Overexpression of a calpastatin transgene in \textit{mdx} muscle reduces dystrophic pathology

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Reduced sarcolemmal integrity in dystrophin-deficient muscles of \textit{mdx} mice and Duchenne muscular dystrophy (DMD) patients has been reported to result in altered calcium homeostasis. Previous studies have shown a correlative relationship between calcium-dependent protease (calpain) activity in dystrophic muscle and muscle necrosis, but have not tested whether calpain activation precedes cell death or is a consequence of it. To test a causal relationship between calpain activation and muscle cell death in dystrophin deficiency, \textit{mdx} mice were generated that overexpress a calpastatin transgene in muscle. Calpastatin (CS) is a specific, endogenous inhibitor of \textit{m-} and \textit{\mu-}calpains that does not inhibit calpain 3 (p94). CS overexpression on a C57/BL10 background produced no phenotype. Transgenic (Tg) mice crossed with \textit{mdx} mice were tested for pathological indicators of necrosis, regeneration and membrane damage. Two lines of mice were examined, with different levels of CS overexpression. Both lines of Tg/\textit{mdx} mice showed reductions in muscle necrosis at 4 weeks of age. These mice had fewer as well as smaller lesions. In addition, one line of mice had significantly less regeneration, indicating a reduction in previous necrosis. The extent of improvement correlated with the level of CS protein expression. Membrane damage, as assessed by procion orange and creatine kinase assays, was unchanged, supporting the idea that calpains act downstream of the primary muscle defect. These data suggest that calpains play an active role in necrotic processes in dystrophic muscle and that inhibition of calpains might provide a good therapeutic option for treatment of DMD.

**INTRODUCTION**

Duchenne muscular dystrophy (DMD) results from mutations in dystrophin, a cytoskeletal, spectrin-like protein that is present at the inner surface of the muscle cell membrane (1). Human patients with DMD and \textit{mdx} mice lacking dystrophin experience progressive muscle cell death characterized by necrosis and regeneration. Dystrophin associates with a large complex of membrane proteins, called the dystrophin glycoprotein complex (DGC), that is believed to be important in cell membrane integrity (2). Since dystrophin binds to actin filaments through its N terminus and to the dystrophin complex through its C terminus, it is an important component of a continuous chain of proteins linking the extracellular matrix (3) to the cytoskeleton, and is believed to provide structural stability to the plasma membrane. Indeed, dystrophin-deficient membranes have been shown to be leaky to extracellular marker dyes (4,5), have been shown to be more susceptible to osmotic stress (6), and have reduced stiffness (7). The compromised plasma membrane integrity is hypothesized to result in an increased efflux of intracellular molecules such as creatine kinase and pyruvate kinase to the systemic circulation.

The compromised plasma membrane of dystrophic muscle could also lead to an influx of ions into the cell. Dystrophic membranes have been shown to have an impaired ability to exclude low-molecular-weight dyes such as Evans blue and procion orange from the bloodstream (5,8). Furthermore, several studies have reported elevated calcium content in dystrophic muscle (9–11) and elevated free calcium in DMD (12,13) and \textit{mdx} myotubes (14–17). In addition, abnormal channel activity has been observed in dystrophin-deficient myotubes (18,19). Whether free calcium is elevated in dystrophic myofibers is still the subject of controversy, and has been difficult to assess due to technical limitations of measuring free calcium in mature muscle cells (20–23). However, while all available data support altered cell membrane integrity as a component of the pathological progression of DMD and \textit{mdx} dystrophies, downstream effector mechanisms have not been elucidated.

Reports of increased cellular free calcium have led to the hypothesis that a cascade of autophosphorylation leads to muscle

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cell death by activation of calcium-dependent proteases (calpains) (24,25). Calpains are a family of cytosolic, non-lysosomal, cysteine proteases defined by their protease domain, which has high homology to the papain protease family (26). Skeletal muscle primarily expresses three isoforms of calpain called \( \mu \)-calpain (calpain 1), \( m \)-calpain (calpain 2) and \( p94 \) (calpain 3)(27). Several other ubiquitous isoforms have been described in the database, including calpains 6, 7 and 10 (26), although their protein products have not been identified in muscle.

