RESEARCH COMMUNICATION

Fibrinogen drives dystrophic muscle fibrosis via a TGF β /alternative macrophage activation pathway

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In the fatal degenerative Duchenne muscular dystrophy (DMD), skeletal muscle is progressively replaced by fibrotic tissue. Here, we show that fibrinogen accumulates in dystrophic muscles of DMD patients and *mdx* mice. Genetic loss or pharmacological depletion of fibrinogen in these mice reduced fibrosis and dystrophy progression. Our results demonstrate that fibrinogen–Mac-1 receptor binding, through induction of IL-1 β , drives the synthesis of transforming growth factor- β (TGF β) by *mdx* macrophages, which in turn induces collagen production in *mdx* fibroblasts. Fibrinogen-produced TGF β further amplifies collagen accumulation through activation of profibrotic alternatively activated macrophages. Fibrinogen, by engaging its α v β 3 receptor on fibroblasts, also directly promotes collagen synthesis. These data unveil a profibrotic role of fibrinogen deposition in muscle dystrophy.

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Duchenne muscular dystrophy (DMD) results from mutations in the gene coding for the protein dystrophin, which localizes at the inner face of the sarcolemma (Campbell 1995). Besides progressive muscle degeneration and inflammation, fibrotic transition of muscle tissue is critical in DMD as it progressively deteriorates locomotor capacity, posture maintenance, and the vital function of cardiac and respiratory muscles. Indeed, DMD individuals have a high degree of fibrosis increasing with age, which is reproduced in the diaphragm muscle of *mdx* mice (the mouse model of DMD) (Stedman et al. 1991). Importantly, the underlying mechanisms of fibrosis development within dystrophic muscle remain largely unknown.

Fibrinogen is a soluble acute phase protein, which is released into the blood in response to stress. Apart from its key role in controlling blood loss following vascular injury, fibrinogen also extravasates at sites of inflammation or increased vascular permeability where it is immobilized and/or converted to fibrin (Rybarczyk et al. 2003) (from hereon we refer to both by the term “fibrin/ogen”). We showed previously that mice with defective fibrinolysis exhibited impaired muscle regeneration after experimental injury (Suelves et al. 2002). In this study, we investigated the role of fibrin/ogen deposition in the development of fibrosis in dystrophic muscle.

Results and Discussion

We first analyzed fibrin/ogen deposition in muscles of DMD patients and its correlation with disease course. Compared with muscles of healthy individuals or of fibromyalgia patients, DMD muscles showed significant fibrin/ogen accumulation (Fig. 1A). Similarly, in *mdx* mice muscles, fibrin/ogen deposits were readily detectable after disease onset, while absent before disease onset (Fig. 1B,C). Thus, fibrin/ogen deposition is associated with muscle dystrophinopathy.

Collagen deposition (fibrosis) was prominent in DMD muscles and particularly found in the same areas occupied by fibrin/ogen (Fig. 1D). To investigate the relationship between the extent of fibrin/ogen deposition and fibrosis in DMD, a total of 39 cases were collected, and the patients were divided into three nonoverlapping groups according to their age: the early group (2–5 yr; $n = 13$), the mid group (6–7 yr; $n = 14$), and the late group (8–11 yr; $n = 12$). Quantitative analysis on muscle biopsies revealed that fibrin/ogen accumulation was already significant at 6–7 yr (mid group) remaining high thereafter. In contrast, collagen deposition increased only at older ages (8–11 yr; late group) (Fig. 1E). Furthermore, a strong correlation was found between fibrin/ogen accumulation and fibrosis in DMD muscles ($P < 0.0007$; Fig. 1F). In *mdx* diaphragm, both fibrin/ogen deposition and fibrosis were consistently increased with age (Fig. 1G). While fibrin/ogen deposition was already considerably elevated at 1 mo of age, collagen accumulation showed a clear increase only from 2.5 mo onward, indicating that deposition of fibrin/ogen precedes that of collagens in *mdx* muscle (Fig. 1G), similar to what we found in DMD muscles. These observations suggest an association between fibrin/ogen accumulation and deposition of collagen in dystrophic muscle.

