
To bring insights on how the lack of dystrophin causes reduced synthesis of NO in ECs, we studied the association of eNOS with dystrophin in ECs isolated from wild-type mice and also looked whether dystrophin and eNOS form a complex with Cav-1 in wild-type ECs because Cav-1 is the principal protein of caveolae in ECs, and it is well known that eNOS is active when located in the caveolae.21 For these experiments, total protein extract was obtained from ECs isolated from wild-type mice and immunoprecipitated with an antidystrophin antibody. Total proteins and immunoprecipitated proteins were then analyzed by Western blot using antibodies against dystrophin, Cav-1, and eNOS. We found increased levels of eNOS and Cav-1 in proteins immunoprecipitated with an antidystrophin antibody compared with total protein extract (Figure 4), indicating that dystrophin forms complexes with eNOS and Cav-1 in ECs.

Aspirin Has Multiple Beneficial Effects on the Angiogenic (and Myogenic) Properties of Dystrophic ECs In Vitro
Aspirin is able to enhance NO release from vascular ECs and has a protective role on ECs via the NO–cGMP pathway.22 For these reasons, we tested the hypothesis that treatment with aspirin may increase the ability of dystrophic ECs to produce NO and form cGMP.

Citrulline synthesis was measured in the protein extracts of ECs isolated from mdx mice and wild-type mice and treated with various concentrations of aspirin or with control vehicle. These studies demonstrated that treatment with aspirin increases eNOS activity in both dystrophic and wild-type ECs in a concentration-dependent manner (Figure 5A). In particular, treatment of dystrophic ECs with 100 μmol/L aspirin increased L-[3H]-citrulline levels to 12.5±0.7 fmol/well compared with 7.1±0.3 fmol/well detected in dystrophic ECs treated with vehicle control (P<0.05). Significantly higher levels of L-[3H]-citrulline were measured in the supernatant of dystrophic ECs treated with progressively higher concentrations of aspirin (16±0.2 fmol/well in cells treated with 300 μmol/L aspirin, P<0.01; 22.5±0.3 fmol/well in cells treated with 500 μmol/L aspirin, P<0.001). In this assay, aspirin-induced citrulline formation was depressed completely by the l-arginine antagonist L-NAME, demonstrating that the observed effect was NO dependent (Figure 5A).

Treatment with aspirin also increased the intracellular levels of cGMP in dystrophic and wild-type ECs compared with cells treated with vehicle control. Such effect was already significant (P<0.01) at aspirin concentrations (100 μmol/L) similar to those used for cardiovascular protection (Figure 5B). Progressively higher levels of cGMP were detected in dystrophic ECs treated with higher concentrations of aspirin.
(7.0±0.5 pmol/well in cells treated with 300 μmol/L aspirin, P<0.01; 10.8±0.7 pmol/well in cells treated with 500 μmol/L aspirin, P<0.001), demonstrating that the observed effect is concentration dependent. To demonstrate that aspirin-induced cGMP accumulation was NO dependent, cells were preincubated with the NO scavenger PTIO, which resulted in complete inhibition of endothelial cGMP stimulation (Figure 5B).

NO significantly contributes to the prosurvival/proangiogenic program of the vascular endothelium via eNOS activation and cGMP-dependent gene transcription. Based on this concept and the evidence that aspirin enhances NO production and cGMP formation in dystrophic ECs, we tested the hypothesis that the impaired angiogenic ability that we observed in the ECs isolated from mdx mice may be rescued by treatment with aspirin. Thus, the assays presented in Figure 2 were repeated in the presence of aspirin supplementation (500 μmol/L). We found significant amelioration in the ability of aspirin-treated dystrophic ECs to migrate (P<0.01), proliferate (P<0.01), and form tubes (P<0.01) compared with cells treated with vehicle (Figure 5C–5E). Importantly, on treatment with 500 μmol/L aspirin, ECs isolated from mdx mice also exhibited significantly increased ability to support myoblast proliferation (P<0.01; Figure 5F).

