Anti-TNFα (Remicade®) therapy protects dystrophic skeletal muscle from necrosis

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ABSTRACT Necrosis of skeletal muscle fibers in the lethal childhood myopathy Duchenne muscular dystrophy (DMD) results from defects in the cell membrane-associated protein, dystrophin. This study tests the novel hypothesis that the initial sarcolemmal breakdown resulting from dystrophin deficiency is exacerbated by inflammatory cells and that cytokines, specifically tumor necrosis factor-α (TNFα), contribute to muscle necrosis. To block in vivo TNFα bioactivity, young dystrophic mdx mice (a model for DMD) were injected weekly from 7 days of age with the anti-TNFα antibody Remicade® before the onset of muscle necrosis and dystropathology that normally occurs at 21 days postnatally. The extent of inflammation, muscle necrosis, and myotube formation was measured by histological analysis from 18 to 28 days and muscle damage was also visualized by penetration of Evans blue dye into myofibers. Data from Remicade®-treated and control mdx mice were compared with mdx/TNFα(−/−) mice that lack TNFα. Pharmacological blockade of TNFα activity with Remicade® clearly delayed and greatly reduced the breakdown of dystrophic muscle, in marked contrast to the situation in mdx and mdx/TNFα(−/−) mice. Remicade® had no adverse effect on new muscle formation. Remicade® is a highly specific anti-inflammatory intervention, and clinical application to muscular dystrophies is suggested by this marked protective effect against skeletal muscle breakdown.—Grounds, M. D., Torrisi, J. Anti-TNFα (Remicade®) therapy protects dystrophic skeletal muscle from necrosis. FASEB J. 18, 676–682 (2004)

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Duchenne muscular dystrophy (DMD) is an early onset lethal X-linked disorder that occurs in ~1 in 3500 boys, resulting in severe skeletal muscle degeneration and death, usually by 20 years of age (1). In DMD and the mdx mouse model for DMD, defects in the subsarcolemmal protein dystrophin initiate muscle pathology (2, 3). There is no successful treatment for DMD. Much research has focused on cell and gene therapies to try and replace the defective dystrophin gene (4, 5). A complementary approach is to try to render the dystrophic myofibers less susceptible to damage using booster genes (6) to salvage the muscle integrity (3). For example, reduction of dystropathology has been demonstrated in mdx mice by overexpression of IGF-1 (7), utrophin (8), cytotoxic T cell GalNAc transferase (9), calpstatin (10), integrin-α 7 (11), or ADAM-12 (12) and in the absence of myostatin (13, 14). Many of these (and other) observations are derived from experiments using genetically engineered mdx mice; difficulties arise in translating these promising observations into the clinical situation. Beyond these strategies designed to alter myofiber characteristics or extracellular interactions is the approach of targeting inflammatory cytokines; this is the focus of the present study.

Damage to the sarcolemma can result in small lesions in the membrane that may normally be repaired rapidly by “patching” to prevent myofiber breakdown, and it has recently been demonstrated that dysferlin, the defective gene in limb girdle type muscular dystrophy, is a key protein involved in such membrane resealing in skeletal muscle (15, 16). We hypothesize that in dystrophic muscle, membrane damage caused by the gene defect can be exacerbated by proinflammatory cytokines, such as tumor necrosis factor-α (TNFα), that tip the balance between patching of the minor lesion or further breakdown leading to focal necrosis of the myofiber. Strong evidence that inflammatory cells can contribute to necrosis of healthy muscle cells comes from studies investigating the role of neutrophils, macrophages, and oxidative damage in vitro (17–20) and in vivo (21, 22). It has been proposed that an inappropriate or excessive inflammatory response can directly damage myofibers in myopathic conditions such as dystrophies or myositis (2, 23–25). TNFα is an early and potent proinflammatory cytokine that stimulates the inflammatory response.

Even minor trauma to muscle will increase levels of TNFα by release from mast cells; TNFα is also produced by neutrophils, macrophages and lymphocytes that accumulate rapidly at the site of injury. TNFα increases rapidly within damaged myofibers and is expressed by myoblasts and myotubes (26–28). TNFα is greatly elevated in damaged myofibers in injured normal (26, 27) and myopathic skeletal muscle (29); it is chemotactic...
for myoblasts (30) and is mitogenic for satellite cells in vivo, suggesting a direct role in myogenesis of regenerating muscle (28).

