Major basic protein-1 promotes fibrosis of dystrophic muscle and attenuates the cellular immune response in muscular dystrophy

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The immune response to dystrophin-deficient muscle promotes the pathology of Duchenne muscular dystrophy (DMD) and the mdx mouse model of DMD. In this investigation, we find that the release of major basic protein (MBP) by eosinophils is a prominent feature of DMD and mdx dystrophy and that eosinophils lyse muscle cells in vitro by the release of MBP-1. We also show that eosinophil depletions of mdx mice by injections of anti-chemokine receptor-3 reduce muscle cell lysis, although lysis of mdx muscle membranes is not reduced by null mutation of MBP-1 in vivo. However, ablation of MBP-1 expression in mdx mice produces other effects on muscular dystrophy. First, fibrosis of muscle and hearts, a major cause of mortality in DMD, is greatly reduced by null mutation of MBP-1 in mdx mice. Furthermore, either ablation of MBP-1 or eosinophil depletion causes large increases in cytotoxic T-lymphocytes (CTLs) in mdx muscles. The increase in CTLs in MBP-1-null mice does not reflect a general shift toward a Th1 inflammatory response, because the mutation had no significant effect on the expression of interferon-gamma, inducible nitric oxide synthase or tumor necrosis factor. Rather, MBP-1 reduces the activation and proliferation of splenocytes in vitro, indicating that MBP-1 acts in a more specific immunomodulatory role to affect the inflammatory response in muscular dystrophy. Together, these findings show that eosinophil-derived MBP-1 plays a significant role in regulating muscular dystrophy by attenuating the cellular immune response and promoting tissue fibrosis that can eventually contribute to increased mortality.

INTRODUCTION

Defects in the expression of dystrophin, a membrane-associated structural protein, underlie the progressive, lethal pathology of Duchenne muscular dystrophy (DMD) and the less severe pathology of mdx mice. Although dystrophin’s primary function is to provide mechanical support to the cell membrane and participate in linking intracellular and extracellular protein assemblies to one another, deficiencies in these functions are insufficient to explain the onset or course of dystrophinopathy. Accumulating experimental evidence indicates that a significant part of the complex pathophysiology of the dystrophin-deficient phenotype may lie in the interactions between the mechanically-injured muscle fibers and the immune cells that invade in response to the injury. For example, depletions of macrophages, cytotoxic T-lymphocytes (CTLs), helper T-cells (Th cells) or neutrophils from mdx mice can each significantly influence the severity of the early stages of the disease (1–4). In addition, the genetic ablation of T-lymphocytes in mdx mice bred onto the nu/nu background greatly reduces muscle fibrosis at later stages of the disease in which there is a chronic but less severe inflammation (3,6). Together, these findings suggest that
immune-based interventions may be valuable for the treatment of muscular dystrophy.

Eosinophilia is a prominent feature of dystrophin-deficient muscles. Although eosinophil invasion of skeletal muscle is an extremely uncommon feature in other muscle injuries or diseases, eosinophils typically reach concentrations that exceed 4000 eosinophils (eos)/mm$^3$ in dystrophin-deficient muscles and their numbers reflect the severity of the muscle pathology (7). Eosinophil functions in other diseases suggest that they would promote the pathology of muscular dystrophy, although their functions in dystrophic muscle have not been examined previously. For example, the release of major basic protein (MBP), eosinophil-derived neurotoxin or eosinophil cationic protein (ECP) from eosinophils is toxic or lytic to neural, cardiovascular and respiratory tissues (8–11), and perhaps these proteins similarly damage skeletal muscle. Eosinophils also release eosinophil peroxidase that can catalyze the production of hypohalous acids and peroxynitrate (12,13), which are potent toxins. In addition, eosinophils can activate mast cells, stimulating the release of histamine (14). Similar activation of mast cells in dystrophic muscle could exacerbate leukocyte extravasation and subsequent damage to the muscle by inflammatory cells.

Eosinophils may also significantly affect the course of disease by influencing the balance between Th1 and Th2 immune responses. Eosinophils release a complex mixture of cytokines that primarily consist of Th2 cytokines that can accelerate the repair of injured tissues, at least in other injuries or diseases. In particular, eosinophils release interleukin-10 (IL-10) (15) that can deactivate classically-activated macrophages which are capable of damaging muscle via free radical production. Eosinophils also secrete IL-4 (15), which promotes the proliferation and activation of macrophages of the Th2 phenotype, that participate in tissue repair. In addition, IL-4 increases the recruitment and fusion of skeletal muscle progenitor cells (satellite cells) (16) that would be beneficial in the regeneration of injured, dystrophic muscle. However, skewing of the immune response toward the Th2 phenotype may also have important negative consequences that frequently underlie eosinophilic disease processes. Most importantly, chronic inflammations that are characterized by a Th2 response and eosinophilia can lead to pathological fibrosis in scleroderma, pulmonary fibrosis, endomyocardial fibrosis and wound healing (17–19). Because fibrosis of skeletal and cardiac muscle of DMD patients and mdx is a major cause of functional defects and death, eosinophils could contribute significantly to the pathology of dystrophin-deficiency if they were to promote fibrosis.