Beneficial Effects of Long-Term Treatment With Low-Dose Aspirin in mdx Mice In Vivo

Based on the in vitro findings outlined above, we tested the hypothesis that treatment with cardiovascular protection doses of aspirin may be beneficial in the setting of muscular dystrophy. Mdx mice were treated with daily low doses of aspirin (10 mg/kg per day), in drinking water for 7 months. An additional set of mdx mice received regular water and were used as controls. The dose that we used was chosen using interspecies conversion factors for equal body surface between humans and mice and corresponds to the range of low-dose aspirin used for the prevention of cardiovascular diseases in
humans. Animals were 4 weeks old at the beginning of the experiments and 8 months old at the end of treatment. At this time point, mice were then challenged on a treadmill and running time, maximal speed, and distance covered were determined. Mdx mice treated with aspirin were able to run on the treadmill for a significantly longer period of time compared with mdx mice treated with control (17.6±1.2 versus 9.9±1.9 minutes; P<0.001; Figure 6A). Likewise, the maximum speed reached on the treadmill was significantly higher in the group of aspirin-treated mice than in the group of control-treated animals (20.1±0.4 versus 14.7±1.0 m/min; P=0.001; Figure 6B). Finally, mice treated with aspirin were able to cover a significantly greater distance than mice treated with control (162.0±7.3 versus 86.5±19.9 meters; P<0.001; Figure 6C).

After completion of the treadmill experiments, mice were euthanized and the adductor and TA muscles were harvested. At the histological level, we first assessed the effect of aspirin treatment on vascular density. Fluorescent staining with BS1-lectin demonstrated significantly higher number of capillaries both in the adductor and in the tibialis anterior muscles of mdx mice treated with aspirin compared with those treated with control (347.3±18.1 versus 218.6±25.3 capillaries/cm², P<0.001 and 344.5±17.8 versus 246.5±22.3 capillaries/cm², P<0.01, respectively; Figure 7A and 7B). Next, we determined the number of regenerating myofibers, identified by the presence of centrally located nuclei. We found that the adductor and tibialis anterior muscles of mdx mice treated with aspirin had a significantly higher percentage of regenerating fibers compared with mdx mice treated with control (52.5±4.2% versus 36.3±2.1%, P<0.01 and 57.5±7.3% versus 46.1±3.2%, P<0.05; Figure 7C and 7D). We also assessed myofiber membrane permeability, by measuring Evans blue dye uptake, and found that the tibialis anterior muscles of mdx mice treated with control exhibited a significant increase in the percentage of Evans blue dye–positive myofibers compared with mdx mice treated with aspirin (2.6±1.8% versus 13.3±0.9%; P<0.001; Figure 7E and 7F). We also evaluated the extent of fibrosis by Van Gieson staining and observed significantly less fibrosis in mice treated with aspirin compared with controls (data not shown).

Discussion

Functional ischemia, impaired vasodilatation in response to flow, reduced NO production, and various types of vascular abnormalities have been described in the setting of muscular dystrophy both in humans and in mice. Recent work demonstrates that improving vascular density and promoting angiogenesis might be beneficial in muscular dystrophy. For these reasons, some studies have tested the ability of angiogenic growth factors to improve the dystrophic phenotype in experimental models of DMD. However, no attention has been paid to the possibility that, in muscular dystrophy, the vascular endothelium might be less responsive to angiogenic stimuli and growth factors and that this deficit might contribute to the development and progression of muscle damage and degeneration.

In this study, we demonstrate that angiogenesis is significantly impaired in mdx mice and that such impairment is detectable in various organs and tissues and under different experimental conditions. We have reported previously that mdx mice exhibit a normal angiogenic response to hindlimb ischemia and even display increased arteriole length density. However, in that study, we used young (8-week-old) mdx mice, which still have very robust regenerative capacities and are not fully representative of the human dystrophic disease. In contrast, in the present study, we used 6-month-old mdx mice, which display more advanced-stage disease, a condition that might also negatively affect angiogenesis. In addition, in our previous study, we only evaluated angiogenesis in the hindlimb ischemic model, without assessing the angiogenic properties of mdx mice at the systemic level. In the present study, we demonstrate that angiogenesis is impaired also in the corneal model, the matrigel angiogenic assay, and the tumor model, thus providing evidence that this phenomenon occurs at the systemic level, is not limited to the skeletal muscle, and does not depend on the peculiar pathobiological environment of the dystrophic musculature. In addition, the fact that angiogenesis is impaired on implantation of pellets or matrigel-containing VEGF, and also during tumor growth,
suggests that the cause of this impairment is not the lack of appropriate angiogenic stimulation or the reduced expression of growth factors. This is also confirmed by the demonstration that, on induction of ischemia, VEGF upregulation occurs normally in mdx mice. Based on these observations, we investigated the hypothesis that the problem lies within the EC compartment and performed experiments that clearly demonstrate that vascular ECs of mdx mice have reduced ability to respond to angiogenic stimulation in terms of migration, proliferation, and tube formation.

