Short Communication

ADAM12 Alleviates the Skeletal Muscle Pathology in *mdx* Dystrophic Mice

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Muscular dystrophy is characterized by muscle degeneration and insufficient regeneration and replacement of muscle fibers by connective tissue. New therapeutic strategies directed toward various forms of muscular dystrophy are needed to preserve muscle mass and promote regeneration. In this study we examined the role of the transmembrane ADAM12, a disintegrin and metalloprotease, which is normally associated with development and regeneration of skeletal muscle. We demonstrate that ADAM12 overexpression in the dystrophin-deficient *mdx* mice alleviated the muscle pathology in these animals, as evidenced by less muscle cell necrosis and inflammation, lower levels of serum creatine kinase, and less uptake of Evans Blue dye into muscle fibers. These studies demonstrate that ADAM12 directly or indirectly contributes to muscle cell regeneration, stability, and survival. (Am J Pathol 2002; 161:1535–1540)

Muscular dystrophy is a group of muscle diseases characterized by accelerated muscle degeneration and ultimate replacement of muscle with fibrotic and fatty tissue.^{1–3} Although information about the genetic causes of muscular dystrophy has increased tremendously, there have been few advances in the treatment of the diseases.^{4,5} Potential treatments for muscular dystrophy may be pursued along several paths, such as replacement of the mutant gene with an autologous, homologous, or related gene;⁶ replacement of dying and dead cells (or lost muscle fibers) with myogenic progenitor cells;^{7,8} phar-

macological stabilization of muscle;^{9,10} inhibition of inflammation;^{11,12} inhibition of fibrosis;¹³ and activation of an intrinsic regeneration program.¹⁴ Much effort has been devoted to gene therapy, muscle cell transplantation, or a combination of the two. However, at this time, these technologies are still hampered by inefficient transfer of genes and cells into muscle, hence our aim is to identify and use factors that normally function in muscle renewal. We hypothesize that such factors could enhance regeneration and counteract insufficient regeneration seen in muscular dystrophy. We have focused on ADAM12 as a potentially important protein in both muscle development and regeneration. ADAM12 is a member of a family of transmembrane glycoproteins with more than 30 members in vertebrates.^{15–18} During mouse embryonic development, ADAM12 is expressed in several tissues, most prominently in muscle.^{19,20} Postnatally, the expression of ADAM12 in skeletal muscle ceases,¹⁹ but reappears transiently during regeneration.^{21,22} Some studies suggest that ADAM12 is a myogenic factor.^{19,23} Here, we generated transgenic mice that continuously express ADAM12 in mature skeletal muscle. The continuous presence of ADAM12 protein had a strikingly beneficial effect on the dystrophin-deficient *mdx* muscle as evidenced by less muscle cell necrosis and inflammation, lower levels of serum creatine kinase, and less uptake of Evans Blue dye into muscle fibers.

Supported by the Neye-Foundation; the Danish Medical Research Council; Novo Nordisk; Haensch; Munksholm; Velux; and by an European Union grant, Quality of Life and Management of Living Resources [contract no. QLGI-CT-1999-00870, designated "Genetic Resolution of Myopathies: European Cluster" (Myocluster) (to U. M. W.)]; the Japanese Society for the Promotion of Science (to N. K.); the Toyota Foundation (to F. C. N.); and the National Institutes of Health (to E. E.).

P. K. and N. K. contributed equally to this study.

Accepted for publication August 8, 2002.