Eosinophils could further worsen the pathology of muscular dystrophy by promoting the cellular immune response. Previous investigators have shown that CTLs in DMD muscle express a highly-conserved epitope in the hyper-variable domain of the T-cell receptor, suggesting that T-cell activation occurs as a result of dystrophin deficiency (20). Furthermore, pathology in mdx muscle is reduced by depletions of CTLs or by null mutation of perforin (1,2), the CTL-derived lytic protein central to target cell death in cellular immunity. Because eosinophils function as antigen presenting cells (21,22), they could feasibly exacerbate muscle lysis through the cellular immune response. However, ECP is also capable of suppressing T-cell activation and proliferation (23), at least in vitro, so that eosinophil inhibition of cellular immune responses is an additional, unexplored possibility.

Many of the apparently conflicting roles of eosinophils in promoting injury or repair may be mediated by MBP. Current speculations note that MBP binds promiscuously to tissue debris at sites of injury or disease, and this may mark the debris for removal to facilitate repair (24). MBP can also bind and inhibit heparanase, which may reduce tissue damage and inflammation (25), and it stimulates IL-6 production by fibroblasts in vitro (26) which could promote tissue repair. Nevertheless, MBP is cytotoxic, it promotes inflammation by activating the release of histamine (14) and it is present at extracellular sites of pathological fibrotic lesions in several disease models (19). Thus, the effects of MBP release by eosinophils can vary dramatically, and may be influenced importantly by the quantity and duration of MBP secretion and the microenvironment into which it is released.

In the present investigation, we test whether eosinophils and MBP play a significant role in the pathology of dystrophinopathy through the analysis of muscles from mdx mice that have been depleted of eosinophils or mdx mice that are null mutants for MBP-1. Specifically, we test the hypothesis that eosinophils and MBP worsen the pathology of muscular dystrophy by promoting the lysis of dystrophic muscles, by increasing skeletal and cardiac muscle fibrosis and affecting the balance between Th1 and Th2 inflammatory responses. Several muscles were selected for analysis because the course or severity of their pathology differs. Limb muscles in the mdx mouse model of DMD show an acute stage of pathology at 4 weeks of age that is characterized by eosinophilia and by muscle fiber lysis that is promoted by a cellular immune response (1–3,7). This acute phase is then followed by attenuation of inflammation that is accompanied by muscle regeneration. However, during the second year of life, mdx mouse limb muscles experience a chronic but less severe inflammation and eosinophilia that are associated with pathological fibrosis of the muscles. Diaphragm muscles are also examined because they experience a continuously progressive inflammation and eosinophilia that accompanies the early, progressive fibrosis of the diaphragm (7,27,28). Finally, hearts are examined because they also experience myocardial cell lysis, invasion by eosinophils and other leukocytes, and a progressive, lethal fibrosis of the myocardium (29). Collectively, our findings show that eosinophil-derived MBP attenuates the cellular immune response at early stages of dystrophinopathy, but significantly elevates the fibrosis of dystrophic limb muscle, diaphragm and heart at later stages of the disease. Our findings suggest that therapeutic approaches that target eosinophils or MBP may ameliorate features of the dystrophic pathology.

RESULTS

Eosinophils promote lysis of mdx muscle fibers in vivo

Previous findings showed that mononucleated cells in dystrophic mouse muscle contained eosinophilic granules, and were present at high concentrations, demonstrating that
Eosinophilia is a component of muscular dystrophy caused by null mutation of dystrophin. Here, we show that these cells express MBP-1, confirming that they are eosinophils, and that they are present at high numbers in dystrophic limb muscles at the acute, early peak of pathology that occurs at 1 month of age. Furthermore, light microscopic and electron microscopic observations confirm that eosinophils expressing MBP-1 are present in foci of muscle damage and necrosis, and that eosinophil degranulation occurs at the site of dystrophic muscle lesions (Figs. 1 and 2). Similarly, immunohistochemical observations show that muscle biopsies obtained from boys with DMD contain MBP-1-expressing eosinophils, and that eosinophils persist in DMD muscle at sites of muscle damage even at later stages of the disease when fibrosis is a major component of muscle pathology (Fig. 1).