The biological foundation of our findings is that dystrophin is physiologically expressed in ECs and is important for proper function of the vascular endothelium. Although the exact role of dystrophin in ECs is not well understood, it has been hypothesized that dystrophin is essential for eNOS to translocate to the caveolae, and several reports have shown that flow-induced dilation and arterial shear-stress mechanotransduction are significantly impaired in the absence of dystrophin. Here, we show that, in wild-type ECs, dystrophin forms a complex with cav-1 and eNOS, and that NO production and cGMP formation are significantly impaired in ECs derived from mdx mice. We also show that NO production in mdx-derived ECs may be enhanced by treatment with aspirin and this corresponds to amelioration of the angiogenic deficits displayed in vitro by these cells. These findings are relevant to muscular dystrophy because, in recent years, NO has emerged as an important messenger not only for angiogenesis but also for myogenesis, especially when this process is required for muscle repair after acute and chronic skeletal muscle injury, as in muscular dystrophy.

If a dysfunctional EC compartment participates to the mechanisms underlying deficient angiogenesis in mdx mice, then there is a need for revising and improving the concept that angiogenic growth factors may be useful to improve the dystrophic phenotype in the setting of muscular dystrophy. Indeed, although it has been reported that VEGF may increase capillary density in the dystrophic skeletal muscle, our findings suggest that treatments that protect the endothelium and correct endothelial dysfunction by donating NO may be potentially beneficial and should be considered as novel therapeutic strategies, alone or in combination with growth factors, in muscular dystrophy. In this respect, a novel finding of this
study is that low-dose aspirin ameliorates the dystrophic phenotype in mdx mice. Although we did not conduct a formal preclinical therapeutic study, these data are important because they provide proof of the concept that an agent that protects the vascular endothelium and increases NO production in ECs, such as low-dose aspirin, may have beneficial effects on the dystrophic phenotype of the skeletal muscle.

About the mechanisms through which aspirin exerts its beneficial effects in mdx mice, our data suggest that enhancement of NO production and intracellular cGMP accumulation in dystrophic ECs may be an important issue. This is consistent with new evidence supporting the use of NO-donating drugs as therapeutic agents for muscle repair and muscular dystrophy.\textsuperscript{8,30,32} It is also consistent with other findings demonstrating that enhancing cGMP signaling improves contractile performance and myocardial metabolic status in the dystrophin-deficient heart.\textsuperscript{33} It has been shown that NO-donating drugs correct functional ischemia in the setting of muscular dystrophy both in animal models and in humans, by restoring normal blood flow regulation and response to vasoconstrictors.\textsuperscript{8,33} These effects are classically attributed to the role played by sarcolemmal neuronal NOS in the modulation of sympathetic vasoconstriction in exercising skeletal muscles.\textsuperscript{3,12} Our data demonstrate that, in the mdx mouse, NO production is deficient also in the EC compartment, thus supporting the concept that NO-donating drugs may also be useful to directly improve endothelial function, increase microvascular recruitment, and enhance perfusion.\textsuperscript{30} In addition, NO is a potent angiogenic factor,\textsuperscript{32} and the fact that treatment with aspirin also ameliorates the angiogenic properties of dystrophic ECs in vitro and increases capillary density in vivo indicates that the beneficial effects of aspirin on the dystrophic phenotype of the skeletal muscle may be, at least in part, also mediated by angiogenesis, which would offer a protective mechanism for contraction-induced damage of the skeletal muscle. Finally, although our data demonstrate that endothelial defects in the mdx mouse are not limited to the muscle, but are present at the systemic level (ie, the cornea and the subcutaneous space), it is possible that aspirin treatment in vivo has beneficial effects on the dystrophic phenotype of the mdx mouse also for reasons that do not depend on angiogenesis or endothelial function. For instance, aspirin might have an effect on the cytokine milieu in the muscle and thus ameliorate endothelial dysfunction also in an indirect manner.

It has also been reported that increased capillary density and enhanced cGMP signaling in dystrophin-deficient animals lead to more stable and less degenerative fibers.\textsuperscript{9,33,34} This is consistent with our observation of reduced myofiber membrane permeability in mdx mice treated with aspirin. About the positive effect of aspirin on the number of regenerating fibers in vivo, further studies are needed to understand whether this will be beneficial in the long term because enhanced regeneration may eventually result in exhaustion of the satellite cell population. However, our in vitro coculture assays show that aspirin improves the ability of ECs to support myoblast proliferation and survival. Because self-renewal capacities are critical to maintain the reserve pool of satellite cells, the fact that aspirin facilitates the interaction between ECs and myoblasts may suggest that treatment with aspirin not only promotes the formation of new myoblasts but also supports the maintenance of the satellite cell population and thus warrants efficient tissue regeneration over time.