To further analyze the role of fibrin/ogen deposition in

Figure 1. Fibrin/ogen accumulates in muscles of DMD patients and *mdx* mice. (A) Staining for fibrin/ogen (brown) in muscle biopsies of DMD and fibromyalgia patients, and healthy subjects. (B) Western blotting of wild-type and *mdx* muscle extracts before and after disease onset (14 and 30 d of age, respectively), with an anti-fibrin/ogen antibody. (C) Fibrin/ogen immunostaining in muscles of wild-type and *mdx* mice of 30 d of age. (D) Representative fibrin/ogen and Sirius Red staining in DMD muscle sections. Bars: A,C,D, 50 μ m. (E) Integrated representation of fibrin/ogen accumulation (red line) and fibrosis (black line) in DMD patients, based on curve fitting of three data points, corresponding to quantitative morphometric results in the early, mid- and late group (see the Materials and Methods). (F) Correlation between fibrin/ogen accumulation and fibrosis in DMD patients (multiple range tests after a significant Kruskal-Wallis test). Samples were divided in three groups: negative/low ($n = 8$), intermediate ($n = 7$), and strong ($n = 11$) immunostaining for fibrin/ogen. (G) Fibrin/ogen and Sirius Red (collagen) staining were quantified as percentage of total area in *mdx* diaphragm sections (at the indicated age in months). *Mdx* values were normalized with age-matched wild-type mice values. Data are presented as fold increase with respect to 0.5-mo-old *mdx* mice.

fibrosis development during muscle dystrophy progression, we bred the *mdx* mice with fibrinogen-deficient mice (Suh et al. 1995) to generate the *Fib*^{-/-}*mdx* double-mutant mice. As expected, after disease onset (at 1 mo of age), muscles from *Fib*^{-/-}*mdx* mice exhibited no fibrin/ogen deposition (Supplemental Fig. 1a). Notably, collagen content in the diaphragm of *Fib*^{-/-}*mdx* mice was found to be significantly reduced compared with *Fib*^{+/+}*mdx* mice (Fig. 2A). To corroborate our genetic experiments using a pharmacological approach, we treated 12-d-old *mdx* mice with saline or with anandrol, an established defibrinogenating agent (Lluis et al. 2001), until the age of 2.5 or 6.5 mo. Compared with saline, anandrol

treatment resulted in a significant reduction of fibrin/ogen accumulation in *mdx* muscles (Supplemental Fig. 1b). Consistent with our findings in *Fib*^{-/-}*mdx* mice, an-

Figure 2. Fibrin/ogen regulates TGF β expression and fibrosis in *mdx* diaphragm. (A) Fibrosis quantification in diaphragm sections of *Fib*^{+/+}*mdx* mice, *Fib*^{-/-}*mdx* mice, and wild-type control mice (3.5 mo of age). (B) Fibrosis quantification in diaphragms of saline- or anandrol-treated *mdx* mice of 2.5 mo of age (left panel) and 6.5 mo of age (right panel). (*) $P < 0.05$ versus *mdx*-saline. (C) qRT-PCR analysis of diaphragms of *mdx* and wild-type mice at indicated ages. Fold induction over wild-type mice. (*) $P < 0.05$ versus wild-type. (D) qRT-PCR analysis in 6.5-mo-old diaphragms of saline- or anandrol-treated *mdx* mice was performed as in C. (*) $P < 0.05$ versus wild-type mice; (#) $P < 0.05$ versus *mdx*-saline. (E) Anti-p-Smad2 and Smad2 immunostaining in diaphragm sections after 6 mo of treatment with saline or anandrol. Bar, 50 μ m. (F) *Mdx* macrophages were treated with fibrinogen (Fg) $\pm$ actinomycin D or cycloheximide for 48 h. TGF β expression was analyzed by qRT-PCR. Values are fold induction with respect to control conditions. (*) $P < 0.05$ versus control; (#) $P < 0.05$ versus Fg. (G) *Mdx* macrophages were treated with Fg $\pm$ an IL-1 β -neutralizing antibody, control IgG antibody, or recombinant IL-1 β for 48 h, and TGF β expression analyzed and represented as in F. (H) Primary fibroblasts from *mdx* diaphragms were incubated with conditioned medium (CM) from *mdx* macrophages treated or not with Fg (as in Fig. 2G) for 24 h, with or without a TGF β -neutralizing antibody or control IgG. Gene expression was analyzed by qRT-PCR. Results are fold induction with respect to control-conditioned medium. (*) $P < 0.05$ versus control CM; (#) $P < 0.05$ versus CM from Fg-treated macrophages. Data are mean $\pm$ SEM. (A) $n = 4$ animals per group. (B) $n =$ animals per group. (C,D) $n = 3$ animals per group. (F-H) $n = 3$ experiments performed in triplicate.

