Amelioration of Duchenne muscular dystrophy in mdx mice by elimination of matrix-associated fibrin-driven inflammation coupled to the $\alpha_M\beta_2$ leukocyte integrin receptor

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In Duchenne muscular dystrophy (DMD), a persistently altered and reorganizing extracellular matrix (ECM) within inflamed muscle promotes damage and dysfunction. However, the molecular determinants of the ECM that mediate inflammatory changes and faulty tissue reorganization remain poorly defined. Here, we show that fibrin deposition is a conspicuous consequence of muscle-vascular damage in dystrophic muscles of DMD patients and mdx mice and that elimination of fibrin(ogen) attenuated dystrophy progression in mdx mice. These benefits appear to be tied to: (i) a decrease in leukocyte integrin $\alpha_M\beta_2$-mediated proinflammatory programs, thereby attenuating counterproductive inflammation and muscle degeneration; and (ii) a release of satellite cells from persistent inhibitory signals, thereby promoting regeneration. Remarkably, Fib-gamma(390-396A) (Fib-$\gamma^{390-396A}$) mice expressing a mutant form of fibrinogen with normal clotting function, but lacking the $\alpha_M\beta_2$ binding motif, ameliorated dystrophic pathology. Delivery of a fibrinogen/$\alpha_M\beta_2$ blocking peptide was similarly beneficial. Conversely, intramuscular fibrinogen delivery sufficed to induce inflammation and degeneration in fibrinogen-null mice. Thus, local fibrin(ogen) deposition drives dystrophic muscle inflammation and dysfunction, and disruption of fibrin(ogen)-$\alpha_M\beta_2$ interactions may provide a novel strategy for DMD treatment.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is one of the most common X-linked lethal diseases, affecting 1 in 3500 newborn males. DMD results from mutations in the gene coding for the protein dystrophin, a cytoskeletal protein localized at the interface of the actin-based contractile apparatus and the sarcolemma. In the absence of a functional dystrophin complex tethering the actin cytoskeleton inside the muscle cell to the extracellular matrix (ECM), forces generated by the muscle fiber contraction result in muscle fiber damage due to shearing of the sarcolemma (reviewed in 1). The mdx mouse strain, which carries a naturally occurring nonsense mutation in exon 2 resulting in loss of dystrophin protein production, is the most widely used animal model for DMD (2,3). DMD patients and mdx mice exhibit progressive muscle degeneration, which is exacerbated by persistent inflammation via the production of free radicals and cytotoxic cytokines...
Myofiber loss is initially compensated by proliferation and fusion of resident myogenic precursor cells (satellite cells) with pre-existing myofibers that thereby enlarge in size. Ultimately, however, after repetitive cycles of muscle degeneration and persistent inflammation, dystrophic myofibers become gradually replaced by fibrotic and fat tissue (5; reviewed in 6,7).

Therapies based on restoration of dystrophin expression or the administration of dystrophin-positive stem cells are promising but still in the preclinical phase (8–14). Mounting evidence indicates a critical involvement of muscle extrinsic factors in DMD disease progression and the recovery of injured muscles. The composition of the basal lamina ECM around the necrotic myofibers can influence the overall repair process. Indeed, immediately after injury, a provisional fibrin-rich matrix and/or fibrin-rich hematoma forms between damaged fibers that provide a scaffold for tissue reorganization/reparative processes resulting in newly formed connective tissue altering contractile function, and a supportive matrix controlling the activity of infiltrating inflammatory cells (especially macrophages). While infiltrating inflammatory cells undoubtedly play a generally positive role in normal repair (e.g. by clearing myofiber debris), exuberant and persistent inflammatory cell action is likely to drive inopportune tissue reorganization in dystrophic muscle. These cells express several cytokines, growth factors and other soluble mediators [e.g. tumor necrosis factor alpha (TNFα), transforming growth factor beta, vascular endothelial growth factor] that modulate the extent of myofiber degeneration as well as satellite cell-mediated regeneration (15–21). Thus, persistent and/or inappropriate ECM deposition around the myofiber is potentially pathogenic, and may promote inflammation in the damaged muscle tissue leading to inopportune tissue reorganization and loss of function. However, the specific matrix components and mechanisms that drive pathological inflammatory cell infiltration in the context of either muscular dystrophies or muscle damage remain largely unexplored.

The local conversion of soluble fibrinogen to a provisional fibrin matrix plays a seminal role in controlling blood loss following vascular injury and is understood to support reparative tissue reorganization (22,23). However, in addition to being a classic acute-phase reactant, fibrin appears to be a potent regulator of the innate immune system by serving as a matrix-associated regulator of inflammatory cell function. In macrophages, immobilized fibrin and fibrinogen (from here on we refer to both by the term ‘fibrin(ogen)’) induce activation of c-Jun N-terminal kinase and NF-κB via the αMβ2 (CD11b/ CD18, Mac-1) integrin receptor, leading to the production of pro-inflammatory cytokines (24; reviewed in 25). Our previous studies showed that fibrin(ogen) and collagen matrices accumulated in diaphragms of aging mdx mice, while pharmacological fibrinogen depletion attenuated muscle fibrosis observed with age (24). However, the precise mechanism(s) by which fibrin(ogen) influences disease progression remain obscure.

In the present study, we show that fibrin(ogen), which is never detected outside of the vascular compartment in healthy muscle, is deposited in the muscle microenvironment immediately after injury, and upon disease onset in mdx mice. Using a combination of genetic and pharmacological approaches focused on fibrin(ogen), we directly tested the hypothesis that the severity of muscular dystrophy in mdx mice is dependent on fibrin(ogen) and that a mechanism supporting disease progression is coupled to the proinflammatory property of fibrin(ogen) linked to the αMβ2-binding motif. These studies establish for the first time that development of muscle pathology in mdx mice can be strongly attenuated by a simple fibrinogen mutation that has no influence on hemostasis and provide a proof-of-principle of the potential utility of interfering with the αMβ2-fibrin(ogen) interactions for treating DMD and other inflammatory myopathies.