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Materials and Methods

Generation of *mdx*/ADAM12 Transgenic Mice

Full-length human transmembrane ADAM12 cDNA (ADAM12-L; GenBank AF023476)²³ was inserted at the *Hind*III site of the pCCLMCK-II.²⁴ This plasmid contains the muscle creatine kinase (MCK) promoter for specific expression in striated muscle. cDNA was injected into fertilized oocytes of C57BL/6J X CBA, F1 mice (M&B, Copenhagen, Denmark). Transgenic founders were bred to C57BL/6J X CBA, F1 or C57BL/6J. All experiments were conducted according to the animal experimental guidelines of the Animal Inspectorate, Denmark. Homozygous female *mdx* mice (C57BL/10ScSn-Dmd<*mdx*>/J) were obtained from Jackson Laboratories and mated with ADAM12 transgenic males to produce F1 males, all of which were dystrophin-deficient (*mdx*) and half of which carried the ADAM12 transgene. The presence of the human ADAM12 transgene was determined by polymerase chain reaction of DNA isolated from tail biopsy using the human ADAM12 forward primer 5'-CCTGGGATCTGCTTGAGAG-3' (nucleotides 1939 to 1958) and backward primer 5'-TCTGCTGATGTCAACATCTCTG-3' (nucleotides 2692 to 2712). The presence of mouse and human ADAM12 mRNA by reverse transcriptase-polymerase chain reaction was detected using mouse ADAM12-L forward primer 5'-CTTGACTGTAGGAATCCTGG-3' (nucleotides 2150 to 2169) and backward primer 5'-CTCACCAAGGCACTAGTGAG-3' (nucleotides 2813 to 2832); human ADAM12-L forward primer 5'-GTTTGGCTTTGGAGGAAGCACAG-3' and backward primer 5'-GCAGCAATCTCCTGGGATTG-3' (nucleotides 2667 to 2686); GAPDH forward primer 5'-AAGGTCATCCAGAGCTGAACG-3' and backward primer 5'-TGTCATACCAGGAAATGAGC-3'.

Immunoprecipitation and Western Blotting to Determine ADAM12 Protein Expression

ADAM12 was extracted from muscle tissue specimens in RIPA buffer containing protease inhibitors (Complete, ethylenediaminetetraacetic acid-free protease inhibitor cocktail tablets; Roche Molecular Biochemicals, Hvidovre, Denmark). After centrifugation supernatants were subjected to immunoprecipitation with monoclonal antibodies (6E6, 8F8, and 6C10) followed by Western blotting using a polyclonal antiserum (rabbit 122 or 109), a horseradish peroxidase-conjugated second antibody and the chemiluminescence SuperSignal West Pico reagent from Pierce. The monoclonal antibodies to human ADAM12 were generated and characterized as previously described^{23,25,26} using purified recombinant full-length human ADAM12²⁷ as an immunogen.

Histological Analysis, Immunohistochemical Staining, Evans Blue Dye Treatment, and Serum Creatine Kinase

Muscle specimens from mice at various ages were fixed in formalin and embedded in paraffin, and 4- μ m sections

were stained with hematoxylin and eosin or with antibodies in immunohistochemistry as described below. The proportion of a given section that contained necrotic fibers surrounded by mononuclear inflammatory cells was estimated morphologically. For immunohistochemistry on frozen sections, polyclonal antiserum to the C-terminal peptide of mouse ADAM12 (rb 109) was used as previously described.²¹ This antiserum reacts to both mouse and human ADAM12. For immunohistochemical staining on paraffin sections, the following antibodies were used: rat anti-mouse macrophage antibody F4/80 (Serotec) at 1:800 and rat anti-mouse CD45 (SRT5, Serotec) at 1:100. The sections were processed for antigen retrieval by either protease pretreatment or by heating in microwave oven. The DAKO ARK (Animal Research Kit K3954; DAKO, Glostrup, Denmark) or the horseradish peroxidase-labeled streptavidin biotin technique (DAKO K5001 with E468) was used as a detection system. Evans Blue dye [10 mg/ml phosphate-buffered saline (PBS); Sigma] was injected intravenously into the tail vein of mice (50 μ l per 10 g body weight) according to Matsuda and colleagues.²⁸ Mice were sacrificed 3 to 4 hours after injection, and hindlimb muscles were dissected and photographed. Frozen sections (7 μ m) of m. quadriceps femoris were rinsed in PBS and examined by fluorescence microscopy at 560 nm. The proportion of dye-positive fibers was calculated. Serum creatine kinase activity was measured using the 520-C kit (Sigma Biotech, Hørsholm, Denmark).