croed significantly decreased the degree of fibrosis in *mdx* diaphragms of 2.5- and 6.5-mo-old mice (Fig. 2B). These data indicate that fibrin/ogen, although not strictly required, does accelerate fibrosis development in *mdx* muscle. Of note, fibrin/ogen depletion also reduced muscle degeneration (Supplemental Fig. 1c,d) and protected *mdx* mice from functional deterioration (Supplemental Fig. 2).

To investigate the mechanisms by which fibrin/ogen might be influencing fibrosis development in *mdx* diaphragm, we first examined the levels of the profibrotic cytokine transforming growth factor- β (TGF β), which is known to promote fibrosis in *mdx* diaphragm (Andreotta et al. 2006). TGF β expression increased with age in *mdx* diaphragm, both at the mRNA (Fig. 2C) and protein levels (mean $\pm$ SD: 110 $\pm$ 39, 210 $\pm$ 50, and 745 $\pm$ 183 pg/mg protein at 2.5, 6, and 12 mo, respectively), correlating with the age-induced fibrosis in *mdx* diaphragm. Importantly, consistent with the observed attenuated fibrosis, fibrin/ogen depletion for 6 mo in *mdx* mice reduced the expression of TGF β and of its target genes collagen I and tissue inhibitor of metalloproteinases-1 (TIMP-1) (Fig. 2D). Moreover, phospho-Smad2 staining was drastically reduced in diaphragms of ancroed-treated (Fig. 2E) and fibrinogen-deficient *mdx* mice (data not shown), indicating that interfering with fibrinogen deposition impaired functional TGF β signaling. These results thus suggest that fibrin/ogen promotes fibrosis by increasing TGF β expression and signaling in *mdx* diaphragm.

Macrophage depletion experiments demonstrated their deleterious role in *mdx* muscular dystrophy (Wehling et al. 2001). Since inflammatory cells are a critical source of TGF β in *mdx* diaphragm (Zhou et al. 2006), the influence of fibrin/ogen on the local inflammatory response was next analyzed. We found that the number of macrophages was reduced in fibrin/ogen depleted *mdx* diaphragm (Supplemental Fig. 3a). Compared with wild-type controls, the levels of proinflammatory cytokines such as TNF α , IL-6, MIP-2 (the functional mouse homolog of human IL-8), and IL-1 β were greatly increased in muscle extracts of *mdx* mice, and significantly reduced when *mdx* mice were treated with ancroed (Supplemental Fig. 3b). We next investigated whether fibrin/ogen had a direct effect on the cytokine production capacity of primary macrophages derived from *mdx* mice. Fibrinogen induced the rapid expression of TNF α , IL-6, MIP-2, and IL-1 β in these cells, as in a classical proinflammatory macrophage response (Supplemental Fig. 3d). In agreement with the capacity of fibrinogen to activate the NF- κ B pathway via engaging its receptor Mac-1 (Supplemental Fig. 3c; Sitrin et al. 1998; Rubel et al. 2003), treatment of primary macrophages with the NF- κ B inhibitor BAY11-7085 abrogated fibrinogen-induced proinflammatory cytokine production (Supplemental Fig. 3d). This induction was also impaired in the presence of a Mac-1-blocking antibody (Supplemental Fig. 3d). Furthermore, *mdx* macrophages responded to fibrin/ogen in a transwell migration assay in a Mac-1-dependent manner (data not shown), confirming the involvement of Mac-1/fibrin/ogen binding in these inflammatory effects (Flick et al. 2004). These results indicate that, by engaging Mac-1 and activating NF- κ B, fibrin/ogen up-regulates the expression of proinflammatory cytokines including IL-1 β .