We tested whether eosinophils contribute significantly to muscle membrane lesions in dystrophic mice by assaying for changes in soleus muscle membrane damage in mdx mice that were depleted of eosinophils by intraperitoneal injections of antibodies to chemokine receptor-3 (CCR3). We confirmed that our depletion protocol was effective by demonstrating a 72% reduction in MBP-1-expressing cells in the muscles of anti-CCR3 depleted mice at 1 month of age (depleted: 1027.1 eos/mm³ of muscle, SEM = 197, n = 7; non-depleted: 3721.5 eos/mm³, SEM = 338, n = 6) (Fig. 3A). Although mast cells can also express CCR3, we observed no change in mast cell numbers in the anti-CCR3 treated animals, which indicated that our depletion protocol was specific for eosinophils (depleted: 276.3 mast cells/mm³, SEM = 55.6, n = 7; non-depleted: 288.8 cells/mm³, SEM = 36.5, n = 6). Accompanying the decline in MBP-1+ eosinophils in treated mice, we observed a significant 67% reduction in necrotic muscle fibers with highly-elevated, cytoplasmic procion orange (depleted: 2.9% fibers injured, SEM = 0.7, n = 7; non-depleted: 8.6% injured, SEM = 1.8, n = 6), as well as a general reduction of cytosolic fluorescence of fibers from procion-orange-treated, eosinophil-depleted muscles, indicating that eosinophils promote muscle membrane damage (Fig. 3A and B). However, eosinophil depletions did not significantly affect muscle regeneration, assessed by the proportion of fibers that were central-nucleated (depleted: 48.8% fibers central-nucleated, SEM = 5.8, n = 5; non-depleted: 51.2% central-nucleated, SEM = 5.7, n = 5; SEM = 0.78). The concentrations of macrophages and CTLs were not reduced as a consequence of eosinophil depletions, which indicates that the reduction in membrane damage was not attributable to a secondary reduction of other immune cells that have been shown previously to kill dystrophic muscle fibers in vivo (1,3). On the contrary, the numbers of CTLs were significantly increased in eosinophil-depleted mdx muscles (depleted: 230.3 CTLs/mm³, SEM = 31, n = 7; non-depleted: 99.6 CTLs/mm³, SEM = 33, n = 6) (Fig. 3A).

**Eosinophils promote muscle cell lysis in vitro through MBP-1-dependent and MBP-1-independent mechanisms**

Eosinophils that were isolated from wild-type or MBP-1 null mice were used in cytotoxicity assays with C2C12 myotubes as targets. Eosinophils from both strains displayed lysis of myotubes that increased with increasing concentrations of eosinophils (Fig. 3C). Addition of platelet-activating factor (PAF) to the cultures to promote eosinophil degranulation increased cytolysis by wild-type eosinophils, but not by MBP-1 null eosinophils, which indicated that muscle cell lysis during eosinophil degranulation in vitro is primarily attributable to MBP-1. However, cytotoxicity assays show that ~30% of cytotoxicity by eosinophils in the presence or absence of PAF is independent of MBP (Fig. 3C).
Eosinophil lysis of mdx muscle fibers in vivo is not mediated by MBP-1

Mdx mice that were null mutants for MBP-1 were also assayed for muscle fiber membrane lesions. Unlike eosinophil-depleted mdx mice, the MBP-1 null mutant mice showed no significant reduction in the percentage of fibers that were necrotic (mdx: 6.6%, SEM = 2.3; n = 13; MBP-1 null mdx: 9.8%, SEM = 3.5, n = 10; P = 0.44). The concentration of creatine kinase in the serum was also unaffected by mutation of MBP-1 in mdx mice (mdx: 6138 U/l, SEM = 606, n = 17; MBP-1 null mdx: 4960, SEM = 636, n = 12; P = 0.34). Similarly, there was no significant reduction in muscle membrane lysis in mdx soleus muscles in which there is an acute onset of membrane damage and eosinophilia at 4-weeks of age (Fig. 4A). We also assayed whether MBP-1 release could exacerbate muscle membrane damage in mdx diaphragms that experience a continuously progressive pathology that includes a continuous increase in eosinophil numbers (7). However, we found that MBP-1 null mutation produced a significant increase in mdx diaphragmatic muscle membrane lesions at 4-weeks of age, and did not affect membrane damage in diaphragms at 3 months of age (Fig. 4B and C). These findings show that eosinophil-mediated damage to dystrophic muscle is not mediated by MBP-1.

MBP-1 null mutation does not affect muscle regeneration in mdx mice

Previous investigators have proposed that MBP-1 binding to extracellular debris could mark the debris for removal and thereby accelerate tissue repair and regeneration (24). We tested whether MBP-1 release could serve this function in dystrophin-deficient muscle by assaying muscle regeneration in MBP+/mdx and MBP−/mdx mice. Muscle regeneration in mdx mice was assessed by the proportion of central-nucleated fibers in cross-sections of entire soleus muscles at 4 weeks of age. However, ablation of MBP-1 had no effect on mdx muscle regeneration (Fig. 5A).

MBP-1 increases fibrosis of mdx limb muscles and diaphragm muscles and heart

Because fibrosis is an important pathological feature of dystrophin-deficiency in mdx heart, diaphragm and limb muscles, and the associations between the presence of fibrosis and eosinophil lysis are well established, we also determined whether MBP-1 release could exacerbate fibrosis of mdx limb muscles and heart. MBP-1 null mutation produced a significant increase in collagen content and fiber diameter in mdx limb muscles (Fig. 5B) and mdx heart (Fig. 5C) at 3 months of age. These findings show that MBP-1 release exacerbates fibrosis of mdx limb muscles and heart.