RESULTS

Genetic or pharmacological depletion of fibrinogen attenuates degeneration, enhances regeneration and preserves function of dystrophic mouse muscle

To investigate whether fibrin(ogen) may directly control dystrophic muscle degeneration, we first analyzed its presence in limb muscles before and after the disease onset in mdx mice. Prior to disease onset (14 days of age), fibrinogen was undetectable by immunostaining in gastrocnemius muscle of mdx mice (Fig. 1A). In contrast, at the first signs of disease (21 days of age), and especially at the disease peak (30 days of age), fibrin(ogen) deposits were readily detectable in areas of muscle degeneration and inflammation (Fig. 1A), remaining elevated thereafter. As fibrin(ogen) deposition was not detected in muscles from healthy age-matched wild-type (WT) mice, these findings suggest that the accumulation of this provisional matrix protein specifically associates with dystrophinopathy in mdx mice.

To explore the importance of fibrin(ogen) on muscle disease, we bred mdx mice onto a fibrinogen-deficient background (Fib−/− mdx mice) and analyzed the consequences of fibrinogen deficiency on dystrophic disease initiation and progression. Prior to 14 days of age, no evidence of muscle dystrophy was observed in cohorts of either Fib−/− mdx or Fib+/− mdx mice, a finding that is consistent with the absence of fibrin(ogen) deposits in mdx muscle before disease onset (data not shown). As expected, at 1 month of age, fibrinogen-sufficient mdx mice, but not Fib−/− mdx mice, exhibited obvious fibrin(ogen) deposition in damaged muscle areas (Fig. 1B). However, more impressively, we observed significantly less muscle damage in Fib−/− mdx mice of 1 and 2.5 months of age in comparison to aged-matched Fib+/− mdx mice tracked in parallel, as indicated by the reduced degenerating muscle area and lower levels of serum creatine kinase (CK) (Fig. 1C, and Supplementary Material, Fig. S1A). Fib−/− mdx mice also exhibited a higher frequency of larger central-nucleated regenerating myofibers (Fig. 1D). In Fib+/− mdx muscles, infiltrating macrophages were found in the areas where fibrin(ogen) deposition was prominent, whereas the number of macrophages was significantly reduced in muscle tissue from Fib−/− mdx mice (Fig. 1E). In a distinct but complementary model of chemically induced muscle injury [cardiotoxin (CTX) challenge], fibrinogen deficiency resulted in a similar dampening of macrophage infiltration (Fig. 1F) to muscle tissue and an increase in the size of regenerating fibers (Fig. 1G) to that
Figure 1. Fibrin(ogen) accumulates in mdx dystrophic muscle, and fibrin(ogen) deficiency results in reduced muscle degeneration and enhanced regeneration. (A) Fibrin(ogen) deposition was analyzed by immunohistochemistry in gastrocnemius muscles of WT control mice and in mdx mice of 14, 30 and 90 days of age. Magnification bar: 50 μm. (B) Immunostaining for fibrin(ogen) of gastrocnemius muscles of 90-day-old mice showing the absence of fibrin(ogen) deposition in Fib²⁻/₋ mdx mice when compared with Fib⁺/+ mdx mice. Bar: 25 μm. (C) Left: reduced percentage of total muscle degeneration area in Fib⁻⁻/₋ mdx versus Fib⁺⁺/⁺ mdx mice at 30 days of age, as determined by morphometric analysis on H&E-stained gastrocnemius muscle sections. Right: reduced muscle membrane damage in Fib⁻⁻/₋ mdx versus Fib⁺⁺/⁺ mdx, as indicated by serum CK measurement. Data are mean ± SEM; n = 5 animals per group (∗P < 0.05, Mann–Whitney test). (D) Increased CSA of growing central-nucleated fibers (CNFs) in muscles of Fib⁻⁻/₋ mdx versus Fib⁺⁺/⁺ mdx mice when compared with Fib⁺⁺/⁺ mdx mice (mean ± SEM, 566.5 ± 5.0 versus 394.5 ± 6.7 μm²; n = 4 animals per group; P < 0.001 Mann–Whitney test); size frequency distribution of regenerating myofibers is represented. (E) Reduced inflammatory infiltration in Fib⁻⁻/₋ mdx muscle. Top: localization of αβ⁺₂ (CD11b/CD18 or Mac-1) positive macrophages in the damaged areas of mdx muscle occupied by fibrin(ogen) is shown by immunofluorescence. Bar: 25 μm. Bottom left: quantification of F4/80-positive cells by flow cytometry analysis. Data are mean ± SEM; n = 3 animals per group (∗P < 0.05, Mann–Whitney test). Bottom right: quantification of macrophages in Fib⁻⁻/₋ mdx versus Fib⁺⁺/⁺ mdx muscles. Data are mean ± SEM; n = 5 animals per group (∗P < 0.005, Mann–Whitney test). (F) CTX injury in muscle of Fib⁻⁻/₋ and Fib⁺⁺/⁺ mice. Quantification of macrophage numbers at 3 days post-injury in degenerating areas showed reduced infiltration in Fib⁻⁻/₋ mice. Data are mean ± SEM; n = 5 animals per group (∗P < 0.05, Mann–Whitney test). (G) At 5 days post-injury, the CSA of growing CNFs was larger in Fib⁻⁻/₋ mice than in Fib⁺⁺/⁺ controls; frequency distribution of myofiber size is represented (mean ± SEM, 141.8 ± 1.9 versus 109.5 ± 1.3 μm²; 4 animals per group; P < 0.001 Mann–Whitney test).
observed in the mdx model. These results suggest that regardless of etiology, the loss of fibrin(ogen) resulted in more limited muscle inflammation and altered regeneration pattern.