The role of the cytokine TNFα in muscular dystrophy has been investigated using mdx/TNFα(−/−) mice, and histopathological analysis has found that the absence of TNFα in vivo resulted in equivocal findings as opposed to amelioration of muscle pathology as predicted (31), although long-term deletion of TNFα appeared beneficial in older (12 months) mdx/TNFα(−/−) mice (32). Cytokine networks are complex, and when TNFα is removed from an in vivo system, as in mdx/TNFα(−/−) mice, other proinflammatory cytokines such as IL-12 and IFNγ (33) may be up-regulated to overcome the TNFα deficiency. The confounding situation of altered cytokine profiles in TNFα knockout mice is further demonstrated by studies of regenerating whole muscle grafts in these mice, where predictions of impaired skeletal muscle regeneration in the absence of TNFα are not supported (26).

As an alternate approach, we have used the antibody Remicade® to neutralize activity of the TNFα protein in mdx mice to assess whether blockade of TNFα in patients with DMD has therapeutic value. It was expected that this strategy to block TNFα activity in vivo would avoid the problems of compensatory up-regulation of other proinflammatory cytokines and silence the inflammatory cell response. Strong support for the in vivo efficacy of Remicade® comes from histological analysis of whole muscle autografts in C57Bl/10SnSc mice (the parental strain for mdx) pretreated with Remicade®, where grafts examined at 5 days after transplantation contain very few inflammatory cells and consist almost entirely of persisting necrotic muscle tissue with no myotubes, in striking contrast to the advanced regeneration seen in control grafts where necrotic tissue has been removed by inflammatory cells and largely replaced by myotubes at 5 days (M. Grounds et al., unpublished results). However, whereas inflammatory cell infiltration and associated regeneration are delayed, they are not prevented since new muscle is formed within 7 days in these Remicade®-treated mice. Clinically, the TNFα neutralizing antibody Remicade® is highly effective at reducing symptoms of inflammatory diseases such as rheumatoid arthritis (34) and Crohn’s disease (35), and is being extended to myositis. The clinical success of Remicade® makes it attractive as a potential drug to treat muscular dystrophies, and so it was tested in the present study on dystrophic mdx mice in vivo. It was hypothesized that TNFα plays a role in the breakdown of dystrophic myofibers and that neutralizing TNFα activity in vivo will result in delayed onset and/or decreased intensity of dystrophopathy in mdx mice.

In mdx mice, the absence of dystrophin results in an abrupt onset of skeletal muscle necrosis at 21 days of age (36, 37), and our studies focus on this early acute transitory phase of dystrophopathy. This critical time provides a sensitive assay for factors that shift the threshold of breakdown of dystrophic muscle. Muscle breakdown peaks at 28 days, then decreases markedly to stabilize around 12 wk of age to a relatively low level of damage (37), with symptoms of dystrophopathy being cumulative and more pronounced in older (15 months) mdx mice (38, 39). Any delay in the acute onset of damage at 19–21 days, indicating a shift in the threshold of muscle breakdown, is readily detected. The onset of damage is assessed histologically (evidence of necrosis and myotubes formed ~3 days after muscle breakdown) and by injection of Evans blue dye (EBD) as a sensitive and early marker of muscle damage/leakiness (40, 41). Although it is often stated that the marked difference in severity of pathology between mdx mice and DMD boys is due to a greater capacity for regeneration in the mice, it would seem that the reduced muscle breakdown in mature mdx mice (after the transitory acute phase has ceased) may be the key issue to consider. For if muscle necrosis does not occur, regeneration is not required.

**MATERIALS AND METHODS**

**Overview**

Mdx mice were injected intraperitoneally once weekly from day 7 after birth with 10 μg (1 μL) Remicade® (Schering-Plough Pty. Ltd., Sydney, Australia) per gram body weight or equivalent volume of control mouse serum albumin (MSA); all mice were weighed before injection. Remicade®-treated and control (MSA) mdx mice were also compared with untreated mdx/TNFα(−/−) mice. At 24 h before sampling, mice were intraperitoneally injected with 1% Evans blue dye (EBD) at 1% volume relative to body mass to identify damaged/leaky sarcolemmal membranes (41). Mice were killed (by halothane anesthesia) and TA muscles sampled from 18 to 28 days of age and at 12 wk. The TA muscles were either frozen or taken for paraffin processing. Frozen sections were analyzed for EBD-positive myofibers. Hematoxylin and eosin (H&E)-stained paraffin sections were analyzed histologically for inflammatory cell infiltration, myofiber necrosis, and myotube formation and, in 12 wk samples, for myofibers with central nuclei.

