Cromolyn administration (to block mast cell degranulation) reduces necrosis of dystrophic muscle in mdx mice

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Introduction

Duchenne muscular dystrophy (DMD) is an X-linked lethal muscle disorder, resulting from mutations in the gene encoding for the skeletal muscle protein dystrophin. The absence of functional dystrophin leaves the muscle membrane vulnerable to damage during contraction. Damage initially occurs as ‘tears’ in the membrane, this damage can be exacerbated by the inflammatory response leading to myofibre necrosis rather than repair. Mast cells resident within skeletal muscle represent an immediate source of pro-inflammatory cytokines. We hypothesise that blockade of mast cell degranulation would reduce the extent of myofibre necrosis in the mdx mouse. Daily cromolyn injections were performed on young and exercised adult mdx mice and histological analysis confirmed that mast cell degranulation contributes to myofibre necrosis. This research identified high biological variation between individual mdx mice in the severity of the dystrophic pathology, and supported a relationship between extent of muscle damage in adult mdx mice and their individual enthusiasm for voluntary wheel running.

Keywords: DMD; mdx; Mast cell; Cromolyn; Myofibre necrosis; Voluntary exercise

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inflammation (Porter et al., 2002, 2003), this highlights the extent of inflammation in mdx mice and supports our hypothesis that inflammation exacerbates damage of dystrophic skeletal muscle.

Tumour necrosis factor alpha (TNFα), a potent pro-inflammatory cytokine, is a chemoattractant for leukocytes and induces further inflammation by increasing the activity of other pro-inflammatory cytokines (Beutler and Cerami, 1988). Antibody (Remicade®) blockade of TNFα activity in young mdx mice showed a striking protective effect on dystrophic myofibres, and prevented the early acute phase of myofibre necrosis (Grounds and Torrisi, 2004). A similar protective effect in dystrophic muscle was demonstrated using Enbrel (a soluble TNFα receptor) (Hodgetts et al., unpublished results) strongly supporting a key role for TNFα and inflammation in the dystropathology of mdx mice. Resident mast cells are an immediate local source of TNFα (Gordon and Galli, 1990) and degranulation of mast cells facilitates one of the earliest responses of the innate immune system that result in a cascade of inflammatory events.

Mast cells initiate many immunoregulatory functions via the release of preformed and newly synthesised mediators (Mekori and Metcalfe, 2000). Of particular interest are the pro-inflammatory cytokines including TNFα, interleukins (IL-1, IL-6), histamine and proteases such as chymase (Wedemeyer et al., 2000). Mast cell proteases are also associated with vasoconstriction and fibrosis; two factors that compound the severity of DMD (McDonald et al., 2001; Takai et al., 2004). Activation (degranulation) of mast cells occurs in response to mechanical trauma, temperature, chemical agents, UV rays, anaphylactic toxins, calcium and basic compounds, in addition to hypersensitivity conditions (reviewed in Maurer et al., 2003). In dystrophic skeletal muscle, mast cells are activated, presumably by mechanical myofibre damage, to rapidly degranulate and release pro-inflammatory cytokines. Leukocytes (neutrophils) accumulate rapidly in response to damage and, along with the damaged myofibre, produce and release TNFα (Collins and Grounds, 2001). Further infiltration of mast cells, neutrophils and other leukocytes occurs approximately 8 h after injury (Gorospe et al., 1996) and macrophages are the major leukocyte present between 24 and 48 h after injury (Fig. 1).

Sarcolemmal lesions initiate calcium leakage and inflammatory mediator release from the damaged tissue; both of which are chemotactic for mast cells (Lefaucheur et al., 1996) causing them to accumulate at high density in dystrophic muscle tissue. More mast cells are present (up to 3-fold) in the muscles of mdx mice and DMD patients, in comparison to age matched controls. This increase in mast cell number is specific to dystrophin-deficient myopathies and not found in other inflammatory myopathies (Gorospe et al., 1996). Increased numbers of mast cells are found in dystrophic tissue from as early as 17 days in the mdx mouse (Lefaucheur et al., 1996) and are localised to areas of myofibre necrosis (Gorospe et al., 1994b; Nahirney et al., 1997).