The presence of elevated calcium in dystrophic muscle suggests the hypothesis that calpains might be more active and unregulated, and may be responsible for initiation of the proteolytic cascade leading to muscle cell death (28). Support for this hypothesis comes from studies in vitro using \( mdx \) myotubes that have demonstrated that elevations in extra-cellular calcium result in increased proteolysis of a calpain substrate (17). Furthermore, studies using leupeptin, a cysteine and serine protease inhibitor, to treat \( mdx \) mice by intramuscular injection showed a reduction in muscle degeneration as assessed by a decrease in centrally nucleated fibers (29). While these findings are generally supportive for a role for calpains in muscular dystrophy, the non-specificity of leupeptin and the 60 intramuscular injections performed over the 30-day time period for this study make interpretation of the results difficult. Needle damage from injection will cause both necrosis and regeneration of muscle, and could therefore confound the data. However, further support for the ‘calpain hypothesis’ has come from examination of whole-muscle extracts that showed an increase in expression of m-calpain in \( mdx \) mice (30,31) and DMD (32). While the latter data are generally supportive for a role for calpains in \( mdx \) dystrophy, they are only correlative and do not experimentally address the question.

Previously, attempts have been made to determine calpain activity in muscle extracts. Calpain assays of whole-muscle extracts are not indicative of \( in vivo \) activity, because of the presence of calpastatin (CS), the specific inhibitor of \( m \)- and \( \mu \)-calpains. Furthermore, measurement of calpain independent of CS does not accurately reflect \( in vivo \) conditions, because it is the ratio of calpain to CS that ultimately determines calpain activity. To overcome these obstacles, examination of the calpain propeptidase was used to assess activation of the calpains \( in vivo \) (25). Cleavage of its propeptide accompanies activation of the protease (33), so that analysis of autoproteolytic cleavage of propeptide provides a good indication of \( in vivo \) activity. This analysis showed that the ratio of cleaved propeptide to intact \( \mu \)-calpain was higher in 4-week \( mdx \) mice than in controls, while \( m \)-calpain propeptidase cleavage remained unchanged, suggesting that \( \mu \)-calpain is specifically activated during the dystrophic process (25). Further analysis demonstrated that calpains have an altered distribution in the cell during muscle necrosis and regeneration of \( mdx \) myofibers (34), which is consistent with altered activation in these muscles. While these studies showed a positive correlation between \( in vivo \) calpain activity and an altered distribution in dystrophic tissues, they did not unequivocally implicate calpains in the process or test whether calpain activation precedes, or is a consequence of, muscle cell death.

To test a causal relationship between calpain activity and the generation of \( mdx \) pathology, we have generated transgenic (Tg) \( mdx \) mice that overexpress CS in muscle. CS has been shown to specifically inhibit \( m \)- and \( \mu \)-calpains, but not to inhibit \( p94 \) (calpain 3) (35). If the conventional calpains play an important role in \( mdx \) pathology, then their inhibition from birth is predicted to reduce cell death in dystrophic muscle. In this study, we have found that high levels of CS can ameliorate dystrophic symptoms. However, this study also showed that calpain inhibition does not repair the primary membrane defect that is common to dystrophic muscles. These data suggest that inhibition of calpain is a potentially promising treatment for DMD, especially if accompanied by therapies directed at repairing membrane damage, such as gene replacement for dystrophin.

RESULTS

Overexpression of CS from a muscle transgene reduces \( m \)- and \( \mu \)-calpain activity

Two lines of Tg mice were analyzed for skeletal muscle-specific transgene activity. Both lines 74.1 and 69.7 showed high levels of CS protein by immunoblot analysis (Fig. 1B). In addition, zymograms were performed using casein as a substrate to examine overall activity in whole-muscle extracts. Normal skeletal muscle produces two bands on a zymogram where \( m \)- and \( \mu \)-calpains migrate in the gel (Fig. 1B: right panel). CS Tg mice showed a strong reduction in the activity of \( m \)- and \( \mu \)-calpains in the zymogram (Fig. 1B). Line 74.1, the line with the highest expression of CS by immunoblotting, showed complete suppression of both calpains, whereas line 69.7 had both lower expression levels of CS by western blot, and reduced calpain suppression in the zymogram. Thus, expression levels of CS correlated with suppression of calpain in the zymogram. The expression of calpains 1, 2 and 3 was not altered by the CS transgene (Fig. 1C) suggesting that suppression of activity in the zymogram is due solely to the overexpressed CS and not to downregulation of calpain. Whether this result is due to CS remaining complexed with calpain after homogenization of the tissue or to co-migration of CS with calpain in the gel is unclear.