MBP-1 null mutation does not affect the morphology of mdx muscle fibers

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Figure 2. Electron micrograph of soleus muscle from 12-month-old mdx mouse. Arrows indicate rods of MBP within vesicles in an eosinophil that is in direct contact with a muscle fiber. Bar = 2 μm.
eosinophils and the occurrence of tissue fibrosis have been noted in other pathologies, we assayed whether ablation of MBP-1 in dystrophic muscles would reduce tissue fibrosis in advanced stages of the pathology. Immunohistochemical observations show that the proportion of mdx limb muscle, diaphragm muscle and hearts occupied by collagens types I, III, IV and V were significantly reduced by ablation of MBP-1 (Fig. 6). Also, using hydroxyproline content as an index of fibrosis, we found that null mutation of MBP-1 significantly reduced the pathological fibrosis caused by dystrophin-deficiency in diaphragms (48% reduction) and limb muscle (36%) at 14 months of age and produced an 89% reduction in pathological fibrosis in 18-month-old mdx hearts (Fig. 5B).

MBP-1 reduces CTL numbers in dystrophin-deficient muscles

The large reductions in fibrosis of dystrophin-deficient muscles in MBP-1 null mutant mice suggested that MBP-1...
increases fibrosis in limb, diaphragm, and heart muscles. The numbers of CTLs in hindlimb muscle increased progressively at 14 months of age, the numbers of CTLs in hindlimb muscle increased tremendously in both MBP-expressing and MBP-null lines of age. The numbers of CTLs in hindlimb muscle increased progressively at 14 months of age, the numbers of CTLs in hindlimb muscle increased tremendously in both MBP-expressing and MBP-null lines. Furthermore, when mdx mice entered the regenerative phase of the pathology (3-months of age), CTLs were still present at significantly higher concentrations in the MBP-null muscles. The elevated numbers of CTLs in eosinophil-depleted mdx muscle and MBP-1 null mutant mdx muscle showed that eosinophils could suppress the cellular immune response through an MBP-mediated mechanism. We tested whether the increase in a cellular immune response reflected a general shift toward a Th1 phenotype in MBP-1-null mice by assaying for indicators of a Th1 inflammatory response. However, quantitative reverse transcription–polymerase chain reaction (RT–PCR) measurements of mRNA levels for inducible nitric oxide synthase (iNOS), tumor necrosis factor-alpha (TNFα) or interferon-gamma (IFNγ) showed no significant change associated with MBP-1 mutation (Fig. 8A), indicating that the MBP-1-mediated effect is not occurring at a level that broadly influences the inflammatory phenotype.

MBP suppresses PHA-stimulated activation and proliferation of lymphoid cells

Because our RT–PCR data indicated that MBP-1 was not influencing CTL numbers in mdx mice by broadly influencing the inflammatory phenotype, we assessed whether MBP could suppress the activation and proliferation of lymphoid cells, and thereby more directly modulate CTL numbers. We tested this by assaying whether administration of exogenous MBP to primary cultures of splenocytes could reduce phytohemagglutinin (PHA)-induced stimulation of T-lymphocyte proliferation. Our findings showed that as MBP concentration increased, 3H-thymidine uptake by splenocytes in primary culture decreased. At 10^{-1} μM MBP, thymidine uptake levels returned to levels of uptake observed in splenocyte cultures that did not receive PHA (Fig. 8B). The data also indicate that the reduction in thymidine uptake reflected a specific inhibition of splenocyte activation that was stimulated by PHA and did not reflect a cytotoxic effect of MBP because MBP had no significant effect on proliferation of cells that were not stimulated by PHA (Fig. 8B).

DISCUSSION

The present investigation establishes a role for eosinophils in the pathology of muscular dystrophy through the release of MBP and shows for the first time that MBP can reduce the cellular immune response and increase fibrosis in vivo. Both of these MBP-mediated effects may have important impacts on the severity of muscle disease in dystrophin-deficiency. Fibrosis of the heart of DMD patients contributes significantly to defects in cardiac function, which can be a proximal cause of death in DMD (30). Autopsies of DMD patients show that myocardial fibrosis is the most common pathoanatomical...
finding in the heart, and the progressive accumulation of collagenous connective tissue that replaces cardiac myocytes and fibers of the Purkinje system may be a primary cause of cardiac involvement for some DMD patients (31). In addition to the fibrotic replacement of contractile tissue, the remaining myocytes in the DMD heart become encased in connective tissue so that the number of intercellular connections (gap junctions) are greatly reduced (32), which may contribute further to conduction defects. Similarly, mdx mice experience defects in cardiac function that progress as myocardial fibrosis increases, and interventions that produce reductions in fibrosis in mdx hearts also improve cardiac function (29).

Skeletal muscle fibrosis is similarly a prominent feature of the pathology of dystrophin-deficiency in DMD patients and
mdx mice, and can lead to skeletal deformities, such as kyphoscoliosis. Between 12 and 24 months, locomotion by mdx mice becomes increasingly and severely impaired, which parallels muscle fibrosis (33). In both DMD and mdx pathologies, progressive fibrosis of respiratory muscles such as the diaphragm leads to impaired respiratory function that can increase morbidity. Thus, previous investigations show that fibrosis has central importance in the pathophysiology of muscular dystrophy and the present investigation shows that MBP is important for promoting the fibrosis of dystrophic hearts and muscles.