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**Disclosures**

None.

**References**


Significance

This study shows that angiogenesis is impaired in the mdx model of muscular dystrophy and this is because of reduced angiogenic properties of dystrophic endothelial cells. NO production and cGMP formation are compromised in these cells but may be enhanced by treatment with aspirin, which also improves multiple functional and histological parameters of the dystrophic phenotype of mdx mice, when administered for a long period of time in vivo. Angiogenesis and vascular endothelium are novel players and potential therapeutic targets in Duchenne muscular dystrophy.
Angiogenic Impairment of the Vascular Endothelium: A Novel Mechanism and Potential Therapeutic Target in Muscular Dystrophy


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Supplementary Figure I. Expression of αVβ3 in the ischemic skeletal muscle of mdx mice and wild-type controls. Ten days after induction of ischemia, αVβ3 expression was determined by immunofluorescent staining in the skeletal muscle of mdx mice and control animals. (a) αVβ3-positive cells were easily detectable in the vasculature of regenerating muscles of wild-type mice, while the vasculature of ischemic muscles of mdx animals was poor in terms of αVβ3-positive cells. (b) A semi-quantitative analysis of αVβ3 expression showed significant decreased expression of this integrin in the vessels of mdx mice.
Supplementary Figure II. VEGF upregulation in the ischemic skeletal muscle of *mdx* mice. VEGF levels were quantified by ELISA in the ischemic and contralateral adductor muscles of *mdx* mice (n=10) and wild-type controls (n=10), 7 days after induction of unilateral hindlimb ischemia. No statistically significant difference was detected between *mdx* mice and controls. Results are expressed as ratio between VEGF levels in the ischemic limb and VEGF levels in the contralateral leg.
**SUPPLEMENTAL TABLE I**

Body weight (g) of individual *mdx* mice in the aspirin and control group at the beginning of experiments.

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**Mean±SD** 23.0±1.6   22.3±1.5  n.s.
MATERIALS AND METHODS

Animals. We used C57BL/10ScSn-Dmd<sup>mdx</sup>/J mice and the appropriate C57BL/10SnJ control mice. Mice were purchased from the Jackson Laboratories. All mice were males and from the same strain. Mdx mice and controls were housed under identical environmental conditions in the Animal Facility of our University Hospital until used for the various experiments described below. Surgical procedures were conducted under general anesthesia. Mice were treated according to international guidelines for animal welfare, surgery, and euthanasia. The experiments were approved by the local Ethics Committee.

Hindlimb ischemic model. A total of 30 mdx mice and 30 controls were used for these experiments. All mice were 6-month-old at the time of surgery, which was performed as previously described<sup>12</sup>. Blood flow was measured by LDPI (Lisca) by two blinded independent investigators. Measurements were carried out at baseline, immediately after induction of ischemia (day 0), and every week for 4 weeks, as established<sup>1</sup>. Upon completion of the LDPI analyses, mice were sacrificed. Thirty minutes before sacrifice, mice received an intra-cardiac injection of BS1-lectin, conjugated with FITC, as described<sup>1</sup>. After sacrifice, the adductor muscles were excised, fixed, embedded in paraffin, sectioned, and analyzed for BS1-lectin staining for quantification of capillary density, as described<sup>1</sup>. An additional set of mice (5 mdx and 5 wild-type) were sacrificed 10 days after induction of ischemia and their muscles used for αVβ3 immunofluorescent staining, which was performed as previously described to identify proliferating ECs<sup>13</sup>. Quantification of αVβ3 expression was performed on immunostained frozen sections by a semiquantitative method (scores from 0 to 3), as described<sup>2</sup>.

Corneal model of angiogenesis. Single pellets containing 0.3 µg of VEGF were implanted in the eyes of 10 mdx mice and 10 controls as previously described<sup>3</sup>. All mice were 6-month-old at the time of the experiment. Mice were sacrificed 6 days after pellet implantation. Thirty minutes before sacrifice, mice received an intra-cardiac injection of FITC-conjugated BS1-lectin. After sacrifice, corneas were dissected and processed for BS1-lectin staining and quantification of corneal angiogenesis, which was performed by two blinded independent investigators, as previously described<sup>3</sup>.