As a complement to these genetics-based studies using mice with life-long fibrin(ogen) deficiency, we explored whether the severity of dystrophic disease was also diminished in mdx mice following the administration of an established pharmacological defibrinogenating agent, ancrod (24,26,27). Here, when ancrod was administered to 12-day-old mdx mice and continued until the age of 2.5 months, the mice exhibited both reduced fibrin(ogen) deposition in muscle tissue relative to vehicle-treated control mdx mice (Fig. 2A) and a significant overall protection against severe dystrophic pathology as shown by: (i) a reduction of 40% in the total area of muscle degeneration (Fig. 2B, left), (ii) a diminution in circulating serum CK (Fig. 2B, right), and (iii) enhanced fiber regeneration (indicated by the larger size of central-nucleated fibers; Fig. 2C). Furthermore, fibrinogen depletion improved the physical performance of mdx mice, as shown by the preserved skeletal muscle strength (assessed by grip strength dynamometer tests) and the capacity of mdx mice to run at increasing high speed prior to exhaustion (Fig. 2D). Thus, the genetic or pharmacological elimination of one regulator of tissue repair and inflammation, fibrin(ogen), revealed a powerful modifier of degenerative muscle disease and protected mdx mice from functional muscle deterioration.

Exogenous fibrinogen is sufficient to induce inflammation and muscle degeneration in fibrinogen-null mice

Fibrin(ogen) can regulate inflammation in various conditions (28–31). Given the reduced presence of infiltrated macrophages in Fib−/−mdx mice (see above), we sought to verify whether pharmacological depletion of fibrinogen exerts its beneficial effect, at least in part, by modulating inflammation. We therefore analyzed the macrophage distribution in the following three distinct types of areas corresponding to the initial stages of the degeneration/regeneration cycle: degenerating areas, characterized by areas of extensive myofiber degeneration/necrosis and ECM-deposition, and absence of regenerating myofibers (phase I); early regenerating areas, with small nascent myofibers and absence of necrotic fibers (phase II), and advanced regenerating areas containing well-defined, larger regenerating myofibers (phase III) (see Supplementary Material, Fig. S2A). Fibrinogen depletion resulted in an overall reduction in infiltrating macrophages (Supplementary Material, Fig. S2B, left). A closer examination revealed that the loss of fibrin(ogen) did not affect macrophage accumulation during the initial stage of myofiber degeneration/necrosis (phase I), but rather it caused a decrease in macrophage number in subsequent phases where fibers are attempting to regenerate (phases II/III; Supplementary Material, Fig. S2B, right). These findings are consistent with the prevailing concept that fibrin(ogen) is not required for leukocyte trafficking per se, but rather controls local leukocyte activation events that alter both cellular function and the expression of

Figure 2. Pharmacological defibrination attenuates dystrophy progression in mdx muscle. Twelve-day-old mdx mice were subjected to a defibrination treatment by daily intraperitoneal injection with ancrod (or with vehicle–control saline solution), up to 2.5 months of age. (A) Fibrin(ogen) staining of muscle sections of vehicle-treated and defibrinated mdx mice. Bar: 50 μm. (B) Left: percentage of total muscle degeneration area. Right: membrane damage indicated by serum CK levels. Data are mean ± SEM; n = 5 animals per group (*P < 0.05). (C) Frequency distribution of regenerating myofiber size (mean ± SEM, 576.4 ± 11.5 mdx defibrinated versus 471.6 ± 12.1 μm² mdx vehicle; 6 animals per group; P < 0.001 Mann–Whitney test). (D) Increased functional muscle strength in defibrinated versus vehicle-treated mdx mice. Left: four limb grip strength. Right: distance to reach exhaustion in treadmill assays. Data are mean ± SEM; 5 animals per group (*P < 0.05 versus mdx vehicle, Mann–Whitney test).
secondary proinflammatory cues. The aggravating effects of fibrin(ogen) on muscle degeneration/regeneration in the context of the chronic insult imposed by the mdx mutation could result, at least in part, from the expression of pro-inflammatory cytokines. Indeed, we found much reduced expression of TNFα, IL-6 and IL-1β in defibrinated mdx muscle compared with vehicle treatment (Supplementary Material, Fig. S2C). A similar association between reduced macrophage infiltration and increased growth of regenerating myofibers was observed after CTX injury in defibrinated WT mice (Supplementary Material, Fig. S2D), supporting the conclusion that reduction in fibrin(ogen) levels attenuates inflammation in degenerating dystrophic muscle.

Although inflammatory macrophage infiltrates are a prominent feature of provisional extracellular matrices early after muscle injury (reviewed in 7,32), neither the specific matrix components that control macrophage function within damaged muscle tissue nor the consequences for muscle disease/repair of limiting function at the level of the provisional matrix-leukocyte interface have been defined. Given that we observed fibrin(ogen) deposition rapidly following muscle damage (Fig. 3A) and that depletion of fibrin(ogen) could ameliorate local muscle damage in both a genetic model of muscle dystrophy and a chemically induced model of inflammatory muscle damage, we hypothesized that fibrin(ogen) could be the early signal that triggers activation of...
macrophages within provisional matrices. To directly determine whether fibrinogen deposition was a specific driver of macrophage activity in muscle, we injected fibrinogen or vehicle into the right and left tibialis muscles, respectively, of FibαMβ2/−/− mice. Challenged muscle was then examined for evidence of both fibrinogen-mediated macrophage activation events as well as a tissue-damage/repair-like response. As shown in Figure 3B, local fibrin deposition was sufficient to induce an acute inflammatory reaction in the FibαMβ2/−/− mouse tibialis muscle as shown by significantly higher macrophage cell numbers within fibrinogen-challenged muscle tissue relative to the saline-injected contralateral tibialis muscle. The fibrinogen-induced inflammation (and ECM accumulation, Fig. 3B) was subsequently followed by degeneration (Fig. 3C), left) and the appearance of small embryonic myosin heavy-chain (eMHC)-positive regenerating myofibers in FibαMβ2/−/− mice (Fig. 3C, right). Thus, fibrinogen deposition is capable of triggering a cascade of events typical of a muscle injury–regeneration response, including inflammatory cell infiltration, ECM accumulation, muscle degeneration and new myofiber formation.