At first view, the large reduction in muscle fibrosis in MBP-1 null mdx mice appears inconsistent with previous inferences that transforming growth factor-β (TGF-β) plays a primary role in promoting fibrosis in muscular dystrophy. TGF-β was first implicated in the fibrosis of dystrophic muscle by immunohistochemical observations that showed TGF-β in DMD muscle (34). Subsequent findings showed elevated levels of mRNA for TGF-β in DMD muscle, but changes in the level of TGF-β mRNA did not correspond to
the time course of muscle fibrosis; TGF-β mRNA in DMD muscle peaked early in the disease and then rapidly declined while fibrosis progressed continuously throughout the disease (35). Similarly, dogs that are null mutants for dystrophin and that experience a progressive, lethal muscular dystrophy that involves extensive, progressive fibrosis show a decline in TGF-β mRNA after 60 days of age, although fibrosis continues to progress (36). Thus, the time course data support the possibilities that either TGF-β promotes fibrosis in early stages of dystrophinopathy, or that it functions primarily in other features of the disease, such as promoting tissue repair. Empirical data support the former possibility. Diaphragms of mdx mice, in which there is a progressive fibrosis that begins early in life, show a transient elevation in TGF-β mRNA concentration at 6–9 weeks of age that then rapidly drops to control levels (37) and then another brief increase at 24 weeks of age that drops rapidly to control levels by 36 weeks of age (38). Treatment of mdx mice with neutralizing antibodies to TGF-β during the first, transient increase in TGF-β mRNA significantly reduced the area occupied by connective tissue in tissue sections of diaphragm from 12-week-old mdx mice, supporting a role for TGF-β in promoting fibrosis early in the disease (38). Those findings together with the results of the present investigation support a model in which the onset of tissue fibrosis in dystrophinopathy reflects elevations of TGF-β expression, but much of the progressive, chronic fibrosis is largely attributable to MBP-1 mediated processes.

The possibility that eosinophil degranulation causes lysis of host cells in vivo has been supported by in vitro observations that have shown the lytic capacity of proteins isolated from eosinophil granules, and by in vivo observations noting correlations between sites of eosinophil degranulation and the death of nearby cells (19,39). Interestingly, previous findings have also suggested that eosinophils can injure muscle fibers in vivo. Riedel’s invasive fibrous thyroiditis (IFT) involves extensive eosinophilia with the extracellular deposition of MBP that is expected to increase fibrosis in the thyroid gland. In addition, eosinophils invade skeletal muscle at sites adjacent to the thyroid in IFT, and those sites show extracellular accumulation of MBP and experience extensive muscle fiber degeneration (40). Similarly, MBP deposition in the myocardia of children with acute necrotizing myocarditis occurs at sites of extensive tissue damage (41). However, whether the deposition of MBP in the necrotic tissue is a cause or consequence of the damage has been contended, and some investigators have speculated that the deposition of MBP serves to promote repair, rather than cause damage (24). The results of the present investigation show that eosinophils can lyse muscle cells in vitro through MBP-1 mediated events, although approximately one-third of the target cell lysis is independent of MBP-1. Furthermore, eosinophil depletions cause a significant reduction of muscle fiber damage in mdx muscles in vivo. However, ablation of MBP-1 from mdx mice does not reduce lysis of muscle cells in the soleus muscle and increases muscle cell lysis in the diaphragm at 4-weeks of age, but not at 3-months. These observations show that MBP-1 does not cause measurable lysis of muscle cells in dystrophic muscle, even though degranulation occurs. Thus, the lysis of mdx muscle fibers that is prevented by eosinophil depletions with anti-CCR3 must occur through MBP-independent mechanisms. Our data also show that ablation of MBP-1 in mdx mice does not influence regeneration of muscle fibers following damage, using fiber central-nucleation as an index of regeneration. We conclude that MBP-1 does not serve an important role in the lysis or regeneration of dystrophin-deficient muscle in vivo.

Because of the growing evidence that eosinophils are not merely end-stage effector cells, but may play a role in modulating the immune response, and because of the important role of inflammatory cells in affecting the severity of pathology in muscular dystrophy, we explored whether perturbations of eosinophils would influence inflammation in mdx muscle. We were particularly stricken by the finding that depletion of eosinophils from dystrophic muscle greatly increased the concentration of CTLs. This finding agreed with a potential role for eosinophils in biasing the inflammatory response toward a Th2 phenotype, presumably through the release of Th2 cytokines such as IL-10 and IL-4. However, we were surprised to find that null mutation of MBP-1 produced a nearly identical increase in CTL numbers, suggesting that MBP itself may serve immunomodulatory roles. Lack of MBP-1 did not produce a general increase in Th1 inflammatory markers; instead the concentrations of mRNAs for iNOS, TNFα and IFNγ were not significantly affected by MBP-1 mutation. However, treatment of splenocytes with MBP decreased their proliferative response following PHA stimulation, which indicates a more direct and specific role of MBP-1 in regulating T-cell function. Previous investigations have shown that eosinophil cationic protein (ECP), another cationic protein in eosinophil granules, can also inhibit PHA-stimulated proliferation of lymphocytes (23), suggesting that the anti-proliferative activity of the two functionally and structurally similar proteins may reside in a shared motif.