**Mice and treatment groups**

Inbred mdx and mdx/TNFα(−/−) mice were used in this investigation. Mdx mice for TNFα neutralization were housed and treated according to the Western Australian Prevention of Cruelties to Animals Act, the National Health and Medical Research Council, and the University of Western Australia Animal Ethics Committee. Remicade®-treated mdx mice were sampled 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, and 28 days after birth with numbers of mice being 4, 2, 4, 5, 4, 3, 9, 7, 7, and 4, respectively, for each time point. Control MSA-injected mdx mice were sampled 19, 20, 21, 22, 23, 24, 25, 26, and 28 days after birth with numbers of mice being 3, 4, 4, 5, 2, 5, 2, 4, and 4, respectively, for each sample point. Four additional mice from both treatment groups were sampled at 12 wk.

Mdx/TNFα(−/−) and control mdx mice for TNFα gene knockout analysis were housed and sampled at the University of California, Los Angeles, using guidelines for animal use in the United States. Duplicate mdx/TNFα(−/−) and control mdx mice were sampled 19, 20, 21, 22, 24, 26, and 28 days after birth.
Histological assay

Inflammatory cell infiltration, muscle necrosis, and myotube formation were analyzed using H&E stained 5 μm frozen and/or paraffin sections using light microscopy. TA muscle for each parameter was examined in transverse section using the following scale: 1 indicating 1–5% of total tissue area affected, 2 representing 6–15%, 3 for 16–30%, 4 for 31–60%, and 5 for 61–100%. Inflammatory cell infiltration was identified by cells with basophilic nuclear staining and little cytoplasm located within the endomysium and/or necrotic tissue. Muscle necrosis was identified by breakdown of sarcolemma and fragmented sarcoplasm, and myotubes (indicating regeneration) were identified as plump cells with central nuclei. In older (12 wk) mice, myofibers with persisting central nuclei are a cumulative index of previous cycles of necrosis/regeneration (39).

RESULTS

Overall, there was no significant difference in weight gain between Remicade® injected and control MSA injected mdx mice during the study period. The mean gram body weight (and standard error) for the Remicade® and control mdx mice, respectively, was 7.25 (+/−0.07) and 8.04 (+/−1.83) on day 7, 8.25 (+/−0.22) and 9.26 (+/−2.27) on day 14, 10.27 (+/−0.30) and 6.62 (+/−1.79) on day 21, 14.44 (+/−0.83) and 10.9 (+/−2.05) on day 28, and 32.03 (+/−0.41) and 30.83 (+/−0.6) at 12 wk. Only day 21 showed significant difference between the two groups, with slightly higher values for the Remicade®-treated mice (t test P<0.05). Therefore, neither the growth rate of the young mdx mice nor the adult body weights were reduced by Remicade® treatment.

The histological appearance of muscles on days 21 and 24 is shown in Fig. 1; the overall patterns of inflammatory cell infiltration, necrosis and myotube formation from days 18 to 28, for control (MSA) mdx, Remicade®-treated mdx and mdx/TNFα(−/−) muscles are summarized in Fig. 2. Detailed quantitation of the tissue area occupied by muscle necrosis and myotubes at 21 and 28 days of age for control (MSA) and Remicade®-treated mdx mice (n=4 mice for each sample) is presented in Fig. 3; these data strongly support the semiquantitative analysis in Fig. 2. Inflammatory cells were conspicuous in control (MSA) mdx muscles at 20 days (Fig. 2a) and pronounced at 21 days throughout >50% of the mdx tissue section (Fig. 1a, Fig. 2a). The marked increase in inflammatory cells at 20 days preceded by 1 day the acute phase of necrosis that occupied up to 30% of the tissue section on day 21 (Fig. 1a, Fig. 2a). In marked contrast, in Remicade®-treated mdx mice, there was little inflammation and minimal necrosis (0–5%) at these times (Fig. 1c, Fig. 2b). The maximum extent of necrosis in control mdx mice (seen on day 21) was much greater than in Remicade®-treated mdx mice (seen on day 25) (Figs. 1 and 2) and the extent of inflammatory cell infiltration were consis-