CS assays of whole-muscle extracts from 4-week 74.1 Tg/\( mdx \) mice (Fig. 1D) showed that Tg muscle contained 2165 units/g muscle compared with age-matched controls that contained 6.3 units/g. Thus, a 340-fold increase in CS units was observed in 74.1 Tg/\( mdx \) muscles, and this level of CS resulted in complete suppression of \( m \)- and \( \mu \)-calpain activity in the zymogram. Figure 1D also shows that transgene activity greatly diminished as the mice aged. By 6 months of age, the transgene was expressed at 50–75% of the 4-week level. The 69.7 line had 10% the number of calpastatin units compared with the 74.1 line (data not shown), and this amount of activity was not sufficient to completely suppress calpain activity in the zymogram (as observed in Fig. 1B). By 6 months of age, the activity of the 69.7 transgene was only 2-fold greater than non-Tg (NTG) controls. Thus, the increase in activity units scaled with both the expression levels on the western blot and suppression of calpain in the zymogram. The decrease in
Figure 1. CS transgene expression suppresses μ- and m-calpain activity as measured by zymogram. (A) Diagram of transgene injected to make Tg mice. The vp1 intron was placed between the promoter and cDNA, containing splice donor and acceptor sites. The SV40 poly(A) signal followed the cDNA. (B) The left panel shows an immunoblot of CS Tg/mdx TA muscles probed with anti-calpastatin. All lanes contained 25 μg of total muscle extract. Tissue CS is ~105 kDa. The right panel shows a casein zymogram of whole-muscle extracts from CS Tg/mdx muscles stained with Coomassie blue. Calpain isoforms are indicated at the right. Tg lines are indicated at the top. Almost-complete suppression of both calpain isoforms is observed in line, 74.1, while line 69.7 shows complete suppression of μ-calpain only. (C) Immunoblot of whole-muscle extracts from CS Tg/mdx and NTG/mdx mice blotted for all calpain isoforms. No difference in m-calpain, μ-calpain or p94 concentrations were observed. (D) Graph of CS activity of whole-muscle extracts. There is a >300-fold increase in CS units in line 74.1. Four animals were analyzed per group. Line 69.7 (not shown) showed an ~30-fold increase in activity. Both lines showed a dramatic reduction in transgene activity with age.
transgene activity with age was observed on both the mdx and C57/BL10 backgrounds.

CS overexpression is not toxic to skeletal muscle

CS Tg mice looked phenotypically normal on both the C57/BL10 and mdx backgrounds. Cross-sections of Tg muscles were analyzed for toxic effects of the transgene by examining for morphological abnormalities in muscle sections of CS Tg/ C57/BL10 mice. Muscles from these mice looked morphologically normal (Fig. 2A and B). Furthermore, CS Tg/mdx mice did not have significantly different body weights than age-matched NTG/mdx mice (Fig. 2C).

Muscle necrosis is reduced in 4 week CS Tg/mdx muscles

The effect of calpain suppression on the prevalence of muscle necrosis in dystrophin deficiency was assessed after crossing CS Tg mice with mdx mice. These mice lack dystrophin, like human DMD patients, and undergo an episode of necrosis at 3–4 weeks of age (36). After this time point, the mice have a low incidence of necrosis and regeneration until around 15 months of age, when they become extremely weak and die prior to their wild-type counterparts (36,37). This early death is presumed to result from a lack of regenerative ability combined with the continual loss of muscle tissue (36,37). We chose to examine the 4-week time point because it is when the first and most significant episode of degeneration and regeneration occurs in mdx muscles. Subsequent to this time point, the muscles look morphologically stable and only low levels of necrosis and regeneration are evident. Thus, at subsequent time points, normal regenerative processes in the muscle can confound the interpretation of a given treatment. In addition, the loss of transgene activity with age would complicate the interpretation of data in older mice.

Muscle necrosis in 28-day CS Tg/mdx mice was examined after staining the tissue for CD11b (Mac1), an integrin expressed on infiltrating immune cells. Mac1 allows for the demarcation of lesions in mdx muscles, because mdx muscle lesions become invaded by immune cells (38). NTG/mdx littermates were used as controls. Following Mac1 staining, the sections were evaluated by a blinded observer and scored for percentage necrosis, lesion size and invaded fibers (39).