In vivo matrigel subcutaneous angiogenic assay. Matrigel (0.4 ml) containing 200 ng of VEGF was injected into the subcutaneous space along the dorsal midline of 6 mdx mice and 6 controls, as described<sup>4</sup>. All mice were 6-month-old at the time of the experiment. Mice were sacrificed 14 days later. After sacrifice, matrigel plugs were harvested, fixed, paraffin embedded, cross-sectioned, and analyzed for CD31 staining by one blinded independent investigator.

Model of tumor growth and angiogenesis. Ten mdx mice and 10 controls were injected subcutaneously in the proximal dorsal midline with 1×10<sup>6</sup> Lewis lung carcinoma cells as described<sup>5</sup>. All mice were 6-month-old at the time of the experiment. Tumor dimensions were measured with calipers every two days for 20 days by one blinded independent investigator. Tumor volume was calculated as previously established<sup>5</sup>. Then, mice were sacrificed and tumors were harvested, fixed, paraffin embedded, cross-sectioned, and analyzed for CD31 immunostaining by two independent investigators.

Quantification of VEGF expression in the skeletal muscle. Ten mdx mice and 10 controls underwent hindlimb ischemia. Mice were 6-month-old at the time of surgery. Mice were sacrificed 7 days later, when the maximal upregulation of VEGF occurs<sup>6</sup>. Adductor muscles were harvested, proteins were extracted, and VEGF levels were measured by ELISA by one blinded independent investigator, as described<sup>6</sup>. Results are expressed as ratio between VEGF levels in the ischemic limb and VEGF levels in the contralateral leg.

EC isolation, migration, proliferation, apoptosis, and tube formation assays. ECs were isolated from the adipose tissues of 20 mdx mice and 20 controls, as described<sup>7</sup>. Mice were 6-month-old at the time of EC isolation. Five isolation procedures were performed using 4 mdx mice.
for each procedure. Likewise, wild-type ECs were obtained by five distinct isolation procedures using 4 wild-type mice for each procedure. This allowed us to obtain five distinct EC lines, each derived from a total of 4 mice. After isolation, ECs were cultured under rigorously standardized conditions. Migration and proliferation of ECs was determined, as previously described, by two blinded independent investigators. For the assessment of apoptosis, we used the TiterTACS Kit (R&D Systems), a quantitative assay for the detection of apoptosis in cells, as per manufacturer’s instructions. Apoptotic cells were determined by fluoro-cytometry at 450 nm. For the tube formation assay, ECs were seeded (5x10^3/well) on matrigel in 96 well plates and cultured in the presence of VEGF (50 ng/ml). Capillary-like structures were quantified 5 hours later, as previously described, by two blinded independent investigators. These experiments were performed in the absence and the presence of aspirin 500 µM. All the experiments were performed in triplicate using ECs derived from five distinct isolation procedures.

Senescence-associated β-galactosidase (SA-βgal) activity. SA-βgal activity was determined as described by Debacq-Chainiaux and coll. β-gal positive cells were visualized by phase contrast microscopy and quantified by two blinded independent investigators.

EC-mediated myoblast proliferation and survival. Satellite cell-derived primary myoblasts were isolated from the lower hindlimb muscles of 2-month-old C57BL/10SnJ mice, as described. ECs were isolated from the adipose tissues of 20 mdx mice and 20 controls, as described. Also in this case, dystrophic and wild-type ECs were isolated by five distinct procedures using 4 mdx mice and 4 control mice for each procedure. Myoblasts were co-cultured with equal numbers of either mdx-derived ECs or wild-type ECs in myoblast growth medium, as described. Myoblast proliferation was assessed by quantifying the number of desmin-positive cells 3 days after co-culture, as described. At least 100 desmin-positive myoblasts were counted for each experiment. These analyses were carried two blinded independent investigators. Experiments were performed in the absence and the presence of aspirin 500 µM. All the experiments were performed in triplicate using ECs derived from five distinct isolation procedures.