A contribution of mast cells to the dystrophic pathology was proposed from studies in adult mice where intramuscular injections of purified mast cell granules (Gorospe et al., 1994a) or Compound 48/80 (a mast cell secretagogue) (Granchelli et al., 1994) induced widespread necrosis in mdx mice, in striking contrast with muscles of non-dystrophic control mice. These data suggest that dystrophic myofibres are especially vulnerable to mast cell mediators. Mast cells have been implicated in the proliferation of connective tissue and fibrosis in numerous pathological conditions (Gordon et al., 1990; Miyazawa et al., 2004; Akgul et al., 2005; Wang et al., 2005) and also related to myofibre remodelling and fibrosis in old mdx mice (Nahirney et al., 1997).

The role of mast cells in dystrophic myopathy and the beneficial effects of Oxatomide (Tinset) a mast cell stabiliser on exercised adult mdx mice (Granchelli et al., 2000) has led to clinical trials of Oxatomide to determine beneficial effects on the

![Fig. 1. Sequence of the inflammatory response in dystrophic skeletal muscle. Resident mast cells act early in the inflammatory cascade and rapidly degranulate in response to damage to release TNFα and many other pro-inflammatory mediators.](image-url)
maintenance of muscle strength in steroid naïve, DMD boys. Oxatomide is an anti-allergic drug, classified as a Histamine (H1) receptor antagonist; however, it is also known to inhibit the release of several mediators (histamine, leukotrienes and prostaglandins) from mast cells and other leukocytes (Ohmori et al., 1998; Shindo and Fukumura, 1998; Marone et al., 1999). Suppressed TNFα release from mast cells is an associated effect after H1 receptor blockade (Xia et al., 2005). These trials were conducted by CINRG (Co-operative International Neuromuscular Research Group-Dr Diana Escolar) and the results are currently under analysis.

Cromolyn (sodium cromoglicate) is another mast cell stabiliser (Edwards and Howell, 2000) that inhibits mast cell degranulation, and thus the release of inflammatory mediators, both in vitro and in vivo (Steiner et al., 2003; Shin et al., 2004) and is a safe and widely used asthma medication (Storms and Kaliner, 2005). Cromolyn has previously been used in mdx mice, as has the mast cell stabiliser Oxatomide, and both maintain whole body strength after exercising adult mdx mice. (Granchelli et al., 1996, 2000). The precise reason for the beneficial effects of these drugs on the dystrophic muscle was not defined. The aim of the present study was to specifically test the role of mast cells in the onset of myofibre necrosis in mdx mice. It was hypothesised that cromolyn treatment, and blockade of mast cell degranulation, would reduce the extent of dystrophic myofibre necrosis in both young and exercised adult mdx mice.

Methods

All experiments were carried out on C57BL/10ScSnmdx/mdx (mdx) mice. Pups used in the onset study were both male and female aged between 19 and 28 days, and adult (6 week) mice used in the exercise study were all female. All mice were obtained from the Animal Resource Centre Murdoch, Western Australia, kept under a 12 h day–night cycle and allowed access to food and water ad libitum. Mice were housed and treated according to the Western Australian Prevention of Cruelties to Animals Act (1920), the National Health and Medical Research Council and the University of Western Australia Animal Ethics Committee.

For the onset study, mdx pups were divided into three experimental groups: cromolyn treated, phosphate-buffered saline (PBS) treated controls and untreated mdx controls. Cromolyn (Sigma C0399) and PBS treated mice were given daily intraperitoneal (IP) injections, from 19 days of age until the day before sacrifice. Cromolyn injections were given at a concentration of 50 mg/kg/day (Granchelli et al., 1996). Three experimental groups were sampled at 21, 23, 24, 26 and 28 days after birth (Table 1).