In the first analysis, a blinded observer analyzed the percent of the cross-sectional area that was necrotic. This analysis showed that a significant reduction in the extent of necrosis was observed in both lines of Tg/mdx mice compared with age-matched controls in the quadriceps muscle (Fig. 3A–E). In the triceps, only line 74.1 showed a significant improvement in pathology (Fig. 3E). Secondly, lesion size was estimated based on the number of fibers that were replaced by necrotic or macrophage-invaded tissue (Fig. 3F). A marked difference in lesion size was observed in the Tg/mdx mice. While the NTG/mdx mice had extremely large lesions that occupied as many as 10 fibers, lesions in Tg/mdx mice mainly involved one or two fibers (Fig. 3F). Finally, the total number of necrotic and immune cell-invaded fibers was assessed in cross-sections of Tg/mdx and NTG/mdx quadriceps muscle. This analysis showed a significant reduction in the number of invaded fibers in both lines of Tg/mdx mice (Fig. 3G). Therefore, these data suggest that suppression of calpain activity is sufficient to reduce the extent of necrosis in mdx mice. They also suggest that very high levels of suppression are beneficial, since a greater improvement in pathology was observed in the line with the highest calpastatin expression (line 74.1).

Regenerative fibers are reduced in 4-week CS Tg/mdx muscles

At 3–4 weeks of age, mdx mouse muscles undergo the first, and most pronounced, cycle of degeneration/regeneration (36). While degenerating areas can be identified by immune cell infiltration, staining for embryonic proteins that are re-expressed in the developing myotube can be used to identify regeneration. Neural cell adhesion molecule (NCAM) is one protein that is highly expressed in development, but is restricted to the neuromuscular junction in mature, fully developed fibers. Thus, staining for NCAM reveals newly regenerating myofibers and is a good marker of regeneration. Regeneration was assessed because it is an indicator of damage previously experienced by the muscle. Since the first evidence of histological necrosis is observed starting at 3 weeks of age, only a small fraction of the muscle will have experienced any necrosis/regeneration at the 4-week time point analyzed.

NCAM analysis was carried out by quantitating the total number of centrally nucleated, NCAM-positive fibers in a cross-section (at the midbelly), and by normalizing for the total cross-sectional area. Both the triceps and quadriceps muscles were analyzed in two different Tg lines by a blinded observer. This analysis showed a significant reduction in the number of NCAM-positive fibers in CS Tg/mdx versus NTG/mdx in both triceps (36%) and quadriceps (27%) in line 74.1 (Fig. 4). Line 69.7 showed an overall reduction in NCAM, but these data were not statistically significant due to high variability (Fig. 4). Since this line expresses only 10% the amount of calpastatin as line 74.1, the data suggest that very high levels of calpastatin activity are necessary to significantly improve the early onset of necrosis in mdx muscle. Thus, suppression of calpain activity reduced both necrosis and the number of actively regenerating fibers in mdx muscle expressing extremely high levels of calpastatin.

Muscle membrane damage is not reduced by overexpression of calpastatin

mdx muscle membranes have an increased uptake of extracellular marker dyes such as Evans blue and procion orange, and an increased loss of cytoplasmic enzymes such as creatine kinase (CK) to the serum (40). Furthermore, calpains have been shown to associate with the plasma membrane in platelets (41) and cardiac tissue (42) in response to increases in intracellular calcium. Thus, it is reasonable to assume that the increased subsarcolemmal calcium coupled with increased membrane-localized calpain might contribute to further membrane damage in mdx muscle. To test whether calpain inhibition improves membrane damage in mdx mice, procion orange uptake into muscle fibers was examined to assess membrane integrity. Procion orange uptake did not differ significantly between the three groups (Fig. 5A, B and C). In addition, creatine kinase efflux was measured in Tg/mdx and NTG/mdx serum (Fig. 5D).
Again, no significant difference was observed between the groups, indicating that overexpression of a CS transgene does not help repair muscle membrane damage in mdx muscles. Thus, the primary defect caused by the dystrophin deficiency is not exacerbated by elevated calpain activity in mdx muscle. Furthermore, suppression of calpains does not improve membrane damage in dystrophic mice.