![Figure 1. Histological appearance of TA muscles from mice aged 21 and 24 days after birth. a, b) Control (MSA injected) mdx mice, c, d) Remicade®-treated mdx mice and e, f) mdx/TNFα(−/−) mice. In control mdx mice on a) day 21 necrosis was predominant and on b) day 24 myotubes were prominent (b, inset: high magnification shows myotubes with central nuclei). In contrast, necrosis is not marked in Remicade®-treated mice on c) day 21 but is apparent at d) day 24. The mdx/TNFα(−/−) mice at e) days 21 and f) 24 were similar to control (MSA injected) mdx mice. Asterisks indicate sites of necrosis. Arrow indicates myotubes. Scale bar, 100 μm.
tently lower in Remicade*-treated mice (Fig. 2b) than in control mdx mice (Fig. 2a). After the initial acute peak of necrosis, there appeared to be a lull in muscle damage; such a cyclic pattern of necrosis/regeneration was also noted in earlier studies in mdx mice (36). The pattern of muscle breakdown in Remicade*-treated mice was markedly different from that seen in mdx/TNFα-/- mice, where the overall extent and pattern of damage resembled the control mdx mice (although the peak of inflammation and necrosis was delayed by several days) (Fig. 2, compare panels b, c, and a). The striking delay in onset of muscle breakdown in the Remicade*-treated mdx mice was further supported by analysis of EBD staining in TA muscles from 22-day-old mice, where many EBD-positive myofibers (indicating membrane damage) were conspicuous in control mdx mice; such myofibers but were rare in the Remicade*-treated mice (Fig. 4). Statistical analysis of the quantitative data related to the area occupied by necrotic tissue (Fig. 3) shows highly significant (P<0.05) reduced necrosis in Remicade*-treated dystrophic muscles at 21 days compared with control (MSA) mdx muscles.

The onset of necrosis was followed ~3 days later by the appearance of myotubes, as expected, since myotubes are not formed until 2–3 days after regeneration starts (42). In control mdx mice (Fig. 2a), myotubes were dramatically increased on day 24, 3 days after the peak of necrosis (at 21 days). In Remicade*-treated mdx mice, myotube numbers increased gradually after day 24 and the rise in numbers seen at 27 days with a further increase at 28 days is 3–4 days after (the much smaller) peaks of necrosis evident at 23 and 25 days. The timing of myotube formation (with respect to myofiber necrosis) appears normal and there is no hint of impaired myogenesis in the Remicade*-treated mdx mice. This is further endorsed by the many regenerated dystrophic myofibers (identified by central myonuclei) in 12-wk-old Remicade*-treated mdx TA muscle.

In 12-wk-old mice, the Remicade*-treated muscles generally had features similar to control mdx muscles with many central nuclei and small areas of necrosis sometimes associated with small myotubes. The extent of necrosis was low and similar in muscles of both groups, being only 0.185% (+/-0.19) and 0.245% (+/-0.294), respectively, for control mdx and Remicade*-treated mice. There was no difference in EBD staining between control and Remicade*-treated mdx mice with only a few individual or small groups of EBD-positive myofibers in TA muscle sections; these

Figure 2. Comparison of graphs summarizing the time course and extent of phagocyte infiltration, necrosis, and myotube formation in a) control (MSA injected) mdx mice, b) mdx mice treated with Remicade*, and c) mdx/TNFα(-/-) mice. No difference in the relative time of onset of myotube formation in response to necrosis was seen between these groups. Each time point shows the average grading for all TA muscles (muscle from both legs was analyzed, n=2, with total n ranging from 4 to 16; see details in Materials and Methods).

Figure 3. Quantitative analysis of the extent of necrosis and myotube formation in TA muscles (n=4 mice) from control and Remicade*-treated mice are shown for samples at 21 and 28 days. These data strongly endorse the semi-quantitative data in Fig. 2. *The lack of necrosis at 21 days in Remicade*-treated compared with control mdx mice is highly significant (P<0.05).
after birth, the TA muscle of mdx and Remicade/H23041 was measured at 28 days and 12 wk of age in control mice. In contrast to massive necrosis and are strongly positive for EBD. This is in fact, overall between the two times of examination is not statistically significant. Whereas these appeared to be fourfold smaller than at 28 days, there was no significant differences overall between the two times of examination (P<0.05). The trend for larger necrotic foci in control mdx vs. Remicade H23041-treated mice at both time points was not statistically significant (t test analysis, P<0.05).