Specific interaction of fibrinogen with αMβ2 integrin receptor on macrophages promotes mdx muscle degeneration

Since leukocyte integrin receptor CD11b/CD18/αMβ2 engagement of immobilized fibrinogen has the potential to regulate cytokine expression in other settings (33–36), we sought to examine the contribution of the fibrinogen–αMβ2 interactions to the inflammatory response and muscle degeneration in mdx mice. To this end, we employed Fib-gamma(390-396A) (Fibγ390-396A) mice expressing a mutant form of fibrinogen that retains full clotting function but lacks the integrin αMβ2-binding motif (33). Here, we intercrossed Fibγ390-396A/− knock-in mice with mdx mice and compared the extent of muscle inflammation and degeneration in cohorts of mdx mice carrying either fibrinogen-γ390-396A or WT fibrinogen. Fibγ390-396A−/−mdx mice were indistinguishable from control mdx mice before disease onset (14 days after birth) (data not shown). However, at 2.5 months of age, Fibγ390-396A−/−mdx mice showed appreciably reduced macrophage infiltration relative to age-matched mdx mice (Fig. 4A and B). Notably, Fibγ390-396A−/−mdx mice exhibited more limited myofiber degeneration area, reduced serum CK levels (Fig. 4B), larger size regenerating fibers (Fig. 4C) and preserved locomotor activity (Fig. 4D). Thus, muscle disease can be attenuated and muscle function preserved despite a fundamental initiating genetic insult for muscular dystrophy by imposing an alteration of fibrinogen that limits αMβ2-mediated leukocyte activation events but does not compromise hemostasis. The general findings were again extended to the model of chemically induced muscle injury where, like fibrinogen-deficient mice, Fibγ390-396 knock-in mice exhibited an appreciable phenotypic benefit relative to control mice in the form of reduced presence of macrophages (Fig. 4E) and larger regenerating myofibers (Fig. 4F).

Treatment with the Fibγ377-395 peptide limits macrophage activation and ameliorates mdx muscular dystrophy progression

Previous studies have shown that a peptide corresponding to Fibγ377-395 is capable of impeding fibrinogen-mediated leukocyte binding via αMβ2 and secondary activation events (34,37,38). We confirmed that a synthetic peptide corresponding to fibrinogen gamma chain residues 377–395, but not a scramble peptide, specifically inhibited the in vitro activation of primary macrophages in response to fibrinogen stimulation, as measured by the expression of TNFα, IL-6 and IL-1β cytokines. Similar inhibitory effects were obtained using a specific blocking antibody against αMβ2 integrin (Fig. 5A). Interestingly, treatment of macrophages with Fibγ377-395 peptide also blocked fibrinogen-induced NF-κB activation in vitro (data not shown), reinforcing the reported induction of cytokines by fibrinogen via an αMβ2-dependent NF-κB axis (24,39,40). As a complement to our studies of Fibγ390-396A mice, we examined whether the Fibγ377-395 peptide could also limit pro-inflammatory processes and the development of dystrophy in mdx mice. We treated 2-month-old mdx mice with a Fibγ377-395 peptide, or a scramble peptide, for a 3-week period. Neither mice treated with the scrambled peptide nor the Fibγ377-395 peptide showed any signs of hemorrhage either grossly or within muscle tissue in microscopic analyses. However, while the scrambled peptide predictably did not alter disease progression, mdx mice treated with the Fibγ377-395 peptide showed reduced inflammation and muscle degeneration, as indicated by diminished macrophage infiltrates (Fig. 5B), lower degenerating areas (Fig. 5C, left) and reduced serum CK levels (Fig. 5C, right). These phenotypic benefits were also associated with larger regenerating myofibers (Fig. 5D), decreased expression of pro-inflammatory cytokines (Fig. 5E) and improved motor performance (Fig. 5F). Collectively, these results show that in vivo treatment with the Fibγ377-395 peptide attenuated the severity of muscular dystrophy without imposing hemorrhagic risk.

Fibrinogen regulates satellite cell-dependent muscle regeneration via direct and indirect mechanisms