eNOS activity (L-citrulline formation assay) and cGMP measurement. Citrulline synthesis and cGMP were measured as described. Briefly, for the L-citrulline assay, ECs isolated from mdx mice or wild-type controls were grown to confluence in 35-mm dishes and incubated for 30 min at 37°C in 1.5 ml of HEPES buffer (pH 7.4) containing 0.25% albumin with or without the NOS inhibitor L-NAME (500 and 1000 µM). Then cells were stimulated with aspirin (100, 300, and 500 µM) in the presence of 10 µM L-arginine and 3.3 µCi/ml L-[3H]-arginine. After 15 min the reaction was stopped with cold PBS containing 5 mM L-arginine and 4 mM EDTA, and the cells were denatured with 96% ethanol. After evaporation, the soluble cellular components were dissolved in 20 mM HEPES sodium salt (pH 5.5) and applied to 2-mL columns of Dowex AG50WX-8 (Na^+ form). The radioactivity corresponding to the [3H]-citrulline content of the eluate was quantified by liquid scintillation counting. Agonist-induced [3H]-citrulline production was expressed in fmol/mg per well. Basal [3H]-citrulline synthesis was determined from the L-NAME–inhibitable radioactivity in unstimulated cells.

For cGMP measurement, ECs isolated from mdx mice or wild-type controls were grown to confluence in 6-well plates and washed twice with 2 mL of a balanced salt solution containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2, 5.5 mM glucose, and 20 mM HEPES-NaOH, buffered to pH 7.3. Cells were exposed for 10 minutes at 37°C to PTIO (50 µM) or vehicle in the balanced solution containing 0.5 mM isobutylmethylxanthine. Aspirin was added at various concentrations (100, 300, and 500 µM) and the incubation was continued for another 10 minutes at 37°C. The final assay volume was 1 mL. Supernatants were aspirated, and after addition of ethanol and subsequent evaporation, cGMP levels were determined by an enzyme-linked immunoassay according to the manufacturer’s protocol (EIA kit, Cayman).

All the experiments were performed in triplicate using ECs derived from five distinct isolation procedures. The analyses were carried out by two blinded independent investigators.

Co-immunoprecipitation assay. ECs isolated from wild-type mice were used for total protein extraction as established. Then, a total of 0.5 mg total proteins was incubated with a polyclonal
anti-dystrophin antibody (ab94752, Abcam). Next, 20 ml of protein G-sepharose (Sigma) were added and the mixture was incubated overnight at 4°C. The immunoprecipitation reaction was centrifuged for 15 min at 12000 x g, and the supernatant was recovered and stored at 4°C. The pellet, containing the immunoprecipitated proteins, was washed three times with extraction buffer 15 min at 12000 x g. Finally, the proteins immunoprecipitated in the pellet and those remaining in the supernatant were analyzed by Western blot using antibodies against dystrophin (ab94752, Abcam), Caveolin-1 (Cav-1) (7C8, Abcam), and eNOS (ab66127, Abcam). Co-immunoprecipitation was performed at least three times with each antibody. Western blots were performed according to previously described procedures.

**Long-term treatment with low-dose aspirin in vivo.** A total of 40 mdx mice were used for these experiments. Animals were randomly assigned to the aspirin treatment group (n=20) or the control group (n=20). Aspirin was administered in drinking water at the daily dose of 10 mg/kg/day. This dose was chosen using interspecies conversion factors for equal body surface between humans and mice and corresponds to the range of low-dose aspirin used for the prevention of cardiovascular diseases in humans. Animals were 4-weeks-old at the beginning of the experiments. The average body weight of the mice in the aspirin group was not statistically different from the average body weight of the animals in the control group when treatment was started (Supplemental Table I). Treatment with aspirin (or control water) was continued for a total of 7 months. Mice were 8-months-old at the end of the experiments. During treatment, mice in the aspirin group and in the control group were housed under identical environmental conditions. At the end of the treatment regimen, mice were challenged on a treadmill. The treadmill protocol consisted of five days of training followed by experimental determination of maximal running time, maximal speed, and maximal distance covered on day 6. On the experimental day, mice were first run at 5 meters/min for 3 minutes. Treadmill speed was then increased by 1 meter/min every minute until mice were exhausted. Continuous nudging was used during treadmill to help mice stay on the track. Exhaustion was defined as spending >10 seconds on the shocker (0.01 mA) without attempting to re-enter the treadmill. These analyses were performed in a blinded fashion by three independent investigators.

The number of capillaries, regenerating fibers, and the extent of fibrosis were assessed as described. For EBD uptake, some mice (5/treatment group) received an intraperitoneal injection of EBD and analyzed as described. These histological studies were performed by two independent investigators blinded to the treatment regimen.

**Statistical analyses.** Results are expressed as mean value±SD. Statistical comparisons between groups were performed by Student’s t-test or one-way ANOVA when appropriate. A P value < 0.05 was considered statistically significant.

**References.**