We next investigated whether fibrin/ogen regulates

TGF β production by *mdx* macrophages. TGF β was induced in these cells 48 h after fibrin/ogen stimulation both at the RNA (Fig. 2F) and secreted protein levels (mean $\pm$ SD, picograms per milliliter: 1414 $\pm$ 270 in control versus 3102 $\pm$ 343 with fibrin/ogen; n = 3; P < 0.05), but not at an early time point (6 h) (data not shown). Consistent with this late induction pattern, the fibrin/ogen-stimulated TGF β mRNA expression in *mdx* macrophages was blocked by actinomycin D and by cycloheximide (Fig. 2F), suggesting the involvement of a newly synthesized protein intermediate. IL-1 β has been shown to induce TGF β gene transcription (Lee et al. 2006), and it is an early Mac-1-fibrin/ogen-inducible gene product (Supplemental Fig. 3d; Perez et al. 1999). When the function of IL-1 β was blocked with an IL-1 β neutralizing antibody, the fibrin/ogen-dependent TGF β expression at 48 h in macrophages was significantly reduced (Fig. 2G), indicating that, in our experimental system, fibrin/ogen induced TGF β expression at least in part through the action of IL-1 β . In addition, and consistent with the observed expression pattern of TGF β , IL-1 β expression was increased in 6.5-mo *mdx* diaphragms compared with wild type, while it was reduced in fibrin/ogen-depleted *mdx* mice (Fig. 2D). Confirming the profibrotic action of IL-1 β via increasing the expression of TGF β in vivo (Bonniaud et al. 2005), treatment of *mdx* mice with an IL-1 receptor antagonist (IL-1ra) for 1 mo reduced both TGF β levels and the extent of collagen deposition in *mdx* diaphragm (data not shown). Taken together, our results suggest that fibrin/ogen induces an early proinflammatory macrophage response that includes synthesis of the intermediate IL-1 β , which in turn will promote the synthesis and secretion of TGF β by macrophages.

The profibrotic activity of fibrinogen-induced TGF β was next examined. Conditioned medium from fibrin/ogen-stimulated *mdx* macrophages induced collagen I expression in *mdx* fibroblasts (Fig. 2H). This effect was specific for TGF β , since collagen I induction was blunted in the presence of a neutralizing antibody against TGF β (Fig. 2H). Similar data were obtained when analyzing the expression of the TGF β target gene TIMP-1 (Fig. 2H). Hence, by promoting TGF β secretion by *mdx* macrophages, fibrin/ogen stimulates the synthesis of collagen by *mdx* fibroblasts and its further accumulation, thereby likely promoting fibrosis development in *mdx* muscles.

Alternatively activated macrophages (as opposed to classically activated, proinflammatory macrophages) have been associated with certain fibrotic pathologies (Wynn 2004). These macrophages are activated by Th2-derived cytokines, such as IL-13, and can be identified by specific cell surface markers such as CD206 (also known as mannose receptor) (Stein et al. 1992). They also express high levels of chitinase-like secretory lectins Ym1 and Ym2, and TIMP-1 (Wynn 2004). However, no link between alternatively activated macrophages and muscular dystrophy has been established. While unchanged in wild-type mice, we found that the number of CD206⁺ cells increased progressively with age in *mdx* diaphragms (Fig. 3A). In addition, quantitative RT-PCR (qRT-PCR) analysis showed an age-dependent increased expression of the macrophage alternative activation markers Ym1 and Ym2 in diaphragms of *mdx* mice (Fig. 2C), phenocopying the age-dependent deposition of fibrin/ogen and collagen in *mdx* diaphragm (Fig. 1G). Im-