Muscles of Fibγ390-396A−/−mdx mice contained larger central-nucleated myofibers compared with aged-matched mdx control mice (Fig. 4C), suggesting that the selective blockade of the fibrinogen/αMβ2-mediated inflammatory/degenerative processes also promoted myofiber regeneration. Since it is well established that muscle regeneration relies on activation and differentiation of satellite cells (41,42), we explored whether cytokines produced in response to αMβ2-mediated macrophage engagement of fibrinogen altered muscle satellite cell functions. As shown in Figure 6A, conditioned medium (CM) from fibrinogen-stimulated macrophages, but not from non-stimulated cells, induced hyperproliferation of satellite cells based on BrdU incorporation assays. Addition of this macrophage, CM also reduced the satellite cell differentiation potential, as shown by analyses of myogenin and eMHC expression (Fig. 6B and C, respectively). Importantly, these effects were significantly reversed by addition of TNFα and...
IL-1β neutralizing antibodies to the CM of fibrinogen-treated macrophages, demonstrating that, indeed, fibrinogen-induced pro-inflammatory cytokines underlie the altered myogenesis in a paracrine fashion (Fig. 6). Together, these results suggest that the proinflammatory milieu generated through persistent fibrinogen within the dystrophic muscle microenvironment indirectly limits myofiber regeneration by inhibiting satellite cell activity. However fibrinogen may also...
Figure 5. Treatment with a fibrinogen-derived $\gamma^{377-395}$ peptide blocks inflammation and disease progression in mdx mice. (A) Activation of macrophages by fibrinogen is reduced by the fibrinogen-$\gamma$-derived $377-395$ peptide. Primary macrophages were treated with fibrinogen (Fg) in the absence or presence of the $\gamma^{377-395}$ peptide or scrambled peptide used as control. Additional experimental controls included treatment with an $\alpha_M\beta_2$ blocking antibody, or IgG control antibody, as indicated. Expression of inflammatory cytokines was analyzed by qRT–PCR. Results are fold-induction values with respect to untreated macrophages. Data are mean ± SEM; $n=3$ experiments performed in triplicate ($^*P<0.05$ versus control conditions, Mann–Whitney test). (B) Inflammation is attenuated by treatment with the $\gamma^{377-395}$ peptide in mdx mice. Mdx mice of 2 months of age were treated with $\gamma^{377-395}$ peptide, or scrambled peptide as control, for the course of 3 weeks. Data are mean ± SEM; $n=5$ animals per group ($^*P<0.05$, Mann–Whitney test). (C) Left: percentage of total muscle degeneration area in mdx mice treated with $\gamma^{377-395}$ or scrambled peptides. Data are mean ± SEM; $n=5$ animals per group ($^*P<0.05$, Mann–Whitney test). Right: extent of muscle membrane damage in mdx mice before and after the treatment with $\gamma^{377-395}$ peptide (or scrambled peptide) as indicated by serum CK levels. Data are mean ± SEM; $n=5$ animals per group ($^*P<0.05$ versus CK values of the same animals before the treatment, Mann–Whitney test). (D) Frequency distribution of the size of regenerating myofibers in mdx mice treated with $\gamma^{377-395}$ peptide (or scrambled peptide) (mean ± SEM, $515.8 \pm 8.4 \text{ mm}^2$ mdx $\gamma^{377-395}$ peptide versus $440.5 \pm 8.4 \text{ mm}^2$ mdx scrambled peptide; 4 animals per group; $P<0.001$ Mann–Whitney test). (E) Reduced cytokine expression in muscles of mdx mice treated with $\gamma^{377-395}$ peptide compared with scrambled peptide-treated mice. Right: improvement of treadmill performance in an exhaustion test of mdx mice treated with a $\gamma^{377-395}$ peptide. Data are mean ± SEM; $n=5$ animals per group ($^*P<0.05$ versus values of the same group before the treatment, Mann–Whitney test).
directly alter satellite cell-dependent muscle tissue repair, and this concept is compatible with immunohistochemical findings showing that fibrinogen deposition in mdx mouse muscle was intimately associated, and often surrounded, activated (MyoD
\textsuperscript{+}) satellite cells (Fig. 7A). To directly test whether fibrinogen might alter directly satellite cell functions, we cultured satellite cells in the absence or presence of fibrinogen and assessed their proliferation and fusion properties (Fig. 7B and C). Treatment of satellite cells with increasing amounts of fibrinogen induced a hyperproliferative effect compared with untreated cells, as shown by satellite cell count following 3 days in culture and by BrdU incorporation (Fig. 7B). Consistent with the reported expression in myoblasts of \(\alpha_v\beta_3\) integrin (43–45) a known fibrinogen-binding receptor (23), we found that incubation of satellite cells with either an arg-gly-asp (RGD) cyclic peptide that specifically interferes with \(\alpha_v\beta_3\) or an anti-\(\alpha_v\) antibody reversed the fibrinogen-induced satellite cell hyperproliferation (Fig. 7B, right). In addition, immunostaining for eMHC in satellite cells cultured in differentiation medium showed reduced myogenic fusion in the presence of fibrinogen (Fig. 7C). These results further support the idea that excessive and/or persistent deposition of fibrinogen might impair regeneration in mdx muscle by both direct and indirect mechanisms acting on satellite cells.

Association of fibrinogen deposition and macrophage infiltrates in dystrophic muscle of DMD patients

Prompted by our findings that fibrinogen regulates inflammation and muscle degeneration/regeneration in mdx dystrophic disease, we analyzed tissue biopsies of degenerating muscle from DMD patients for the presence of fibrinogen deposits and inflammatory cell infiltrates. Compared with muscle biopsies of healthy subjects, in which fibrinogen positivity was only observed in capillaries surrounding myofibers, DMD muscles showed significant fibrinogen accumulation correlating with muscle damage. Immunohistochemical analyses using an \(\alpha_M\beta_2\) antibody also showed a prominent presence of macrophages in the same degenerating areas occupied by fibrinogen (Fig. 8A and Supplementary Material, Fig. S3) in human dystrophic muscles, but were absent in biopsies from healthy individuals. Thus, a linkage between exuberant and recurrent fibrinogen deposition and secondary inflammatory changes driving muscle disease progression may be a common feature of both mdx mice and human DMD.

DISCUSSION

Despite intense research efforts, DMD is still an incurable and fatal degenerative muscle disorder that demands novel experimental and therapeutic advances. The present study explores a new mechanistic and therapeutic dimension by examining the molecular link between fibrinogen deposition within persistently challenged muscle and inflammation-driven muscle damage, tissue reorganization and function loss. We provide direct evidence that fibrinogen, which is never present in normal muscle but rapidly accumulates in dystrophic muscle secondary to persistent tissue disruption and vascular leak, is a critical factor for inflammation-mediated DMD progression. Imposing either a genetic or pharmacological depletion of fibrinogen reduced macrophage numbers and activation in injured and dystrophic muscle and enhanced repair, while preserving locomotor capacity. However, more detailed studies revealed that muscle dystrophy pathology could also be ameliorated and muscle function preserved by interventions at the
level of fibrinogen that do not impose any hemostatic alteration or hemorrhagic risk. Specifically, the simple elimination of the \( \alpha_\text{M}\beta_3 \)-binding motif on the fibrin(ogen) gamma chain that drives pro-inflammatory functions, but is irrelevant to fibrin polymer formation, sufficed to alleviate disease severity in mdx dystrophic muscle. Conversely, delivery of exogenous fibrinogen into intact muscle of fibrinogen-null mice caused a classical inflammatory tissue repair response. The relevance of fibrinogen...
these findings to human disease is underscored by the finding that fibrin(ogen) accumulation was positively associated with inflammation in degenerating muscles of DMD patients. Hence, selectively targeting fibrin(ogen) inflammatory functions may represent a promising therapeutic strategy for DMD. The fact that fibrin(ogen) depletion affected several distinct aspects of muscle disease in mdx mice implies that recurrent fibrin deposition within the ECM may influence the progression of muscle disease through multiple mechanisms (Fig. 8B). Vascular damage early after disease onset in dystrophic muscle and secondary fibrin(ogen) accumulation in the ECM may be an early driver of the inflammatory changes known to exacerbate muscular dystrophy (17,20,21,46–49). Under this working hypothesis, intramuscular fibrin(ogen) deposits serve as temporospatial cue controlling local activation events in macrophages and other inflammatory cells that express αMβ2 (reviewed in 50), and recurrent and/or exuberant fibrin(ogen) within diseased muscle tissue promotes counterproductive inflammatory tissue degeneration. Our results demonstrate that upon fibrin(ogen) engagement, macrophages increase the expression of pro-inflammatory cytokines, including IL-1β, TNFα and IL-6 in vitro and in vivo, in an αMβ2-dependent manner. The presence of fibrin(ogen) appears to regulate the expression of cytokines known to promote muscle degeneration and impair regeneration, such as TNFα (17,49). Limiting these responses via experimentally imposed suppression of fibrin(ogen)-αMβ2 engagement by macrophages and other inflammatory cells may largely explain the reduced pathological changes observed in mdx muscle. Our data do not exclude
other fibrinogen-mediated effects on inflammatory cells such as \( \alpha_5\beta_3 \)-mediated inhibition of inflammatory cell apoptosis (39,40) and the increased release of free radicals (47,51,52).