Results

ADAM12 is expressed in developing muscle cells during both embryonic (Figure 1A) and early postnatal (Figure 1B) development but not in the normal adult mouse muscle (Figure 1C). To determine whether enhanced expression of ADAM12 might be beneficial for the *mdx* dystrophin-deficient muscle, we generated ADAM12 transgenic mice, which continuously overexpress ADAM12 in mature muscle under control of the MCK promoter and bred them to the *mdx* mice. We used human ADAM12 cDNA to distinguish between the endogenous gene and the transgene. ADAM12 was detected by immunohistochemical staining in the cytoplasm as well as at the cell membrane in adult skeletal muscle of the transgenic mice (Figure 1D). Immunoprecipitation and Western blot analysis confirmed the presence of the expected 110-kD proform and the 90-kD mature form of ADAM12 protein in adult transgenic mouse muscle. No ADAM12 protein was detected in adult nontransgenic littermate controls (Figure 1E).

Reverse transcriptase-polymerase chain reaction analysis of muscle RNA showed that *mdx* mice expressed endogenous mouse ADAM12 (Figure 2A, lane 5), and that the *mdx*/ADAM12 mice expressed both mouse and human ADAM12 mRNA (Figure 2A, lane 6). The morphological analysis was performed on muscle specimens (hindlimb and diaphragm) from 6-week-old *mdx* ($n = 12$) and *mdx*/ADAM12 ($n = 14$) mice. *Mdx* hindlimb muscle showed numerous large areas with muscle fiber necrosis, inflammatory cell infiltration, and muscle fiber regenera-

Figure 1. Characterization of ADAM12 transgenic mouse muscle. **A–D:** Immunohistochemical demonstration of endogenous and transgenic ADAM12 on frozen sections of the hindlimb muscle using a polyclonal antiserum to the C-terminal part of ADAM12 (rb 109). **A:** Wild-type normal mouse at embryonic day 18 (E18). **B:** Wild-type normal mouse at postnatal day 4. **C:** Wild-type normal adult mouse at 2 months. **D:** Adult mouse expressing transgenic ADAM12 (6 months old). **E:** Immunoprecipitation and Western blot of ADAM12 in transgenic (TG) mice and wild-type normal littermate controls (LC) showing the expected 110-kD proform and the 90-kD mature form of membrane-anchored ADAM12. **Lanes 1 and 2** are from hindlimb muscle and **lanes 3 and 4** are from diaphragm. **Lane 5** represents COS-7 cells transiently transfected with a full-length human ADAM12-L expression construct as a control. **A and B** are cross-sections, and **C and D** are obliquely cut sections. Scale bars: 20 μ m (**A–D**).

tion (Figure 2, B and D). In contrast, the hindlimb muscle in *mdx*/ADAM12 transgenic mice had an estimated 80% reduction of areas of necrosis, and the diaphragm had an estimated 60% reduction with areas of necrosis, inflammation, and regenerating fibers (Figure 2; C, E, and H). Furthermore, immunostaining with antibodies to CD45 (not shown) and F4/80 revealed an overall decrease in the number of inflammatory cells in *mdx*/ADAM12 mice compared to the *mdx* mice (Figure 2, G and F). The reduced inflammatory response was confirmed by microarray experiments (manuscript in preparation). Serum creatine kinase values were also reduced in 4- to 6-month-old *mdx*/ADAM12 mice (4440 ± 2516 U/L; $n = 21$) compared to *mdx* mice ($10,816 \pm 6830$ U/L; $n = 22$; $P < 0.0005$, Mann-Whitney test) (Figure 2I). These results demonstrate that continuous and enhanced expression of ADAM12 in *mdx* muscle reduces the extent of necrosis and subsequent inflammation. The reduction in necrosis and serum creatine kinase values suggests that ADAM12 acts in stabilizing the dystrophin-deficient muscle.