DISCUSSION

The pathogenesis of DMD has long been speculated to involve the activation of calcium-dependent proteases as a result of calcium dysregulation at the cell membrane and increases in cellular free calcium; however, this hypothesis had not been adequately tested (9,11,43). Previous studies have shown correlations between elevated calpain concentration and dystrophy, or correlations between elevated N-terminal auto-lysis of calpain and dystrophy (25,30). While these data are supportive of the ‘calpain hypothesis’, testing experimentally whether calpains were active participants in promoting the dystrophic process or whether they were activated as bystanders following increases in calcium during cell death had not been attempted. The data presented here are the first to experimentally test this hypothesis in vivo, by using a CS transgene to suppress calpain activity in mdx mice from birth, prior to the first necrotic episode. The data show that suppression of calpain activity is sufficient to reduce the histologically discernable features of dystrophin deficiency.

It has previously been speculated that dystrophic muscle cell death is initiated by increases in intracellular calcium, although the downstream effectors that lead to cell death have not been identified. Our data suggest that unregulated activation of calpains is one consequence of the elevated intracellular calcium. The normal physiological role of calpains is unknown; however, previous studies have demonstrated that they may regulate cytoskeletal remodeling in platelets (41), cardiac muscle (44) and brain (45). Calpains may also play a role in remodeling the cytoskeleton of skeletal muscle, and may be responsible for as much as 60% of normal muscle cell protein degradation in vitro (46). In pathological conditions such as muscular dystrophy where there is widespread proteolysis, this figure is likely to be higher. This observed degradation is not likely to be solely due to calpains, since these proteases only degrade substrates in a limited fashion. Most likely, activation of calpains and cleavage of substrate is the first in a series of proteolytic degradation steps, involving other cellular proteases, that ultimately results in death of the cell. A candidate downstream proteolytic system that could potentially be involved in mdx dystrophy is the ubiquitin–proteasome system (47). We speculate that proteins that are succinctly cleaved by calpain are targetted by ubiquitination and degraded in the proteasome. At this time, the role of the proteasome in mdx dystrophy has not been addressed experimentally.

The speculation that calpain activation results in unregulated, generalized proteolysis is just one mechanism to explain the role of calpains in mdx dystrophy. Another hypothesis to explain calpain involvement is that a more specific, unregulated,
Figure 3. CS transgene reduces muscle necrosis in mdx mice at 4 weeks of age. (A)-(D) Micrographs of cross-sections of NTG/mdx (A and B) and CS Tg/mdx (C and D) quadriceps muscles at 4 weeks of age (line 74.1). (A) and (C) were photographed with a 20× objective, and (B) and (D) with a 40× objective. Sections were stained with Mac1 (red) and counterstained with hematoxylin (blue). Dark blue-stained structures are nuclei, light blue-stained areas are cytosol. (E)-(G) Quantitation of the data assessing necrosis. (E) Graph showing percent necrosis in Tg/mdx and NTG/mdx mice for triceps and quadriceps. For the triceps, ten 74.1/mdx, seven 69.7/mdx and ten NTG/mdx mice were analyzed, and for quadriceps, thirteen 74.1/mdx, eleven 69.7/mdx and seven NTG/mdx mice were analyzed. (F) Graph showing the distribution of lesion size in line 74.1/mdx (black diamonds) and NTG/mdx (solid yellow) quadriceps. This analysis was performed on six 74.1/mdx and six NTG/mdx quadriceps. (G) Graph showing the number of necrotic or invaded fibers in a cross-section of Tg/mdx and NTG/mdx quadriceps. For this analysis, only the quadriceps was utilized, and nine 74.1/mdx eleven 69.7/mdx and seven NTG/mdx mice were analyzed. *P < 0.05 indicates statistical significance by t-test. Vertical bars indicate standard error.
Calpain-mediated proteolysis may be occurring that disrupts normal cellular processes and causes ultimate failure of the muscle cell. For example, many different transcription factors in muscle have been shown to be substrates of calpains, including c-Fos and c-Jun (48). Unregulated degradation of either of these transcription factors could cause a fatal reduction in muscle gene expression, or satellite cell proliferation, and result in cell death. Calpain has also been shown to regulate IκB degradation, which indirectly affects the ability of the transcription factor NFκB to travel to the nucleus (49). Loss of nuclear NFκB could have an impact on cell survival, since NFκB has been shown to be protective against apoptosis, one of the mechanisms by which dystrophic muscle cells die (50). Lastly, calpain has been shown to cleave YY1, a transcription factor that negatively regulates dystrophin transcription during myogenesis (51). Constitutive degradation of YY1 might promote transcription of embryonic forms of normal muscle genes that are less capable of force generation, and might result in muscle cell failure.