Further quantitative analysis of the area occupied by myofibers with central myonuclei (a cumulative measurement of muscle damage and repair) indicated less

stained myofibers generally corresponded to necrotic foci visualized by H&E staining. To test the possibility that the size of necrotic lesions might be more limited in Remicade H23041-treated mice, the area of all necrotic foci was measured at 28 days and 12 wk of age in control mdx and Remicade H23041-treated mdx mice. Visually there was no striking difference in necrotic foci size between the two groups of mice. Morphometric analysis (ImagePro Plus 4.0 software, Microsoft) showed that at 28 days the mean size of the necrotic foci (expressed as percentage of total section area) was small being only 0.08% (+/−0.008%) (n=5) and 0.19% (+/−0.12%) (n=4) for Remicade H23041-treated and control mdx mice, respectively; the relatively high variation in the 28 day controls was accounted for by one of the four mice having significantly larger necrotic foci (maximum value was 1.04%) than the other three mice (maximum of 0.32%). At 12 wk, the mean size of the necrotic foci was 0.02% (+/−0.001) (n=4) and 0.04 (+/−0.014) (n=5) for Remicade H23041-treated and control mdx mice, respectively. Whereas these appeared to be fourfold smaller than at 28 days, there was no significant differences overall between the two times of examination (P<0.05). The trend for larger necrotic foci in control mdx vs. Remicade H23041-treated mice at both time points was not statistically significant (t test analysis, P<0.05).

DISCUSSION

The reduced dystropathology in Remicade H23041-treated mdx mice in the acute phase from 21 to 28 days supports the hypothesis that TNFα contributes to the early breakdown of dystrophic muscle. It is concluded that although a lack of dystrophin results in sarcolemmal weakness leading to initial breakdown of dystrophic muscle (2), the extent of necrosis is exacerbated by TNFα, which likely triggers the associated inflammatory cascade (23, 25).

The reduced inflammatory response did not appear to have any adverse effects on regeneration since myotube formation was not impaired in Remicade H23041-treated mdx mice in response to necrosis during the acute phase. Therefore, whereas the presence of central myonuclei indicates that regeneration has occurred, it does not indicate how many times this occurs.

Figure 4. Evans blue dye (EBD) staining as a marker of muscle damage is shown in TA muscles of day 22 a) control (MSA injected) and b) Remicade H23041-treated mdx mice. On day 22 after birth, the TA muscle of a) control mdx mice show massive necrosis and are strongly positive for EBD. This is in contrast to b) Remicade H23041-treated mice where the onset of necrosis is delayed and only a few myofibers show weak staining. Scale bar, 50 μm.
individual myofiber has been subjected to repeated necrosis/regeneration, and this should be taken into account. Furthermore, although the presence of EBD within myofibers certainly indicates sarcoclemmal damage and leakiness, there is not always a close correlation with histological myofiber necrosis (Shavlakadze et al., unpublished results) (40, 41). There is also great variation between the pathology of different muscles and individual mdx mice (40, 44, 45), and therefore a detailed study of many muscles up to 20 wk of age is in progress to thoroughly assess the impact of Remicade®. On the basis of this available information in the mdx mouse (where the pathology is mild), it is difficult to predict the potential overall benefit of Remicade® on the severe pathology seen in the dog models of DMD (46, 47) or in clinical dystrophies and dysferlinopathies (48).

The reduced breakdown of dystrophic muscle with Remicade® treatment suggests that this highly specific anti-inflammatory drug may be a useful clinical intervention to treat DMD and other myopathies with a pronounced inflammatory component such as dysferlinopathies (15, 48). The high specificity of Remicade® is advantageous compared with the existing use of anti-inflammatory corticosteroids such as Prednisolone and Deflazacort to treat DMD as these steroids are associated with severe adverse side effects such as weight gain and osteoporosis (49, 50), and steroids should be avoided for dysferlinopathies due to unrecoverable loss of strength (48). The precise mode of action of these steroids—whether they act by preventing muscle breakdown, suppressing inflammation, increasing the size and strength of myofibers, enhancing regeneration, or reducing fibrosis—remains unclear (50). In contrast, the precise mode of action of Remicade® is known: it does not cause generalized suppression of the immune system and has been used clinically to treat inflammatory disorders (34). Although it can have mild adverse effects such as rash and infusion-related reactions, there are few instances of severe reactions such as sepsis or pneumonia (35, 51). Remicade® has been used in pediatric patients to treat disorders such as juvenile idiopathic arthritis (52, 53), and Crohn’s disease (54, 55) in children as young as 3 years (53), and children as young as 18 months are now being treated.

The present study shows that treatment of mdx mice with Remicade® reduces the onset and intensity of necrosis and the dystrophathy. This presents a new approach for using highly specific anti-cytokine therapies to treat DMD. Further studies are required to evaluate long-term benefits of Remicade® treatment and to compare them with other clinically relevant anti-cytokine drugs (34) such as the use of soluble receptors that bind TNFα (Enbrel®) or interleukin-1 (56, 57). This striking protective effect combined with high specificity and few serious adverse side effects for Remicade® makes this simple strategy a serious contender for the clinical treatment of dystrophopathies.

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REFERENCES