Previous reports have shown that macrophage depletion or interference with pro-inflammatory cytokines transiently attenuated muscle dystrophy in mdx mice (20,53,54; reviewed in 7,32,55). Our present findings further underscore the relevance of the \( \alpha_5\beta_3 \) integrin receptor in macrophage activation in DMD and directly establish for the first time that the engagement of \( \alpha_5\beta_2 \) with one of its ligands, fibrinogen, an early component of the muscle provisional matrix after injury, is a critical regulatory event in dystrophic muscle pathology. We show that mice bearing an endogenous fibrinogen \( \gamma \) chain gene with a specific mutation in the \( \alpha_5\beta_3 \)-binding region (Fib\( ^{390-396A} \)) mice, which does not affect the clotting function of fibrinogen and does not limit the engagement of \( \alpha_5\beta_2 \) with other ligands (33,34), are significantly protected from acute muscle injury and dystrophic disease progression. Furthermore, the effective amelioration of muscle dystrophy in mdx mice treated with the Fib\( ^{377-395} \) peptide demonstrates that inflammatory functions of fibrinogen can, at least in principle, be pharmacologically suppressed, thus presenting an alternative treatment paradigm for impeding DMD disease progression.

Impeding macrophage \( \alpha_5\beta_2 \)-fibrinogen binding could also enhance muscle regeneration, which is primarily mediated by activation of satellite cells, localized along the basal lamina. This effect might again be related to the inhibition of macrophage activation. Interestingly, we also found that fibrinogen was deposited in the fibronectin \(^{+} \) and laminin \(^{+} \) ECM basal lamina of regenerating mdx muscle, where it physically entrapped satellite cells. Further, we demonstrated that fibrinogen regulates the proliferation and differentiation potential of satellite cells in culture through an \( \alpha_5\beta_3 \)-dependent mechanism. Thus, fibrinogen depletion may promote regeneration at least in part by indirectly attenuating inflammation, and possibly by diminishing deleterious effects of persisting fibrinogen on satellite cell functions.

Collectively, we propose that the development of exuberant and recurrent fibrin-rich matrices within challenged muscle tissue establishes a counterproductive inflammatory environment through \( \alpha_5\beta_3 \)-mediated leukocyte activation events as well as a counterproductive environment for muscle tissue regeneration. Consistent with this, our analysis on DMD patient samples suggests an association between fibrinogen deposition and the presence of macrophages in degenerating dystrophic muscle. Interestingly, interference with the fibrinogen/macrophage axis was recently shown to ameliorate neurodegeneration and halt demyelination in a mouse model of multiple sclerosis (34), to protect from collagen-induced arthritis (35) and colitis-associated colon cancer in mice (36). Alzheimer disease has also been associated to fibrinogen deposition and inflammation (56,57). It is therefore possible that targeting fibrinogen–\( \alpha_5\beta_3 \) interactions could represent a quite broad strategy for inhibiting macrophage activation in neurodegenerative diseases coursing with inflammation. In this regard, our study provides a compelling proof-of-principle that the sole blockage of fibrinogen binding to \( \alpha_5\beta_3 \) on macrophages via therapeutic administration of the Fib\( ^{377-395} \) peptide improved muscle function in mdx mice. Although the activation of macrophages plays a central role in the pathogenesis of muscular dystrophies (reviewed in 6,7), to the best of our knowledge agents that selectively inhibit macrophage inflammatory activation have not been developed and tried in this pathology. A potential advantage of a fibrinogen/macrophage interaction-based approach over current anti-inflammatory strategies is that interfering with \( \alpha_5\beta_3 \)-fibrinogen (i.e. dystrophin replacement or rescue) is likely to impose only a local block on the activation (and recruitment) of macrophages, i.e. within the dystrophic muscle where fibrinogen is deposited. Since no therapies correcting the primary defect in DMD (i.e. dystrophin replacement or rescue) are currently available, strategies selectively targeting fibrinogen/inflammatory cell signaling, without affecting its pro-coagulant properties, may constitute an attractive alternative for DMD disease treatment.