Figure 3. Fibrin/ogen enhances alternative macrophage activation in *mdx* diaphragm. (A) Immunohistochemical quantification of CD206⁺ cells in diaphragms of wild-type and *mdx* mice of different ages (fold increase with respect to wild-type mice). (B) Alternatively activated (AA) macrophages (F4/80⁺CD206⁺) in diaphragm muscle sections of *Fib*^{+/+}*mdx* and *Fib*^{-/-}*mdx* mice (left panel) and saline- or anicord-treated mice of 6.5 mo of age (right panel). (C,D) Double-positive cells for CD206 (red) and Arginase I (green) (C), and for CD206 (red) and TGFβ (green) (D) in an 8-mo-old *mdx* diaphragm. Bars, 50 μm. (E) Mdx macrophages were treated with recombinant TGFβ, IL-13, or both for 24 h. Gene expression was analyzed by qRT-PCR. Results are fold induction with respect to control conditions. (*) *P* < 0.05 versus control conditions; (#) *P* < 0.05 versus TGFβ and IL-13 alone. (F) *mdx* fibroblasts were treated with Fg ± an αv-neutralizing antibody or control IgG for 5 h. Gene expression as analyzed by qRT-PCR. (*) *P* < 0.05 versus control conditions; (#) *P* < 0.05 versus Fg alone. Data are mean ± SEM. (A,B) *n* = 6 animals per group. (E,F) *n* = 3 experiments performed in triplicate.

portantly, fibrin/ogen depletion in *mdx* mice reduced significantly the number of CD206⁺ alternatively activated macrophages (Fig. 3B) and the expression of Ym1 and Ym2 (Fig. 2D) in the diaphragm. By double-immunostaining, CD206⁺ macrophages coexpressed both arginase I (Fig. 3C) and TGFβ (Fig. 3D), thus suggesting that fibrin/ogen regulates the presence of alternatively activated profibrotic macrophages in *mdx* diaphragm.

TGFβ, in conjunction with IL-13, may amplify the expression of arginase I—a key enzyme in the initiation of collagen synthesis by fibroblasts (Wynn 2004; Bronte and Zanovello 2005)—in alternatively activated macrophages (Boutard et al. 1995). We found that simultaneous

treatment of *mdx* macrophages with TGFβ and IL-13 resulted in the synergistic induction of the alternatively activated gene products arginase I, Ym1, and Ym2 (Fig. 3E), suggesting that fibrin/ogen, by inducing TGFβ (see above), might further stimulate collagen synthesis through amplifying the alternative activation of arginase-producing macrophages.

Additionally, fibrin/ogen also directly induced the expression of collagen by *mdx* fibroblasts (Fig. 3F), and this stimulation was independent of new protein synthesis (data not shown). Consistent with the reported binding of fibrin/ogen to fibroblasts via engaging αvβ3 integrins (Rybarczyk et al. 2003), we found that incubation of *mdx* fibroblasts with an anti-αv antibody abrogated the induction of collagen expression by fibrin/ogen (Fig. 3F). Similar effects were obtained for TIMP-1 (Fig. 3F). Thus, these results further support the idea that fibrin/ogen might promote fibrosis in *mdx* diaphragm by direct and indirect fibroblast activating mechanisms.

Muscle degeneration in DMD patients has been associated with alterations in coagulation and fibrinolysis (Saito et al. 2001), thrombosis being a frequent complication in DMD. In addition, impaired fibrinolysis was also shown recently to exacerbate degeneration in *mdx* mice (Suelves et al. 2007).

In this study, we unveil a new role for fibrin/ogen deposition in promoting the fibrotic transition of dystrophic muscle by demonstrating that blunting fibrin/ogen accumulation reduces fibrosis in *mdx* mice. Through binding its receptor Mac-1, fibrin/ogen can induce a pro-inflammatory response that subsequently leads to TGFβ production by macrophages, which in turn may potenti-

Figure 4. Proposed model for the role of fibrin/ogen in the muscle dystrophy-associated fibrosis. Fibrinogen promotes collagen production by three distinct but possibly overlapping mechanisms in *mdx* muscle. (1) TGFβ is produced in response to fibrinogen stimulation of proinflammatory, classically activated *mdx* macrophages (through Mac-1 binding) and via the intermediate induction of IL-1β (a direct fibrinogen-inducible gene), as an anti-inflammatory counteracting mechanism involving a macrophage activation switch. Macrophage-secreted TGFβ is free to bind and activate TGFβ receptors expressed by fibroblasts from *mdx* diaphragms, and directly induce collagen synthesis. (2) TGFβ can also promote the alternative activation of macrophages initiated by IL-13 Th2 cytokine. By up-regulating arginase activity in these cells, TGFβ (in conjunction with IL-13) is known to promote fibroblast proliferation, collagen production, and ultimately, fibrosis. (3) Fibrin/ogen might also directly activate the collagen-producing machinery in fibroblasts by binding to αvβ3 receptors.