Twenty-four, 6 week old adult female mdx mice were used in the exercise study. These mice were divided into three groups. The first group (n = 8) was exercised voluntarily for 48 h and given daily IP cromolyn injections (50 mg/kg/day) 1 day prior to exercise and on both days of exercise. The second group (n = 8) was exercised for 48 h and the third group (n = 8) was not exercised or injected. While an extended period of voluntary exercise would presumably lead to increased muscle necrosis, it also allows for myofibres that became necrotic at the beginning of the exercise regime to commence regeneration, making interpretation difficult against the background pathology. For this reason, exercise was limited to 48 h to allow for analysis shortly after the onset of exercise induced necrosis.

Mice were voluntarily exercised using a metal mouse wheel placed inside the cage for 48 h. Exercise data were collected via a small magnet attached to the mouse wheel and a sensor from a bicycle pedometer attached to the back of the cage. The sensor recorded single wheel revolutions, allowing total distance (km) run by an individual mouse to be determined. Of the 16 mice exercised, 4 were exercised at the Australian Neuromuscular Research Institute, Perth, where the elaborate exercise equipment allowed for detailed temporal running patterns over the 48 h period to be recorded (Fig. 6).

All mice were sacrificed using halothane anaesthesia followed by cervical dislocation. The tibialis anterior (TA) and quadriceps muscles were sampled and prepared for paraffin processing. For the onset study, muscles from both legs were analysed, and therefore n = number of muscles, for the exercise study muscles from only the left leg were analysed and therefore n = number of mice (Fig. 7). Slides were stained with H&E for morphometric analysis and toluidine blue for quantitative mast cell analysis. Non-overlapping tiled images of transverse muscle sections provided a picture of the entire muscle cross section. Images were acquired using a Leica DM BPE microscope, a personal computer, a Hitachi HVC20M digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software. Tiled images were taken at 10× magnification. Muscle morphology was drawn interactively by the researcher using Image Pro Plus 4.5.1 software and specific histological features measured as a percentage (area) of the whole muscle section. Mast cell counts were performed manually by the researcher on the Leica DM BPE microscope (×10 magn. All image analysis was done blind. Significant difference (P < 0.05) between cromolyn treated and control groups was determined using unpaired Student’s t tests.

Results

Onset of muscle necrosis in mdx pups aged 21–28 days

The onset of muscle necrosis was assessed on H&E stained transverse sections of TA and quadriceps muscles: necrotic areas were identified by the presence of infiltrating inflammatory cells (basophilic staining) and degenerating myofibres with fragmented sarcoplasm. Regeneration occurs in response to necrosis and results (2–3 days later) in myotubes that then mature into plump myofibres with central nuclei (Fig. 2). Cumulative skeletal muscle damage in young mdx mice consists of active myofibre necrosis plus the areas of subsequent regeneration.

At all times between day 21 and day 28, except day 24, there was a trend for less cumulative muscle damage in the cromolyn treated mice in comparison to untreated mdx mice, in both the TA and the quadriceps muscle (Fig. 3). At 28 days of age, there was significantly less cumulative damage in cromolyn treated TA in comparison to untreated mdx mice (P = 0.006). There was no significant difference between untreated and PBS injected control mdx mice.

| Table 1 |
The number (n) of muscles analysed for the three treatment groups in young mdx mice (21–28 days of age) |
<table>
<thead>
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<tr>
<td>n = number of muscles</td>
</tr>
<tr>
<td>Cromolyn treatment</td>
</tr>
<tr>
<td>Untreated (mdx)</td>
</tr>
<tr>
<td>PBS treated</td>
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</table>

The number (n) of muscles Day 21 Day 23 Day 24 Day 26 Day 28
Cromolyn treatment 8 8 8 8
Untreated (mdx) 8 8 6 6 7
PBS treated 7 5 6 4 4
Further examination of cumulative damage revealed high biological variation between individual mice within sample groups, as shown for days 21 and 23 (Fig. 4). Many of the muscles at these two times showed no evidence of necrosis, despite an expected onset of necrosis at day 21. Myotubes with central nuclei, indicating myofibre regeneration, appear approximately 3 days after necrosis (Straub et al., 1997; Shavlakadze et al., 2004). Cromolyn treatment did not prevent necrotic muscle from regenerating, as myotubes were seen in cromolyn treated mice from day 24 onwards.