These findings provide further evidence of the complex interactions that occur between CTLs and eosinophils in diseased tissue. Previous work has shown that the adoptive transfer of mdx splenocytes into wild-type mice induces eosinophilia in the muscles of the recipient mice, indicating that T-cells promote eosinophilia in muscular dystrophy (7). This effect of T-cells on eosinophilia has been demonstrated in other disease models. For example, mice that are infected with respiratory syncytial virus experience airway eosinophilia and bronchial hypersensitivity, but CTL depletion following infection prevents eosinophilia and the associated hypersensitivity from developing (42). Also, the adoptive transfer of splenocytes from mice with experimental autoimmune encephalomyopathy into healthy recipients induces eosinophilia in the recipient mice (43), further supporting a role for T-cells in promoting eosinophilia. Furthermore, null mutation of the CTL-specific, cytolytic protein perforin from mdx mice reduces eosinophilia (7). Thus, CTLs can promote eosinophilia in dystrophin-deficient muscles and this role is mediated by perforin. Together, these data support a model for CTL and eosinophil involvement in muscular dystrophy in which there is an initial, cellular immune response driven by an unidentified antigen that subsequently promotes eosinophilia. The invasive eosinophils then decrease CTL proliferation to attenuate the cellular immune response via an MBP-1 mediated mechanism. However, the persistent eosinphilia...
promotes tissue fibrosis that is directly or indirectly attributable to MBP-1 release. Further validation of this model would support developing therapeutic strategies for DMD that would target eosinophils and MBP, and thereby reduce cardiac and muscle fibrosis that can be life-threatening for DMD patients. However, such interventions may also present dangers, if they were to increase a cellular immune response to the dystrophin-deficient tissue, and thereby increase tissue damage.

**Methods**

**Mouse lines used in the investigation**

All animal experimentation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the University of California, Los Angeles Institutional Animal Care and Use Committee. Mice were maintained in an accredited animal care facility and examined daily for signs of distress, injury or disease. At the end of experimentation, prior to tissue collection, animals were sacrificed by intraperitoneal injection with an overdose of sodium pentobarbital, according to the Panel on Euthanasia of the American Veterinary Medical Association.

C57BL/6J mice (wild-type) and dystrophin-deficient, mdx mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). MBP-1 null mice were generated previously by targeted deletion of exons 2, 3 and 4 of MBP-1 in C57BL/6J blastocysts, and then bred onto a 129/SvJ background (44). The MBP-1 null mutants were then back-crossed for at least six generations onto a C57 background and used to generate MBP-1 null, dystrophin null (MBP-1−/−/mdx) mice using a previously described breeding scheme (1). Homozygous mutants for MBP-1 were identified by isolating genomic DNA from tail tissue that was then screened for the mutation by PCR using a common upstream primer (5'-ATG CCA CTG TGA GAC AGG AGG-3'), a wild-type downstream primer (5'-CAG ATG AAG AGG AGA CGC TC-3') and a downstream primer for the neomycin cassette (5'-GAA CCA GCT GGG GCT CGA G-3'). Null mutation for dystrophin was confirmed by mdx-amplification-resistant mutation system PCR (45). Mice were used at 1, 3, 14 or 18 months of age. The hindlimb musculature is at the early acute peak of pathology in 1-month-old animals but has then regenerated at 3 months of age (46). Diaphragm pathology is also apparent at 1 month of age, but it continues to progress throughout the life of the mdx mice (27). At 14 months of age, both limb and diaphragm muscles are in the chronic, progressive stage of pathology that involves extensive muscle fibrosis. At 18 months of age, mdx hearts are significantly fibrotic and display functional defects (29).

**Human tissue samples**

All human tissue samples were obtained from a referral muscle biopsy service (Children’s National Medical Center, University of Pittsburgh School of Medicine). Biopsies were obtained in accordance with the Institutional Review Boards requirements following informed consent.

**Antibody depletions of eosinophils**

A monoclonal antibody to CCR3 (mab 6S2-19-4; kindly provided by Dr D.L. Coffman, DNAX Corporation) was used for in vivo depletion of eosinophils. Mdx mice received daily, intraperitoneal injections of 100 μg of anti-CCR3 in sterile PBS (50 mM sodium phosphate buffer pH 7.2 containing 150 mM sodium chloride), beginning at 14 days of age and continuing until 28 days of age when the mice were euthanized and tissue was collected. Littermates were used as control mice, which received 100 μg of isotype control rat IgG in sterile PBS by intraperitoneal injections on the same schedule.

**Quantitative immunohistochemistry**

At the time of tissue collection, muscles that were to be used for microscopic examination were rapidly frozen in isopentane cooled with liquid nitrogen. Muscle cross-sections 10 μm thick were fixed in acetone and then immunolabeled for eosinophils (using rabbit polyclonal antiserum to MBP; 47), macrophages [using rat anti-mouse F4/80 purified from supernatant of rat anti-F4/80 hybridoma cultures (ATCC)], and CTLs [using rat anti-CD8 purified from hybridoma supernatant (ATCC)]. Sections probed with antibodies were processed as described previously (3) and immunoreactive cells were identified using a biotinylated mouse anti-rat IgG second antibody and horseradish peroxidase conjugated avidin before reaction with aminoethylcarbonyl (Vector). Because mast cells can also express CCR3, we also tested whether anti-CCR3 treatments affected mast cell numbers in muscles of eosinophil-depleted mice by staining tissue sections for mast cells according to the protocol of Gersch et al. (48).