Recent reports have suggested that the slow leakage of calcium across the impaired plasma membrane of mdx muscle might activate a calcium-dependent protease that subsequently cleaves a calcium leak channel, leading to further increases in cellular calcium (17,52). Support for this hypothesis comes from studies using patch-clamped mdx membranes and subsequently measuring leak channel activity. Leupeptin, a serine and cysteine protease inhibitor, prevented the increase in leak channel activity (52). Lowered free calcium was also observed in mdx myotubes after incubation with the calpain inhibitor calpeptin after hypo-osmotic shock (12). These studies taken together suggest that calpain may be specifically involved in the regulation of a calcium channel, thus exacerbating the levels of intracellular free calcium and contributing to further cell damage. If this is calpain’s role in the pathological process, then the downstream effectors that are being activated by calcium, and are ultimately responsible for dystrophic muscle cell death, remain unidentified.

While calpains are apparently one component of the pathophysiological progression of mdx dystrophy, they certainly cannot be the only mechanism contributing to cell death in dystrophic muscle, since the loss of dystrophin from the cell membrane impacts many aspects of cell homeostasis. This list includes processes such as calcium overload of the mitochondria and free-radical generation resulting in increased lipid peroxidation in prenecrotic mdx muscle (53) and increased susceptibility to oxidative stress in mdx cells in vitro (54). Equally important is the loss of the dystrophin-associated proteins (2), loss of connection to the extracellular matrix (3) and loss of signalling through this complex (55). In addition, loss of nitric oxide synthase results in loss of protection against muscle inflammation and damage (8) and a loss of vasodilatory response to α-adrenergic stimulation (56). Finally, apoptotic cell death contributes a minor amount of cell death, independent of calpain activation (38,50).

The identification of calpains as components of the pathophysiological progression of mdx dystrophy leads to a new avenue of therapeutics for DMD. Albuterol is one drug that can increase muscle calpastatin, and may prove valuable as a therapy for DMD. This drug is a β2-adrenergic agonist,
normally used to treat asthma because of its bronchodilatory effects. Muscle also has β2 receptors, and it has been previously shown that β2-adrenergic agonists increase muscle CS, which results in increases in muscle mass and strength in healthy animals and humans (57). Furthermore, treatment of mdx mice with clenbuterol, a long-acting β2-adrenergic agonist, has also been shown to improve muscle mass and strength (58–60). Clinical trials are now ongoing to test whether DMD patients will also benefit from albuterol therapy. As more specific inhibitors of calpains are being developed, the potential to treat human patients with pharmacological agents to target calpains becomes more promising. Most likely, the earlier the intervention, the more that patients will benefit. The primary obstacle to treating DMD patients with calpain inhibitors pharmacologically is the diverse number of physiological processes that involve calpains. For example, calpains play an important role in platelet aggregation and the immune defense against pathogens. Inhibition of either of these processes might be deleterious for the patient; thus, it will be important to develop drugs with tissue specificity if calpain inhibition and suppression of dystrophy is to be accomplished successfully.

**MATERIALS AND METHODS**

**Generation of CS transgenics**

The full-length human CS gene (kindly donated by Dr Masatoshi Maki) was subcloned behind the human skeletal actin promoter generously supplied by Dr Jeffrey Chamberlain (Fig. 1A) (61). This promoter was first described in 1987 (62), and was characterized in a transgenic mouse by Brennan et al. (63). After removal of most vector sequences, the linearized, gel-purified DNA was microinjected into F2 hybrid zygotes from C57BL/6J × Balb/c matings at the University of California, Irvine transgenic mouse facility. Founders were identified by PCR and mated to mdx mice. Only males from the N1 generation were analyzed. All comparisons were made with age-matched, male, dystrophin-deficient littermates that were non-transgenic (NTG). Calpastatin protein levels were verified by immunoblotting for all mice used for analysis.

**Animals**

CS Tg/mdx and non-Tg (NTG/mdx) littermate controls were analyzed at 28 days of age, the first peak of disease activity. Mice were housed in the Life Science vivarium at UCLA, and all methods and procedures were carried out according to the protocol approved by the Animal Research Committee.