Mast cell density (per mm\(^2\) of muscle cross sectional area—manual count) and mast cell morphology were analysed to determine the effects of cromolyn on resident mast cells in vivo. Quantitative analysis of mast cells (Table 2) in the TA and quadriceps of mdx mice aged between 21 and 28 days showed no significant difference in total mast cell number/mm\(^2\) between cromolyn treated and control groups. Total mast cell numbers ranged from 1 to 13 cells/mm\(^2\) in TA and from 1 to 9 cells/mm\(^2\) in quadriceps muscles. Significantly fewer mast cells were found in the quadriceps muscle in comparison to TA in mice sampled between days 21 and 28 regardless of treatment (Table 2). The reduced number of mast cells correlates with the trend for less cumulative damage in quadriceps muscle, in comparison to TA, in young mdx mice (Fig. 3) and is in accordance with previous studies that show less cumulative muscle damage in the quadriceps muscle in comparison to the TA (Shavlakadze et al., 2004).

Analysis of the number of intact (non-degranulated) mast cells compared with degranulating mast cells (Fig. 2) confirmed that cromolyn blocks mast cell degranulation in dystrophic skeletal muscle in vivo. The reduced number of mast cells that had degranulated in cromolyn treated mice (Fig. 5) demonstrated significant mast cell blockade in the TA on days 21, 23 and 24 ($P < 0.05$) and quadriceps muscle on days 21, 23 and 28 ($P < 0.05$).

When skeletal muscle necrosis was plotted in relation to mast cell degranulation, both groups of control mdx mice (untreated and PBS injected) showed a high percentage of degranulated mast cells at day 21 (Figs. 5A–D) followed by high myofibre necrosis. This initial bout of degranulation is absent in cromolyn treated mice (Figs. 5E, F) and, as a result, myofibre necrosis that follows is reduced. This effect is clearly demonstrated when comparing untreated TA and cromolyn treated TA (Figs. 5A, E).

**Exercise induced muscle necrosis in adult mdx mice**

In adult (6 weeks) exercised mdx mice, the area occupied by necrotic myofibres, regenerating and regenerated muscle and unaffected/intact myofibres was measured. Necrotic tissue was identified by the presence of infiltrating inflammatory cells (basophilic staining) and degenerating myofibres with fragmented sarcoplasm; regenerating muscle was identified by myotubes, regenerated muscle by plump myofibres with central nuclei, and unaffected/intact myofibres were normal looking myofibres with peripheral nuclei. 100% of muscle tissue cross-sectional area (CSA) is made up of necrotic tissue, regenerating tissue (recently necrotic), regenerated tissue (central nuclei), unaffected tissue (never been necrotic) and connective tissue. Detailed exercise data for individual mdx mice over the 48 hour exercise period highlighted the biological variation and difference in enthusiasm for exercise between age and gender matched inbred adult mdx mice (Fig. 6).

Voluntary exercise (wheel running) over 48 h resulted in a significant ($P < 0.05$) approximately 2-fold increase in skeletal...
muscle necrosis in the quadriceps muscle of untreated adult (6 weeks) female mdx mice in comparison to age-matched unexercised mdx mice. The extent of exercise induced damage was insignificant in the TA and therefore only the quadriceps muscles, where induced damage had occurred, were analysed. Cromolyn treatment significantly reduced \((P < 0.05)\) the extent of exercise induced necrosis in the quadriceps muscle (Fig. 7). Cromolyn treatment also significantly reduced \((P < 0.01)\) the extent of mast cell degranulation in the quadriceps muscle of exercised adult mdx mice (data not shown). Cromolyn treatment had no effect on unexercised adult mdx mice \((n = 8, \text{see Fig. 7})\). The percentage of muscle necrosis seen in unexercised adult mdx mice was similar regardless of cromolyn treatment (approximately 7% necrosis). These data highlight the need to exercise adult mdx mice as otherwise the extent of active muscle damage (necrosis) in old mdx mice is usually very mild.