The concentrations of specific cell populations in the muscle were expressed as the number of cells/volume of each section. The total volume of each section was determined by measuring the area of each section using a stereological, point-counting technique (2), and multiplying that value by section thickness (10 μm). One-way analysis of variance was used to test whether anti-CCR3 treatments affected mast cell numbers in muscles of eosinophil-depleted mice by staining tissue sections for mast cells according to the protocol of Gersch et al. (48).

**Electron microscopy**

Hindlimb muscles from 1- and 3-month-old mdx mice were fixed at physiological length using 1.4% glutaraldehyde in 0.2 M Na cacodylate pH 7.4 for 30 min. The soleus muscles were then dissected free from the hindlimb and fixed for an additional 30 min in glutaraldehyde. The muscles were sliced into small strips and placed in 1.0% osmium tetroxide for 60 min, after which they were rinsed in 0.2 M sodium cacodylate, dehydrated in a series of ethanol, followed by propylene oxide and then embedded in epoxy resin. Thin sections were cut at 70 nm, stained with uranyl acetate and lead citrate and viewed in a transmission electron microscope.
Assays of muscle membrane lesions in vivo

Muscle membrane lesions were assessed by measuring the concentration of muscle creatine kinase in the sera of mice. Blood was collected by cardiac puncture from euthanized animals, and creatine kinase concentration measured using a commercial kit (Sigma) according to the manufacturer’s directions.

Membrane lesions in soleus muscle fibers were assayed by measuring the relative concentration of the fluorescent, extracellular tracer dye, procion orange, in the cytoplasm of soleus fibers. Procion orange dye solutions normally remain in the extracellular space unless membrane lesions are present. Soleus muscles were collected from euthanized mice and then incubated in 0.5% procion orange dye solution in Krebs ringer solution for 1 h followed by washes with Krebs ringer solution. The muscles were then frozen in isopentane and cross-sections were cut at 10 μm thickness from the mid-belly of each muscle examined. Fiber membrane lesions were then assayed by counting the number of brightly fluorescent, injured fibers in each section of each muscle and expressing that value as a percentage of the total fibers that were present in the section.

Muscle membrane lesions were also assayed in diaphragm muscle fibers. However, we observed that diaphragm fiber membranes were frequently damaged during dissection before incubation in procion orange, giving artefactually high values for membrane damage. As an alternative procedure, mice were injected intraperitoneally with 5% Evans blue dye in 50 mM sodium phosphate buffer containing 150 mM sodium chloride (PBS), pH 7.5, to yield 100 mg Evans blue/kg body weight at 24 h before sacrifice and tissue collection. Evans blue dye is also a fluorescent, extracellular marker dye that permitted measurement of cytosolic fluorescence as an index of fiber damage. Fluorescence intensity of each, individual fiber in every muscle section was measured in an 8-μm diameter circle that was sampled at the center of each fiber using a digital imaging system (Bioquant, Nashville, TN, USA). The fluorescence intensity value for each fiber was attained below signal saturation levels and intensity was corrected for background levels, by measuring signal from an area of the slide that contained no tissue, and subtracting that background value from the cytosolic fluorescence measurements.

In vitro cytotoxicity assays

Eosinophils were isolated from peritoneal exudates of wild-type and MBP-1 null mice for use in cytotoxicity assays. Mice were injected intraperitoneally with 0.5 ml of 10 μg/ml of trypstat, 10 μg/ml of histamine in sterile PBS. The following day, peritoneal fluid was collected, cells were separated by centrifugation and red cells lysed in 0.85% ammonium chloride solution. Cells were preplated for 30 min at 37°C on plastic Petri dishes. Non-adherent cells were then placed on a discontinuous metrizamide gradient using metrizamide in Tyrode’s solution at pH 7.3 containing 0.03% Tyrode’s gel DNAse, 0.3 g/l Bloom’s gelatin, and 14 mg/l deoxyribonuclease-1, and centrifuged at 600 g for 40 min at 5°C. Cells were collected from the 20/22% metrizamide interface and then washed in Hanks balanced salt solution (HBSS). The proportion of the cell population that was eosinophils was assessed by Wrights stain of cytospin preparations of an aliquot of the isolated cells. The remaining cells were suspended in HBSS containing 0.25% fetal bovine serum before their use in cytotoxicity assays.