**MATERIALS AND METHODS**

**Generation of double-mutant mice**

Fibrinogen (Fib) knock-out male mice (58) were crossed with mdx female mice, Jackson Laboratories (USA). Male F1 mice were bred with mdx female mice, and their F2 heterozygous (Fib\(^{+/−}\)) male and female offspring was intercrossed. The resulting F3 generation showed the expected Mendelian distribution of Fib\(^{+/+}\), Fib\(^{+/−}\) and Fib\(^{−/−}\) genotypes, all of them in an mdx background. The Fib genotypes were confirmed by polymerase chain reaction (PCR) of ear biopsy genomic DNA, as previously described (58). The mdx genotype was confirmed by western blotting of muscle biopsies, using an anti-dystrophin antibody (Novocastra, UK, 1:200). Fib\(^{390-396A} \) knock-in mice (33) were generated from double heterozygous matings to produce homozygous Fib\(^{390-396A} \) mice. Male Fib\(^{390-396A} \) mice were sequentially intercrossed with mdx female mice, as described above, to generate double-mutant mice. All animal experiments were approved by the Catalan Government of Animal Care Committee.

**Morphometric analysis**

At selected times, muscles of vehicle and ancrod-treated mdx mice, Fib\(^{+/+}\), Fib\(^{−/−}\), Fib\(^{390-396A} \), Fib\(^{+/−}\)mdx, Fib\(^{−/−}\)mdx and Fib\(^{390-396A} \)mdx mice were removed after cervical dislocation, frozen and stored at −80°C prior to analysis. Ten micrometer sections were collected from the mid-belly of muscles and stained with hematoxylin/eosin (H&E). The percentage of muscle degeneration was determined by morphometrical analysis of digital photomicrographs of H&E-stained cryosections. Areas of necrosis and degeneration were identified by the presence of pale-stained myofibers, with irregular shape and often fragmented sarcoplasm. These areas contained few myonuclei and inflammatory cells were not conspicuous. Evans Blue Dye staining, a vital stain of myofiber permeability (59), showed that degenerating areas identified with H&E were composed of damaged fibers that had become permeable owing to muscular dystrophy (Supplementary Material, Fig. S1). The areas of degeneration on each individual sample were measured using the public domain ImageJ software on calibrated micrographs and were expressed as a percentage of the total muscle cross-sectional.
area (CSA). Myofiber CSA was determined as an indicator of muscle growth and regeneration. All analyses and photography were performed on a Leica DC 500 microscope equipped with a video camera. All parameters relative to myofiber CSA were measured using the ImageJ program.

**Biochemical and functional assessment of muscle**

Serum CK was measured with the indirect CK colorimetric assay kit and standards (Thermo Electron, USA). Grip strength assay: Limb grip strength was measured as tension force using a computerized force transducer (Grip Strength Meter, Bioseb). Three trials of three measurements per trial were performed for each animal with a few minutes resting period between trials. The average tension force (grams) was calculated for each group of mice. Treadmill assay: the treadmill apparatus (Treadmill, Panlab) consisted of a motor-driven belt varying in terms of speed and slope. At the end of the treadmill, an electrified grid was placed, on which footshocks (0.6 mA) were administered whenever the mice fell off the belt. The last week before sacrifice, after one daily acclimation session of 20 min at 6 m/min for 2 days, all mice were subjected to exhaustion treadmill tests at 5° inclination. Each test was repeated twice at a temporal distance of 4 days and results averaged. The following scheme was adopted: 5 min at 5 m/min followed by incremental increase of speed of 1 m/min every minute until exhaustion. Exhaustion was defined as spending time on the shocker plate without attempting to re-engage the treadmill within 20 s. The individual and the average times (min) and distances (meters) to exhaustion of running were measured.

**Induction of muscle regeneration**

Regeneration of skeletal muscle was induced by intramuscular injection of 300 μl of 10⁻⁵ μ CTX (Latoxan, France) in the gastrocnemius muscle group of the mice, usually of 8–12 weeks of age. This concentration and volume were chosen to ensure maximum degeneration of the myofibers. The experiments were performed in right hindlimb muscles, and contralateral intact muscles were used as control. Morphological and biochemical examinations were performed at the indicated days after injury.

**Systemic defibrination**

Twelve-day-old mdx mice were daily injected intraperitoneally with ancord (Sigma, 1–3 U ancord/day) or with a saline solution for 60 days, and killed at 2.5 months of age.

**Systemic delivery of Fibγ377-395 peptide**

Fibrinogen γ377-395 peptide (Y S M K E T T M K I I P F N R L S G; Azco Pharmchem) or scramble peptide (K M I S Y T F P I E R T G L I S N K; Azco Pharmchem) was resuspended in 0.9% NaCl at a concentration of 3 mg/ml, as described (34). Peptides were delivered intraperitoneally every other day into 2-month-old mdx mice over the course of 3 weeks.

**Intramuscular fibrinogen delivery**

Fib<sup>−/−</sup> mice of 8–12 weeks of age were used for intramuscular injection of fibrinogen or saline. Fibrinogen (Calbiochem) was dissolved in endotoxin-free distilled water, diluted to 9 mg/ml with saline, aliquoted and kept at 37°C. Fibrinogen (50 μl of 9 mg/ml) or saline was injected with a 10 μl Hamilton syringe attached to a 33-gauge needle into mouse tibialis anterior muscles. Samples were obtained at the indicated time points.

**Analysis of muscle macrophages by FACS**

Hindlimb muscles were collected and weighed. Muscles were dissociated by incubation in Dulbecco’s modified Eagle’s medium (DMEM) containing pronase 0.05% (Calbiochem) at 37°C for 45 min twice, filtered and counted. Cells were separated using a Percoll gradient and stained with fluorescein isothiocyanate-conjugated anti-CD45, allophycocyanin-Cy7-conjugated F4/80 antibody (BD Pharmingen). Cells were sorted using a cell sorter [fluorescence-activated cell-sorting (FACS) Aria II; BD]. Total number of cells was normalized by milligram of wet tissue.

**Immunohistochemistry**

The following primary antibodies were used for immunohistochemistry: rat anti-F4/80 (Serotec, 1:200), goat anti-fibrinogen antibody (Accurate Chemical & Scientific Corporation, Westbury, NY, USA), clone NYBT2G1, 1:200, and rat anti-mouse αM/CD11b (ebioscience 1:100), eMHC (F1.652, neat hybridoma supernatant; Developmental Studies Hybridoma Bank). Depending on the antibody, immunohistochemistry was performed with the tyramide signal amplification Cyanine 3 system (PerkinElmer Life Sciences) or as previously described (60). Control experiments without primary antibody demonstrated that signals observed were specific (data not shown).