**PCR screening**

PCR of tail-chop DNA was used to identify Tg mice (38) using primers in the 3′ end of the HSA promoter (5′-ccc gag ccc aga gta gca gtt-3′) and the vpl intron (5′-ccc ttc cct gtt ggc tac t-3′) (61).
Tissue preparation and immunohistochemistry

Tissue preparation and immunohistochemistry were performed as described previously (64). All tissues were frozen-sectioned at 10 μm thickness and stored at −20°C until used. Staining was performed using either rat anti-NCAM (Chemicon; mAb 310, 1:100) or rat anti-Mac 1 (CD11b, Pharmingen, 1:100) following acetone fixation.

Immunoblots

Immunoblotting was performed as previously described (25). Blots were probed with anti-calpastatin (Takara) or anti-total calpain (clone 11B3, Novacastra) for 1 h at room temperature. Secondary antibody was HRP-labeled anti-mouse (1:5000) from Amersham. Reactions were carried out using an enhanced chemiluminescent (ECL kit, Amersham) and exposed to autoradiographic film.

Casein zymography

Zymograms were performed according to the protocol of Croall et al. (65). Gels were overrun for 1 h at 80 V to increase separation of the isoforms. Five hundred micrograms of whole-muscle lysate was loaded per lane of the gel, and gels were incubated overnight in 5 mM CaCl₂.

Calpastatin assays

CS was assayed by its ability to inhibit calpain-generated trichloroacetic acid-soluble peptides from 14C-methylated casein as previously described (66), with minor modifications. Frozen 50–250 mg muscle samples were quickly minced with a scalpel blade, and immediately diluted in 2.5 volumes of ice-cold homogenization buffer: 50 mM imidazole–HCl, 5 mM EGTA, 100 mM NaCl, 0.5% Triton X-100 and 1 mM dithi- toreitol, pH 7.4 measured at 23°C. The samples were homogenized on ice for 20 strokes in an ~2 ml frosted glass homogenizer, and immediately heated to 95°C for 10 min. The heated samples were re-homogenized, and centrifuged at 10 000 g for 10 min to obtain clarified supernatants containing CS. Aliquots of the heat-treated supernatants were included in the standard calpain caseinolytic assay, utilizing 10 nM purified human erythrocyte μ-calcin, and 0.5 mg/ml 14C-methylated casein as the substrate. One unit of CS produces 50% inhibition of calpain.

Assessment of muscle membrane damage

Assessment of muscle membrane damage was assessed by procion orange assays of the soleus muscle and analysis of serum creatine kinase as previously described (8). Data were analyzed by Student’s t-test with P < 0.05 considered statistically significant.

Assessment of muscle regeneration

Cross-sections of quadriceps and triceps from Tg/mdx and NTG/mdx mice were stained for NCAM, a protein expressed highly in regenerating muscle fibers. A blinded observer assessed two sections from each mouse. The entire cross-section, taken at midbelly, was analyzed for each muscle. The total number of NCAM-positive fibers was assessed. Data were normalized by cross-sectional area, determined using an eyepiece micrometer (Olympus). Two sections per animal were analyzed, and significance of differences was assessed by Student’s t-test, with P < 0.05 considered statistically significant.

Assessment of muscle necrosis

Cross-sections taken from the midbelly of quadriceps and triceps from Tg/mdx and NTG/mdx mice were stained for CD11b, a marker of inflammatory cells, because lesions from mdx mice contain high numbers of inflammatory cells (39). For each sample, a blinded observer made three different measurements. First, the entire cross-section of both quadriceps and triceps muscles were examined using a 10× objective and scored for the percentage of the total cross-section that was necrotic. Significance of differences was assessed by Student’s t-test, with P < 0.05 considered statistically significant. Secondly, the size of the lesions was considered. The number of fibers considered necrotic or immune cell-invaded was scored. The lesions were put into the following categories: (1) 1 cell/lesion; (2) 2–5 cells/lesion; (3) 6–10 cells/lesion; (4) >10 cells/lesion. Lastly, the number of necrotic or immune-cell invaded cells was quantified per cross-section. For this analysis, only the quadriceps was utilized. Significance of differences was assessed by Student’s t-test, with P < 0.05 considered statistically significant.

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