Figure 2. Enhanced expression of ADAM12 reduces necrosis and inflammation in the *mdx* mouse muscle. **A:** Reverse transcriptase-polymerase chain reaction analysis of mouse and human ADAM12 expression in tissue from m. quadriceps of littermate control mice (LC) (**lanes 1 and 3**); ADAM12 transgenic mice (TG) (**lanes 2 and 4**); *mdx* mice (**lane 5**); and *mdx*/ADAM12 mice (**lane 6**). Expression of GAPDH served as an internal control. **Lanes 1 and 2** represent samples from E17 mice; **lanes 3 to 6** are from adult (6 to 8 weeks old) mice. **B:** Morphological analysis of 6-week-old *mdx* mice quadriceps muscle with large areas of necrosis and regeneration (**arrows**) and of a *mdx*/ADAM12 muscle showing decreased pathology (**C**). **D:** Larger magnification of *mdx* mice and the *mdx*/ADAM12 quadriceps muscle (**E**). **F and G:** Immunohistochemical analysis using antibodies to the F4/80 macrophage marker (**arrows**) demonstrates the presence of numerous macrophages (**F**) in *mdx* muscle and a reduction of macrophages (**G**) in the *mdx*/ADAM12 mice. **H:** Estimate of the morphological changes (%) including necrosis, inflammation, and regenerating fibers. *, Statistically significant by the Mann-Whitney test, $P < 0.0001$. **I:** Measurements of the serum creatine kinase (U/L). Although the SD is rather big, the difference (*) between the *mdx* and the *mdx*/ADAM12 mice is statistically significant by the Mann-Whitney test at $P < 0.0005$. Scale bars: 60 μ m (**B and C**); 16 μ m (**D and E**); and 20 μ m (**F and G**).

Figure 3. Expression of ADAM12 in the *mdx* mouse muscle reduces muscle necrosis. To compare the degree of muscle membrane lesions between *mdx* and *mdx/ADAM12* mice, Evans Blue dye was injected and the hindlimbs analyzed macroscopically and by fluorescence microscopy. **A:** *mdx* mice exhibited large areas of intense blue coloration of the muscle, whereas the *mdx/ADAM12* mouse exhibited much less staining, and the wild-type (Wt) mouse showed almost no coloration. **B** and **C:** Frozen sections of the quadriceps muscle were examined by fluorescence microscopy at 560 nm. **D:** Estimate of dye uptake. *, Statistically significant by the Mann-Whitney test at $P < 0.002$. Scale bars, 40 μ m (**B** and **C**).

To test more directly the potential effect of ADAM12 on muscle membrane integrity/stability, we compared the extent of Evans Blue dye uptake in muscle in 4-month-old *mdx* and *mdx/ADAM12* mice. This low-molecular weight diazo dye forms a complex with serum albumin that is excluded from normal skeletal muscle fibers but is taken up by damaged fibers in dystrophin-deficient muscle.^{28,29} Macroscopic examination (Figure 3A) of muscle after intravenous injection of Evans Blue dye demonstrated much less uptake in the hindlimb muscles of *mdx/ADAM12* mice muscles than that seen in *mdx* mice. Microscopic examination showed that the number of dye-positive fibers in the *mdx/ADAM12* quadriceps muscle was significantly lower than in the *mdx* muscle, and that the pattern of dye uptake was different (Figure 3; B, C, and D). In *mdx* mice, groups of fibers contained the dye (Figure 3B), whereas in *mdx/ADAM12* mice only occasional fibers were positive for the dye (Figure 3C).

These results indicate that overexpression of ADAM12 stabilizes the plasma membrane and consequently increases cell survival of dystrophin-deficient muscle fibers. The MCK promoter used in the present study drives transcription in both skeletal and cardiac muscle. Because it has been shown that *mdx* mice have pathological changes in cardiac muscle, we compared cardiac muscle morphology of 6-month-old *mdx* mice ($n = 13$)

and their ADAM12/*mdx* littermates ($n = 10$). We found that the extent of necrosis was decreased in the *mdx/ADAM12* mice ($7.5 \pm 6.4\%$ and $2.6 \pm 2.6\%$; $P < 0.05$), suggesting a beneficial effect of ADAM12 also on cardiac muscle.

Discussion

Factors that function during normal skeletal muscle growth and regeneration may be the source of candidate drug targets for treatment of muscular dystrophy and aging.^{4,5} In this regard ADAM12, a disintegrin and metalloprotease, may offer promise. ADAM12 is highly expressed in muscle during development. Although its expression ceases after birth, it is re-expressed in regenerating muscle cells.^{19,21,22} Here we demonstrate that overexpression of ADAM12 in *mdx* muscle alleviates the pathology of the dystrophin-deficient *mdx* mice. The transgenic *mdx/ADAM12* mice show less muscle fiber necrosis and inflammation, significantly lower creatine kinase values, and reduced Evans Blue dye uptake. As less necrosis and inflammation was seen in both skeletal muscle and heart muscle, and as heart does not normally regenerate, it seems that the action of ADAM12 is on survival of muscle cells and possibly also on regeneration.