**Cell culture and isolation of primary cells**

Isolation and culture of primary macrophages. Bone-marrow-derived macrophages (BMDMs) were obtained from mdx mice and cultured as previously described (24,61). In brief, bone-marrow cells were flushed from the femurs of mice. Cells were differentiated in DMEM supplemented with 20% heat-inactivated fetal calf serum (FCS) and 30% L929 supernatants containing macrophage-stimulating factor (MCSF) for 5–7 days. Cells were harvested and plated at a density of 2–4 × 10⁵ per ml in RPMI supplemented with 5% fetal bovine serum. At 24–48 h after being replated, cells were stimulated for various times with lipopolysaccharides (10 ng/ml; Sigma). CM from WT BMDMs was obtained stimulating cell cultures with for fibrinogen (0.5 mg/ml; Sigma). When indicated, fibrinogen-stimulated macrophages were also treated with 200 μM of γ377-395 peptide [a concentration shown to inhibit adhesion of α<sub>Mβ2</sub>/Mac-1-overexpressing...
cells to immobilized fibrinogen (62)] and 200 μM of scramble peptide. When indicated, CM was supplemented with blocking antibodies anti-IL-1β (30 ng/ml; R&D Systems) or anti-TNFα (50 ng/ml; BD Pharmingen) or unspecific IgG.

Isolation and culture of muscle satellite cells. Hindlimb muscles from mice were excised, separated from adipose and connective tissue, minced into coarse slurry and then digested for 1 h with 0.1% pronase (Sigma) in DMEM at 37°C with mild agitation. The digest was then mechanically dissociated by repeated trituration followed by filtration through a 100 mm vacuum filter (Millipore). The filtered digest was centrifuged through an isotonic Percoll gradient (60% overlaid with 20%). Mononucleated cells in the Percoll interface were collected and resuspended in Ham’s F10 medium supplemented with 20% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.001% Fungizone and 5 ng/ml bFGF [growth medium (GM)]. Primary satellite cell cultures were maintained on a routine basis on collagen-coated dishes in GM. The medium was changed daily and cultures were passaged 1:3 as they reached 60–70% confluence. Experiments were performed by plating cells on Matrigel (BD Biosciences) Basement Membrane Matrix-coated dishes. To maintain the primary characteristics of the cells, all experiments were performed using cultures that had undergone between four and seven passages. All experiments were performed with independent cell isolates from at least three different animals for each genotype. To induce muscle differentiation and fusion, GM was replaced by differentiation medium DM (DMEM supplemented with 2% horse serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.001% Fungizone) at satellite cell subconfluence.

For detection of S-phase cells, satellite cells were cultured in GM, and cultures were pulsed with bromodeoxyuridine (Brdu, Sigma) for hours prior to fixation in 3.7% formaldehyde for 10 min and were immunostained using anti-Brdu antibody (Oxford Biotech) and a specific secondary biotinylated goat anti-rat antibody (Jackson Immunoresearch Laboratories), followed by quantification (63). For fusion analysis, cells were immunostained with anti-eMHC antibody and nuclei within the eMHC-positive myofibers were quantified (63). When indicated, CM of cultured macrophages, control non-specific RGD peptide (GRGDPN; Biomol) or the cyclic RGD peptide (OpgnGRGD; Bachem; 100 nm) or an alphaV neutralizing antibody (20 μg/ml; Chemicon) was added.

RNA isolation and quantitative reverse transcription–polymerase chain reaction

RNA was analyzed by quantitative reverse transcription–polymerase chain reaction (RT–PCR). Total RNA was isolated from cells or muscle tissue using Tripure reagent (Roche Diagnostic Corporation). DNase digestion of 10 μg of RNA was performed using 2 U DNase (Turbo DNA-freeTM, Ambion). Complementary DNA was synthesized from 2 μg of total RNA using the First-Strand cDNA Synthesis kit (Amersham Biosciences). PCRs were performed with a LightCycler® 2.0 or in a LightCycler® 480 System using a Light Cycler® FastStart DNA Master PLUS SYBR Green I or Light Cycler® 480 SYBR Green I Master (Roche Diagnostic Corporation), respectively, and specific primers shown in Supplementary Material, Table S1. Thermocycling conditions were as follow: initial step of 10 min at 95°C, then 45 cycles of 15 s denaturation at 94°C, 10 s annealing at 60°C and 15 s extension at 72°C. Reactions were run in triplicate, and automatically detected threshold cycle (Ct) values were compared between samples. Transcripts of the ribosomal protein L7 gene were used as endogenous normalization control. qPCRs primers: eMHC, 5′-AAAAAGCCCATCACTGAGC-3′ and 5′-CAAGCTCTCTGACTCCGTCTC-3′ myogenin, 5′-GGTG TGTAAGAGGAACTGTG-3′ and 5′-TAGGGCCTCAT GTACCTGAT-3′ TNFα, 5′-CGCTCTTCTGTCTACTGAAC TT-3′ and 5′-GATGAGAGGAGGCCATT-3′ IL-1β, 5′-CCA AAATACCTGTGGCCTTGG-3′ and 5′-GCTTGTCCTCTTGCTTGTGAG-3′ IL-6, 5′-GAGTACCATCTCGCAGACGA CC-3′ and 5′- AAGTGCATCATCGTTGTG-3′ L7, 5′-GAAGCTCATCTATGAGAGCC-3′ and 5′-AAGACGAGGAGCTGCAAC-3′.

Statistical analysis

Data are presented as mean ± SEM. All quantitative data were analyzed with Prism software (GraphPad Software) for statistical analyses. The one-way analysis of variance and Dunnett’s test were used for multiple comparisons. The Non-parametric Mann–Whitney test was used for single comparisons of unmatched groups. P < 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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