Two non-dystrophic (C57BL/10) mice were also exercised and showed no histological evidence of muscle damage after running up to 10 km in 24 h (data not shown), confirming an increased susceptibility to muscle damage after exercise only in mdx mice.

It was expected that all mdx mice (cromolyn treated and untreated) would run comparable distances (with less muscle necrosis anticipated in the cromolyn treated mdx mice). However, as illustrated in Fig. 6, there was much individual variation and the total distance run for all 16 mice over 48 h was plotted against myofibre necrosis in the quadriceps muscle (Fig. 8). The average distance run by all 16 mice (both cromolyn treated and untreated) was 6.45 km, and the average area of myofibre necrosis seen in all mice was 10.7% of the quadriceps in cross-section. In untreated control exercised mice, mice numbers 1 and 2 ran a relatively high total distance (above average 6.45 km) and showed high muscle necrosis (above average 10.7%), supporting a relationship between distance and muscle necrosis. However, control mouse number 3 exhibited a low level of muscle necrosis (below average 10.7%) after running a relatively high distance (9.4 km), suggesting a possible resistance to muscle necrosis in this individual, and mouse number 6 seemed particularly vulnerable to the dystrophic myopathy, showing a high level of muscle necrosis (above average 10.7%) after running only a short total distance (5.7 km). Overall, the total distance (km) run by an individual mouse did not correlate with the amount of necrosis seen in the quadriceps muscle.

Cromolyn treated exercised mice, numbers 1, 2 and 3 (Fig. 8—arrows), showed a relatively low level of muscle necrosis (below average 10.7%) in response to a relatively high total distance run (above average 6.45 km) suggesting a striking protective effect of cromolyn against the onset of exercise induced necrosis in these 3 cromolyn treated mice. However, mouse number 6 showed no beneficial effects of cromolyn treatment and mice numbers 4 and 8 suggested a direct relationship between total distance run (km) and extent of muscle necrosis (%) without the protective effects of cromolyn. Cromolyn treatment protected some, but not all, mice necrosis in the quadriceps muscle of untreated adult (6 weeks) female mdx mice in comparison to age-matched unexercised mdx mice. The extent of exercise induced damage was insignificant in the TA and therefore only the quadriceps muscles, where induced damage had occurred, were analysed. Cromolyn treatment significantly reduced \((P < 0.05)\) the extent of exercise induced necrosis in the quadriceps muscle (Fig. 7). Cromolyn treatment also significantly reduced \((P < 0.01)\) the extent of mast cell degranulation in the quadriceps muscle of exercised adult mdx mice (data not shown). Cromolyn treatment had no effect on unexercised adult mdx mice \((n = 8, \text{see Fig. 7})\). The percentage of muscle necrosis seen in unexercised adult mdx mice was similar regardless of cromolyn treatment (approximately 7% necrosis). These data highlight the need to exercise adult mdx mice as otherwise the extent of active muscle damage (necrosis) in old mdx mice is usually very mild.

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individual mice and marked variation in the severity of the dystrophic myopathy was identified. Untreated exercised mice ran significantly more \((P < 0.05)\) than cromolyn treated mice; however, there appears to be no direct relationship between the total distance (km) run and muscle necrosis in an individual mouse (Fig. 8).