There is ample evidence in mouse and human that disturbing the adhesion of muscle cells to the extracellular matrix makes the cells susceptible to injury and prone to cell death by apoptosis and necrosis.¹ Several ADAMs have been shown to support cell-substrate and cell-cell interactions.¹⁸ ADAM12, for example, supports (or mediates) cell adhesion through its disintegrin and cysteine-rich domains.^{25,26,30,31} It is possible that ADAM12 could strengthen the muscle cell membrane in *mdx* mice through its interaction with syndecans and/or integrins and thereby act as a survival factor. It is also possible that it acts as a survival factor by the generation of trophic factors such as heparin-binding epidermal growth factor (HB-EGF)³² via its metalloprotease activity.

The molecular mechanisms by which ADAM12 affects *mdx* muscle are not yet clear. The multidomain protein ADAM12 could exert several different activities at different times such as protease, cell adhesion, and cell signaling activities, suggesting that ADAM12 has complex direct and indirect effects on the muscle. With regard to protease activity, several studies indicate that ADAMs can regulate the processing, or shedding, of growth factors and receptors.^{15,17} Recently ADAM12 was proposed to cleave HB-EGF in the heart.³³ We have previously shown that ADAM12 can cleave insulin-like growth factor (IGF)-BP3 and -5^{27,34} and in this way promotes the biological activity of IGF-1 and -2, which are positive regulators of muscle growth, survival, and regeneration.³⁵ IGF-BP3 binds the IGFs and prevents the activation of the IGF-1 receptor, which normally mediates cell signals to promote myogenesis. IGF-BP3 degradation by ADAM12 could thus result in enhanced IGF signaling. This hypothesis is particularly attractive in the light of a recent study showing that high levels of muscle-specific expression of IGF-1 in *mdx* muscle leads to increased muscle mass

and strength and decreased muscle cell pathological changes.³⁶

A number of different strategies for alleviating the muscle pathology seen in dystrophin-deficient *mdx* mice or the laminin $\alpha 2$ -deficient *dy* mice have been reported. For *mdx* mice these include compensating the dystrophin-deficiency through overexpression of utrophin,³⁷ or compensating the secondary dystroglycan-deficiency by overexpression of integrin $\alpha 7$,³⁸ or by reducing inflammation by overexpression of nNOS.¹¹ In case of *dy* mice, an artificial miniagrin was used to cross-link proteins into a basement membrane-like matrix to compensate for lack of laminin-2 and -4. Surprisingly, overexpression of proteins that are normally confined to the neuromuscular junction such as utrophin,³⁷ $\alpha 7$ integrin,³⁸ GalNAc transferase,¹² and the agrin minigene³⁹ have localized these proteins all along the plasma membrane with subsequent benefit for the diseased muscle. The exact molecular mechanisms for this distribution and beneficial effect, however, are not clear. We now add to this list a new strategy for how to alleviate the *mdx* pathology, ie, overexpression of ADAM12, a disintegrin and metalloprotease.

In summary we show here that enhanced expression of ADAM12, a protein associated with muscle development and regeneration, can positively modulate the pathology associated with dystrophin-deficiency. Future studies will be needed to determine precisely how ADAM12 benefits muscle. Overexpression of ADAM12 in mice of different genetic backgrounds and with other forms of muscular dystrophy may give clues to the function of ADAM12. For example, the *dy* laminin-deficient mouse suffers from a form of muscular dystrophy, which is very different from that of the *mdx* dystrophin-deficient mouse. In the *dy* mice, apoptosis rather than necrosis is a characteristic feature, and regeneration is particularly compromised.⁴⁰ The *dy* muscle may benefit even more than the *mdx* muscle from enhanced expression of ADAM12 if ADAM12 acts to promote survival and regeneration. On the other hand, it will benefit less than *mdx* if ADAM12 acts to stabilize the cell membrane, as the *dy* mouse has a basement membrane defect but not a cell membrane defect. In the future it will be necessary to address the function of ADAM12 in muscle through combinations of *in vitro* and *in vivo* experiments to determine precisely how ADAM12 benefits muscle and to what extent the different domains of ADAM12 contribute to muscle cell regeneration, stability, and survival.

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