ate their alternative activation state. Apart from reinforcing the emerging link between tissue inflammation and fibrosis, we provide evidence for a fibrin/ogen/Mac-1-IL-1 β -TGF β axis, which can drive distinct pathways leading to fibrotic transition of dystrophic muscle. Fibrin/ogen also directly stimulates the expression of collagen by *mdx* fibroblasts via binding its receptor $\alpha\text{v}\beta 3$ integrin. Taken together, these findings introduce the integrative concept that fibrin/ogen promotes tissue inflammation and fibrosis in *mdx* muscular dystrophy (see Fig. 4).

Given the importance of preserving fibrinogen-clotting function, strategies targeting only fibrinogen signaling without affecting its procoagulant properties might be suitable for human therapeutic intervention. *Fib γ ^{390-396A}* knock-in mice produce a modified fibrinogen protein incapable of binding Mac-1, but preserving its clotting capacity (Flick et al. 2004; Adams et al. 2007). We therefore comparatively analyzed the inflammatory response and connective tissue-remodeling in *Fib^{-/-}* and *Fib γ ^{390-396A}* mice in experimentally injured muscle. Importantly, both *Fib^{-/-}* and *Fib γ ^{390-396A}* mice showed reduced macrophage infiltration and less collagen deposition compared with wild-type mice (Fig. 5), corroborating the idea that blocking fibrin/ogen-Mac-1 interaction might be of therapeutic relevance in muscle dystrophy.

A potential advantage of a fibrin/ogen-based therapeutic approach over current anti-inflammatory/fibrotic strategies is that targeting fibrin/ogen interactions is likely to impose a block on the activation and recruit-

ment of leukocytes only locally, i.e., within the dystrophic muscle where fibrin/ogen is deposited. Indeed, anti-TGF β antibody therapies in muscle dystrophy reduce fibrosis but also increase inflammation (Andreetta et al. 2006). Hence, by targeting fibrinogen, one might prevent both inflammation and fibrosis in dystrophic muscle.

Importantly, muscle fibrosis not only accelerates the clinical decline in young DMD patients, but also represents a major obstacle for successful engraftment of stem cells in ongoing experimental cell-based therapies in DMD aimed at correcting the primary defect (i.e., dystrophin replacement or rescue). Thus, reducing inflammation-mediated fibrosis by targeting fibrin/ogen might also facilitate the homing of donor stem cells to muscle, and hence, the success of these primary therapies.

Materials and methods

Mice, induction of muscle regeneration, and muscle analyses

Mouse lines; genotyping protocols; systemic defibrinogenation with anacrod; induction of muscle regeneration; morphometric, immunohistochemical, and collagen content (fibrosis) analyses of muscle sections; and functional muscle force assays are described in the Supplemental Material.

Cell culture, RNA, and protein analyses

Culture conditions for primary macrophages and fibroblasts, qRT-PCR, and antibodies for protein immunodetection are described in the Supplemental Material.

DMD patient study

Muscle biopsies were analyzed as described in Supplemental Material.

Statistical analysis

Quantitative data were analyzed by Student's *t*-test, unless otherwise specified. *P* < 0.05 was considered statistically significant.

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Figure 5. Cardiotoxin-injured *Fib^{-/-}* mice or *Fib γ ^{390-396A}* mice have diminished inflammation and reduced collagen deposition. Muscle injury was performed by cardiotoxin (CTX) injection in gastrocnemius muscles of *Fib^{-/-}*, *Fib γ ^{390-396A}*, and their respective wild-type controls. (A,B) The Macrophage number per square millimeter of damaged area at 3 and 5 d post-injury (dpi) in *Fib^{+/+}* and *Fib^{-/-}* mice (left panels) or wild-type and *Fib γ ^{390-396A}* mice (right panels). (C) Sirius Red staining quantification in the described mice. (*) *P* < 0.05 versus wild-type mice. *n* = 4 animals per group.

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