Table 2
Average total numbers of mast cells/mm\(^2\) (mean ± SD) in mdx mice

<table>
<thead>
<tr>
<th>Day (TA)</th>
<th>Cromolyn</th>
<th>PBS</th>
<th>Untreated</th>
<th>Day (Q)</th>
<th>Cromolyn</th>
<th>PBS</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>6.3 ± 3.6</td>
<td>7.6 ± 1.7</td>
<td>4.8 ± 1.7</td>
<td>21</td>
<td>5.4 ± 2.5</td>
<td>4 ± 1.5</td>
<td>4.3 ± 1.3(^a)</td>
</tr>
<tr>
<td>23</td>
<td>9.5 ± 3.7</td>
<td>7.2 ± 1.5</td>
<td>8.8 ± 2.2</td>
<td>23</td>
<td>4.8 ± 1.4(^a)</td>
<td>1.8 ± 0.9(^a)</td>
<td>5.1 ± 1.1(^a)</td>
</tr>
<tr>
<td>24</td>
<td>7.8 ± 2.7</td>
<td>5 ± 1.8</td>
<td>4.4 ± 1.1</td>
<td>24</td>
<td>4.1 ± 1.6(^a)</td>
<td>5.4 ± 1.1</td>
<td>4.1 ± 3.3</td>
</tr>
<tr>
<td>26</td>
<td>6.2 ± 3.8</td>
<td>5.6 ± 4.7</td>
<td>7.7 ± 3.6</td>
<td>26</td>
<td>3.4 ± 1.1(^b)</td>
<td>2.0 ± 1.15(^a)</td>
<td>2.4 ± 0.6(^b)</td>
</tr>
<tr>
<td>28</td>
<td>6 ± 3.2</td>
<td>8.9 ± 3.1</td>
<td>6.5 ± 6.2</td>
<td>28</td>
<td>2.5 ± 1.2(^a)</td>
<td>2.9 ± 0.2(^a)</td>
<td>2.1 ± 0.95(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Indicates a significantly higher number of mast cells/mm\(^2\) in the TA in comparison to quadriceps muscle for the same treatment \((P < 0.05)\).

\(^b\) Indicates a trend for higher number of mast cells/mm\(^2\) \((P = <0.09)\) in the TA.

Fig. 5. Cumulative damage in individual muscles compared with mast cell degranulation, for TA (A, C, E) and quadriceps muscles (B, D, F) in young mdx mice. * indicates significantly less mast cell degranulation in cromolyn treated mice compared to untreated mice and ^ indicates significantly less degranulation in cromolyn treated mice compared to PBS treated mdx mice. Error bars represent the SEM for mast cell degranulation. For error bars of cumulative damage see Fig. 3.
In an attempt to determine why some mdx mice voluntarily ran more than other age and sex matched mice, regardless of treatment, it was hypothesised that the extent of background (pre-exercise) muscle pathology for an individual mouse might indicate the susceptibility to muscle damage and thus determine the enthusiasm for exercise and the total distance run by that mouse. The severity of background (pre-exercise) pathology for an individual mouse was determined by the percentage of muscle tissue that had not been affected by the disease, i.e. the percentage of myofibres with peripheral nuclei and a normal appearance, in the quadriceps muscle (Table 2). The average area of unaffected myofibres in both cromolyn treated and untreated adult mice was 12.45% by area of the quadriceps, but ranged from 2 to 48% immediately identifying high biological variation in the extent of dystrophic pathology in 6 week old female mdx mice. In 6/8 untreated exercised mice (numbers 3–8), the extent of background pathology related to the total distance (km) run (Fig. 8) i.e. an individual mouse with a high percentage of unaffected muscle tissue (low level of background pathology) ran a greater total distance (km) than a mouse with little unaffected muscle tissue (high level of background pathology).

Cromolyn treated mice numbers 1, 5, 6 and 8 (Fig. 8) showed the same pattern. There were exceptions to this trend as cromolyn treated mice numbers 2, 3 and 4 (Fig. 8) all appeared to have a high susceptibility to muscle damage as indicated by high background pathology (low unaffected tissue %) yet were able to run a high total distance (above average 6.45 km), suggesting a strong protective effect of cromolyn in these three mice.

An additional possible explanation for the distance run by an individual mouse is that cromolyn treatment caused mdx mice to run less. When considering adverse background pathology (Cromolyn treated = 10% vs. Controls = 15%), cromolyn treated mice might be expected to run more than the untreated controls—but they did not.