Muscle cell cultures for cytotoxicity assays used C2C12 myotubes as targets (49). Myotubes were incubated in HBSS containing 51Cr and 0.25% FBS for 2 h and then washed twice with HBSS before addition of eosinophils. Cytotoxicity assays were conducted for 18 h in the presence or absence of 1.0 μM PAF, to increase release of cytolytic granules (50). At the end of the co-culture period, the media was collected and the content of 51Cr in the media was determined by scintillation counting. Cytotoxicity was expressed as a percentage of total lysis by setting 0% as chromium released spontaneously by myotubes in the absence of eosinophils. 100% cytotoxicity was set at the chromium release by myotubes incubated with 0.1% Triton X-100 in the absence of eosinophils. Cell numbers were not expressed as effector-to-target ratios because target cell numbers cannot be determined in these preparations. During myoblast proliferation, differentiation and fusion to form myotubes, large syncytia are formed so that the number of separate myotubes is unknown. Instead, the numbers of target cells were normalized by the surface area of the culture dish to which they adhere and form a continuous monolayer.

Assays of fibrosis

Fibrosis of muscles was determined by assaying for changes in the concentration of hydroxyproline, using the colorimetric technique of Kivirikko et al. (51). Frozen sections of skeletal and cardiac muscle samples were also qualitatively assayed for fibrosis by immunohistochemistry following the techniques described above. Sections were incubated with primary antibodies for 3 h and with host-appropriate fluorescent-conjugated secondary antibodies (Vector) for 1 h. Primary antibodies used were rabbit anti-rat collagen type I (Chemicon, Temecula, CA, USA), rabbit anti-rat collagen type III (Chemicon), and goat anti-human collagen type IV (Southern Biotech, Birmingham, AL, USA).

Purification of MBP

MBP was purified using modifications of a previously described technique (52). Eosinophils (CRL-1964; ATCC) were grown in Roswell Park Memorial Institute (RPMI) media supplemented with 10% FBS. They were then collected by centrifugation at 600 g for 10 min, and then resuspended in 0.25 M sucrose. Cells were lysed by repeated trituration through a 18 gauge needle and unlysed cells removed by centrifugation. Eosinophil granules were then separated from the supernatant by centrifugation at 10 000 g for 10 min. Pelleted granules were resuspended in PBS and then centrifuged at 40 000 g for 15 min. The pellet was then extracted by repeated trituration in 0.01 M HCl, and insoluble material removed by centrifugation at 20 000 g for 10 min. MBP was then isolated by gel filtration chromatography using 0.025 Na acetate buffer pH 4.2 containing 150 mM NaCl (Superose
MBP effect on mitogen-stimulated splenocytes

Previous investigators demonstrated that ECP can inhibit proliferation of mitogen stimulated lymphoid cells (23). We assayed whether MBP could similarly affect lymphoid cell proliferation to test whether that function could contribute to changes in lymphoid cell populations in muscles of mdx mice that were null mutants for MBP or that were depleted of eosinophils. Splenocytes were isolated from adult C57 mice (2) and then cultured at $1 \times 10^6$ cells/ml in RPMI 1640 containing 10% fetal bovine serum and 50 $\mu$M $\beta$-mercaptoethanol in 24-well plates. PHA was added to some wells at $2.5 \mu$g/ml to stimulate proliferation. MBP was added to PHA-stimulated and non-stimulated (control) cultures at $10^{-3}$, $10^{-2}$ or $10^{-1}$ $\mu$M, and the cells were then incubated for 24 h at $37^\circ$C in a 5% CO$_2$ incubator. Five $\mu$Ci of $^3$H-thymidine were then added to each well, followed by additional incubation for 18 h at $37^\circ$C. Cells were then collected by centrifugation, rinsed three times in PBS and then radioactivity was assayed with a scintillation counter as a measure of cell proliferation.

Relative levels of transcripts for selected Th1 and Th2 cytokines

The relative transcript levels for selected cytokines were assayed by quantitative, real-time PCR to test whether null mutation of MBP-1 produced an increase in Th1 cytokines in mdx muscle. Frozen muscles were homogenized with a mortar and pestle while partially submerged in liquid nitrogen. One milliliter of Trizol (Invitrogen) per 50 mg of tissue was used to quantitatively measure the expression levels of iNOS, IFN$\gamma$, and TNF$\alpha$. Relative transcript levels for selected cytokines were assayed by determining 28S and 18S ribosomal RNA integrity. Additional incubation for 18 h at 37°C was required to be able to measure gene expression using real-time PCR (Table 1).

Table 1. Real-time PCR primers

<table>
<thead>
<tr>
<th>Gene (accession no.)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS (NM_010927)</td>
<td>5'-CTCATGGGCTCTGAG-3'</td>
<td>5'-CGTACAGCCTATGAG-3'</td>
<td>124 bp</td>
</tr>
<tr>
<td>IFN$\gamma$ (NM_008337)</td>
<td>5'-GACACAGGGCATCAC-3'</td>
<td>5'-CAGATGAGCTTGGAG-CCTT-3'</td>
<td>161 bp</td>
</tr>
<tr>
<td>TNF$\alpha$ (NM_013693)</td>
<td>5'-CTCTGCTGCTACGATCGG-3'</td>
<td>5'-CATTTGGTTTGTAGAC-3'</td>
<td>163 bp</td>
</tr>
</tbody>
</table>

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REFERENCES

characterization of pathways available to eosinophils for generating reactive nitrogen species. J. Immunol., 166, 5763–5772.