Discussion

The results demonstrate that cromolyn treatment, that blocks mast cell degranulation, reduces the extent of muscle necrosis and supports the original hypothesis that mast cell mediators contribute to the breakdown of dystrophic muscle.
In comparison to non-dystrophic control C57BL/10ScSn mice, mast cells are elevated approximately 3-fold in dystrophic mdx skeletal muscle (Gorospe et al., 1996). The earliest age at which mast cells have been quantified in the mdx mouse is 17 days where numbers ranged between 2 and 8 mast cells/mm² in the TA in comparison to negligible numbers in normal controls (Lefaucheur et al., 1996). Similar results with quantitation of mast cells in mdx mice where numbers ranged from 1 to 13 mast cells/mm² in the TA and 1 to 9 mast cells/mm² in quadriceps muscles of mdx mice sampled between 21 and 28 days of age were obtained (Table 2). Greater number of mast cells/mm² in the TA, compared with the quadriceps, correlated with more muscle necrosis present in this muscle between 21 and 28 days (Fig. 3). These observations support previous reports of more necrosis in the TA compared to both the diaphragm and quadriceps muscle in young mdx mice (Shavlakadze et al., 2004). The extent of muscle necrosis in the mdx mouse between days 21 and 28 also correlates with more mast cell degranulation in the TA (compared to the quadriceps muscle) of untreated mdx mice at day 21 (Figs. 5A–D), linking mast cell degranulation with myofibre necrosis.

Earlier quantitative mast cell studies in mdx mice report localisation of mast cells in areas of myofibre necrosis (Gorospe et al., 1994b; Lefaucheur et al., 1996). However, in the present study, mast cell location did not appear to be closely associated with muscle necrosis (data not shown) and this accords with observations reported by others (Roig et al., 2004).

All mdx pups began daily cromolyn treatment at 19 days of age, 2 days prior to the expected onset of acute muscle necrosis. The sampling of young mdx mice at various ages (d21–d28) results in different length of cromolyn exposure at each sample time-point (e.g. d21 = 2 days exposure, d28 = 9 days exposure). The variation in cromolyn exposure might explain the increased beneficial effects seen in later time-points (d28) when cromolyn exposure was the longest. Studies in humans suggest a possible 3 week delay in the onset of action of cromolyn (Sinha and David, 2003); however, the protective effects of cromolyn were seen after just 3 injections (3 days) in the cromolyn treated adult exercised mdx mice (Fig. 7), demonstrating benefits after short term administration.

Voluntary exercise (wheel running) induces a significant (P < 0.05) increase (approximately 2× fold) in myofibre necrosis in the quadriceps of adult mdx mice (whereas the TA showed relatively insignificant exercise induced damage). Cromolyn treatment, and thus blockade of mast cell degranulation, significantly reduced exercise induced myofibre necrosis in the quadriceps of adult mdx mice (n = 8) (Fig. 7).

The use of voluntary wheel running in the present study provided insight into why some mdx mice run less than others. While the genetic background of mice is known to have significant effects on total distance, intensity and duration of exercise (Lerman et al., 2002; Lightfoot et al., 2004), the adult mdx mice in this exercise study were inbred, age and sex matched, yet they exhibited large variation in enthusiasm for exercise. We identified an underlying trend that the extent of background (pre-exercise) dystropathology in each individual mouse determines the extent of participation in voluntary exercise. While a range of interest in downhill eccentric exercise (Brussee et al., 1997) and an intermittent running pattern (Hara et al., 2002) in mdx mice has been reported, this particular trend has not been previously documented. Due to the biological variation reported within sample groups, it was necessary to examine each mouse as an individual for background muscle pathology (indicating severity of pre-existing myopathy) and relate this to the capacity for exercise that appeared to reflect the inherent susceptibility to muscle damage.
The biological variation documented between individual mdx mice, in both young (onset study) and adult (exercise study) mice is a much greater problem than anticipated. Despite the same genetic background of the inbred mdx mice, it appears that the onset and progression of dystrophic myopathy can differ markedly between individual mdx mice. Analysis of soleus muscles of mdx and normal mice surprisingly show considerable variation between satellite cell frequencies of age-matched mice (Schafer et al., 2005). The reasons for such high variation are unknown, but such inherent variation needs to be taken into account and necessitates large sample sizes to show statistically significant effects of treatments. The exact timing of the onset of necrosis is extremely sensitive, and variation in expression of the myopathy is possibly explained by differences in gestation length, litter size, birth weight and behaviour of the individual mdx mice. To minimise effects of gestation and litter size on inter-litter variation, treatments should be compared between litters. The biological variation evident in young mdx mice is also manifested in older mdx as demonstrated by extraordinary variation in detailed analysis of running patterns for 4 untreated adult mdx mice over 48 h (Fig. 6).

The data reveal an interesting relationship between the extent of pre-existing muscle pathology, the enthusiasm for exercise and the susceptibility to muscle damage.

Numerous different muscles, including the soleus, gastrocnemius, extensor digitorum longus, diaphragm, muscles of the forelimb and whole body strength (Weller et al., 1990; Brussee et al., 1997; Granchelli et al., 2000; De Luca et al., 2005), have been found that both the biceps brachii and gastrocnemius in the lower limb showed reasonable amounts of damage (both approximately 10–15%) after voluntary exercise (data not shown).

Two previous studies have used mast cell blockade to reduce the deterioration of skeletal muscle in exercised mdx mice (Granchelli et al., 1996, 2000). Both concluded that mast cell blockade maintained whole body strength after numerous forced exercise sessions; however, the mechanism of protection was not defined. The present detailed histochemical analysis of short-term cromolyn administration in young (21–28 days) mdx mice combined with the effects of short-term (48 hours) voluntary exercise on adult mdx mice is novel. These data strongly support the observations in previous studies which concluded (but did not histologically confirm) that mast cell blockade by cromolyn treatment reduced myofibre necrosis in mdx mice.

Exercise protects against chronic systemic inflammation and thus is beneficial in numerous cardiovascular and metabolic disorders. This may be due, in part, to the anti-inflammatory properties of Interleukin 6 (IL-6) produced by skeletal muscle (Reviewed in (Petersen and Pedersen, 2005) as circulating IL-6 exerts anti-inflammatory effects by inducing the production of IL-10, IL-1ra, sTNF-r and by reducing circulating TNFα (Steensberg et al., 2003). During exercise, IL-6 is produced and released by contracting myofibres within 30 min and plasma levels of IL-6 increase exponentially up to 100-fold (Pedersen et al., 2001; Steensberg et al., 2002). The systemic and local benefits of exercised muscle (Timmons et al., 2005) must be considered against the damage to fragile dystrophic myofibres that can result from exercise.

TNFα is a major inflammatory cytokine produced and released by mast cells during degranulation (Gordon and Galli, 1990). Previous studies with the blockade of TNFα activity in mdx mice in vivo, using specific antibodies (Grounds and Torrisi, 2004) or soluble receptors (Enbrel; Hodgetts et al. unpublished) strongly implicate TNFα as a major factor that exacerbates the initial sarcolemmal damage and results in muscle necrosis (at least in part by the recruitment of neutrophils). While there is evidence that TNFα plays a role in myofibre necrosis, there are clearly many other potent factors (interleukins, histamine, proteases) released during mast cell degranulation and the role of these factors in the dystrophic process remains to be elucidated. Mast cell degranulation and the release of pro-inflammatory cytokines occur in the early stages of the inflammatory response (Fig. 1). The blockade of mast cell degranulation by cromolyn should restrict initial cytokine (TNFα) release from mast cells and delay the initial signalling in onset of inflammation in dystrophic skeletal muscle. As demonstrated previously, blockade of TNFα activity and reduced inflammation results in an improvement of dystrophic muscle pathology.

In conclusion, three observations support the hypothesis that mast cells are involved in the onset of muscle necrosis in the mdx mouse: (1) In comparison to the TA, the quadriiceps muscle contains significantly fewer mast cells (Table 1), less mast cell degranulation and has less muscle necrosis (Fig. 3). This implicates mast cell involvement in the muscle necrosis of young mdx mice. (2) Cromolyn treatment reduces cumulative muscle damage in young mdx mice (significant at day 28). (3) Cromolyn treatment significantly reduces exercise induced necrosis in adult mdx mice.

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